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EFFECT OF GLYCOLYSIS INHIBITORS ON CYCLIC AMP SYNTHESIS IN RAT ADIPOSE TISSUE

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Abstract—The effect of two glycolysis inhibitors, sodium fluoride and *monoiodoacetate*, on the level of cyclic AMP in rat epididymal fat was studied *in vitro* with cyclic AMP-dependent protein kinases.

The large increase (about 200 times in respect of the control level) of cyclic nucleotide concentration induced by noradrenaline plus theophylline, was strongly inhibited by 0·04 M sodium fluoride and by 0·001 M monoiodoacetic acid. The same inhibitors decreased the rate of hormone-induced lipolysis to a similar extent. Cyclic AMP level inside the adipose tissue, not treated with noradrenaline plus theophylline, as well as spontaneous lipolysis, were modified in the same way by fluoride and iodoacetate.

These data emphasize the relationship between lipolysis and energy metabolism and, particularly, the importance of the glycolytic pathway for cyclic AMP synthesis.

THE RELATIONSHIP between hormone-induced lipolysis and the main metabolic sources of ATP, was investigated by the use of some specific inhibitors of oxidative phosphorylation[1–9] as well as of glycolysis.[1,3] The inhibition induced by all these compounds seems to indicate that the lipolytic process requires a continuous supply of energy and that both glycolysis and oxidative phosphorylation have an essential role on the hormonal action. However, the kinetic study of the aforesaid inhibitory effects[3] gave no indication on the relative significance of the two metabolic pathways in the process studied. That is, it was not possible to discriminate the importance of glycolysis and of oxidative phosphorylation in successive stages of the lipolytic process, before and after cyclic AMP synthesis.

This problem led us to investigate the effect of two glycolysis inhibitors, sodium fluoride and monoiodoacetate, on the level of cyclic AMP in rat epididymal fat, in the absence and in presence of a lipolytic hormone, noradrenaline, together with an inhibitor of phosphodiesterase, theophylline.

The increase of cyclic AMP induced by noradrenaline plus theophylline, was strongly inhibited by fluoride and monoiodoacetate. The degree of this inhibitory effect was finally compared with that exerted by the same drugs on hormone-stimulated lipolysis.

MATERIALS AND METHODS

Preparation and incubation of rat epididymal fat pads

Treatment of adipose tissue and determination of lipolysis, were the same as previously described.[3] Free fatty acids were titrated in the incubation medium according to Dole[10] and glycerol according to Korn.[11]

Fat pads removed from rats weighing 200–250 g and pooled to maximize random distribution, were weighed and placed (200 ± 5 mg) into 1·90 ml of Krebs–Ringer

bicarbonate pH 7·2 containing bovine albumin (2·5 per cent) and, where indicated, the glycolysis inhibitors. After a preliminary incubation in a metabolic shaker at 37° for 30 min, noradrenaline and theophylline were added (each dissolved in a volume of 50 μl of 9% NaCl) and the assays were further incubated for 15 min at 37°, under air phase in a shaking apparatus. At the end of the incubation period, tissue and medium were immediately separated by filtration and washed (1 ml of 9% NaCl) under vacuum.

Isolation of cyclic AMP from tissue and medium

The fat was rapidly put inside 1 ml of ice-cold 3% TCA and homogenized by a Potter homogenizer with Teflon pestel. Cyclic AMP (100 *p*moles) was added to a control sample for determining its recovery. The assays were then neutralized by the addition of 0·2 ml of 1 M Tris and centrifuged for 20 min at 10,000 *g*. The supernatant represented the starting material for purification of cyclic AMP. Ice-cold TCA 50% (0·15 ml) was added to the incubation medium, the precipitate removed by centrifugation and the pH of the supernatant neutralized by the addition of 0·4 ml of 1 M Tris. Before centrifugation, 100 *p*moles of cyclic AMP were introduced into a control sample for determining recovery.

Cyclic AMP was purified from the neutralized supernatants, both of tissue and medium, by the $BaSO_4$ method of Krishna, Weiss and Brodie.[12] To the tissue extracts 0·5 ml each of 5% $ZnSO_4$ and 2·6% Ba $[OH]_2$, and to the medium extracts 1·5 ml each of the same solutions, were added. After the removal of the $BaSO_4$ precipitate by centrifugation, cyclic AMP was further purified by ion exchange chromatography according to Kuo and Greengard.[13,14] One ml aliquots of the supernatant solutions were loaded into AG 50W-X8 columns (0·5 × 5 cm) and eluted with water. Cyclic AMP appeared in the 3rd through 6th ml of the column eluate; 2 ml of the cyclic AMP fraction from the columns were lyophilized together with 0·5 ml of histone solution (0·343 mg/ml water). Lyophilized residue was dissolved in 300 μl of distilled water and cyclic AMP assayed in different amounts of this solution. In the samples where cyclic AMP was contained in a very high concentration, the nucleotide was assayed directly in the column eluate.

Assay for cyclic AMP. The nucleotide levels were measured by the method of Kuo and Greengard[13,14] based upon the ability of a protein kinase from bovine heart to catalyse the transfer of ^{32}P to histone from $\gamma^{32}P$-ATP in a reaction dependent on the presence of cyclic AMP. Protein kinase was prepared according to Kuo and Greengard[15] and to Kuo *et al.*[16] The enzyme preparation was that obtained after the DEAE–cellulose step of purification and was stored at −50° in small lyophilized portions. Histone-bound ^{32}P was measured in a Beckman liquid scintillation counter. The scintillation solvent solution consisted of 5 g of PPO (2,5-diphenylisoxazole) dissolved in 1000 ml of a mixture (50%, v/v) of toluene and methyl cellosolve. The amounts of synthetic cyclic AMP used in each experiment for obtaining the standard curve were from 0·5 to 12 *p*moles. In our experimental conditions, the slope of the curve relating the activity of the protein kinase to the concentration of cyclic AMP was constant between 0·5–10 *p*moles and the apparent K_m value for cyclic AMP was $1{\cdot}2 \times 10^{-8}$ M. Overall recovery of synthetic cyclic AMP added to the control samples, was between 60 and 75%. The data have been corrected in each experiment for the recovery.

Materials. DEAE–cellulose (medium mesh, 0·85 m.eq. per g), histone (Type II), cyclic AMP and bovine serum albumin (Fraction V) were purchased from Sigma. γ^{32}P-ATP was obtained from Radiochemical Centre, Amersham. AG 50W-X8 resin (200–400 mesh) purchased as analytical grade from BioRad Laboratories in the hydrogen form, was washed repeatedly in distilled water to remove fins and kept as a stock at 4° as a 50% (v/v) suspension in distilled water. Noradrenaline bitartrate monohydrate was from Recordati, theophylline from C. Erba, sodium fluoride from Merck and monoiodoacetic acid from British Drug Houses. N^6 C_2'-dibutyryl cyclic 3',5'-AMP was a generous gift of Dr. M. Carissimi (Maggioni, Milan, Italy).

RESULTS

Effect of sodium fluoride and monoiodoacetate on cyclic AMP level in adipose tissue and in its incubation medium

Sodium fluoride (Table 1) at a concentration of 0·04 M did not induce a significant variation on the level of cyclic AMP in the tissue nor in the medium. In contrast, iodoacetate 0·001 M increased the cyclic nucleotide concentration inside adipose tissue (Table 1). The same was shown also in the case of spontaneous lipolysis, as indicated in Table 2 by the glycerol release from epididymal fat in the absence and in presence of the metabolic inhibitors.

TABLE 1. EFFECT OF GLYCOLYSIS INHIBITORS ON CYCLIC AMP LEVELS IN RAT EPIDIDYMAL FAT AND IN ITS INCUBATION MEDIUM

Drugs in the medium M conc.	Cyclic AMP *p*moles/g fresh tissue		
	Tissue	Medium	Tissue + Medium
	36·71 ± 4·06	263·06 ± 19·06	299·77
NaF 4×10^{-2}	37·36 ± 6·30 (P > 0·90)	215·16 ± 13·90 (P > 0·05)	252·52
Iodoacetate 10^{-3}	92·98 ± 9·88 (P < 0·001)	311·78 ± 20·84 (P > 0·10)	404·76

Rat epididymal fat (200 ± 5 mg) was incubated in 2 ml of Krebs–Ringer bicarbonate containing bovine albumin, for 45 min at 37°, with glycolysis inhibitors present where indicated.

At the end of incubation, tissue and medium were treated as indicated in the method. Cyclic AMP was then extracted purified, and titrated according to Kuo and Greengard[13] by the use of bovine heart protein kinase (8 μg). The data are the means (±S.E.) of six determinations from three experiments.

This similarity between the variations of cyclic AMP and lipolysis is of interest, although the experimental conditions were different regarding the incubation time (180 min for lipolysis, 45 min for cyclic AMP). The data of Table 2 represent the control assays of experiments concerning the effect of noradrenaline on lipolysis and on cyclic AMP. The different incubation times are therefore related to the maximum effect induced by catecholamines on lipolysis (150 min) and on cyclic AMP (15 min). The preincubation of fat in the medium plus inhibitors, was of 30 min in both cases.

TABLE 2. EFFECT OF GLYCOLYSIS INHIBITORS ON THE SPONTANEOUS GLYCEROL RELEASE AND CYCLIC AMP LEVEL IN RAT EPIDIDYMAL ADIPOSE TISSUE (*in vitro*)

Drugs in the medium M conc.	Glycerol (medium) μmoles/g fresh tissue	Cyclic AMP (tissue) pmoles/g fresh tissue
	2·12 ± 0·10	36·71 ± 4·06
NaF 4×10^{-2}	2·63 ± 0·13 (P > 0·01)	37·36 ± 6·30 (P > 0·90)
Iodoacetate 10^{-3}	3·80 ± 0·26 (P < 0·001) (+79%)	92·98 ± 9·88 (P < 0·001) (+151%)

Rat epididymal fat was incubated in Krebs–Ringer bicarbonate containing 2·5% bovine albumin, with glycolysis inhibitors present where indicated, at 37° in a metabolic shaker, for different incubation periods: for 180 min the assays where FFA and glycerol were titrated in the incubation medium, and for 45 min the assays in which CAMP was determined. The experimental conditions applied for separating tissue from medium, and for extracting, purifying and titrating cyclic AMP, were as indicated under Table 1. The data are the means (±S.E.) of six determinations from three experiments.

Effect of noradrenaline and theophylline on cyclic AMP level in adipose tissue and medium

The effect of noradrenaline 10^{-5} M on cyclic AMP level of adipose tissue and medium, was determined in the presence of theophylline 0·003 M for preserving cyclic AMP from hydrolysis (Table 3). After incubation with these agents for 15 min, cyclic AMP increased in respect to its initial level of about 200-times inside the tissue and of 13-times in the medium. The increase of the cyclic nucleotide was therefore mostly evident inside the tissue, in agreement with previous results[17] obtained in the same experimental conditions. When present alone, noradrenaline or theophylline induced only a small and somewhat variable increasing effect on cyclic AMP level.[13,17–19] Therefore, only the contemporaneous presence of noradrenaline and theophylline, acting sinergistically on the cyclic nucleotide synthesis and accumulation, was the ground for the experimental conditions suitable for investigating the effect of glycolysis inhibitors on the process.

TABLE 3. EFFECT OF NORADRENALINE AND THEOPHYLLINE ON THE LEVELS OF CYCLIC AMP IN RAT EPIDIDYMAL FAT AND IN ITS INCUBATION MEDIUM

Drugs in the medium M conc.	Cyclic AMP pmoles/g fresh tissue		
	Tissue	Medium	Tissue + Medium
	37·22 ± 6·00	263·06 ± 19·06	300·28
Noradrenaline 10^{-5} + Threophylline 3×10^{-3}	6997·75 ± 121·50	3375·57 ± 227·50	10373·32

Rat epididymal fat (200 ± 5 mg) was preincubated in 2 ml of Krebs–Ringer bicarbonate containing bovine albumin, for 30 min at 37°. At that time, theophylline and noradrenaline were added and the assays further incubated for 15 min. The experimental conditions for separating tissue from medium, and for extracting, purifying and titrating cyclic AMP, were as described under Table 1. The data are the means (±S.E.) of four determinations.

Effect of glycolysis inhibitors on the increase of cyclic AMP level induced by noradrenaline and theophylline

In the presence of 0·04 M sodium fluoride and of 0·001 M iodoacetate, the effect of noradrenaline plus theophylline in elevating cyclic AMP was inhibited (Table 4) both inside the tissue as well as in the medium. The total amount of cyclic AMP which accumulates in tissue plus medium is reported in the last column of Table 4; the whole inhibitory effect was of 96 per cent in the presence of sodium fluoride, and of 66 per cent in the presence of iodoacetate.

TABLE 4. EFFECT OF GLYCOLYSIS INHIBITORS ON THE INCREASE OF CYCLIC AMP LEVEL INDUCED BY NORADRENALINE AND THEOPHYLLINE IN ADIPOSE TISSUE

Drugs in the medium M conc.	Cyclic AMP *p*moles/g fresh tissue		
	Tissue	Medium	Tissue + Medium
Noradrenaline 10^{-5} + Theophylline 3×10^{-3}	6997·75 ± 121·50	3375·57 ± 227·50	10373·32
Noradrenaline 10^{-5} + Theophylline 3×10^{-3} + NaF 4×10^{-2}	113·07 ± 13·10* (−98%)	289·07 ± 46·25* (−91%)	402·14 (−96%)
Noradrenaline 10^{-5} + Theophylline 3×10^{-3} + Iodoacetate 10^{-3}	3215·37 ± 149·25* (−54%)	339·06 ± 47·00* (−90%)	3554·43 (−66%)

Rat epididymal fat (200 ± 5 mg) was introduced in 2 ml of Krebs–Ringer bicarbonate containing bovine albumin and, where indicated, the glycolysis inhibitors. After a pre-incubation for 30 min at 37°, theophylline and noradrenaline were added and the assays further incubated for 15 min. The experimental conditions applied for separating tissue from medium and for extracting, purifying and titrating cyclic AMP, were as described under Table 1. The data are the means (±S.E.) of four determinations.

* $P < 0·001$.

The inhibitory effect of NaF on cyclic AMP synthesis induced by noradrenaline, was found by Kuo and DeRenzo[20] also in intact adipocytes. At this point, it is interesting to note the well known fact[21–28] that sodium fluoride stimulates cyclic AMP formation in homogenates, membrane and particulate adenyl cyclase preparations of many tissues. However, in intact adipose tissue the effect of sodium fluoride on cyclic AMP synthesis is well in accordance with its action on basal and hormone-induced lipolysis (Tables 2 and 5). Table 5 shows the comparison between the effect of glycolysis inhibitors on the rate of lipolysis stimulated by noradrenaline and by dibutyryl cyclic AMP, and on the level of cyclic AMP increased by noradrenaline plus theophylline. The parallel variations are interesting, although the experimental conditions are different regarding the incubation time and the presence of theophylline.

TABLE 5. COMPARISON BETWEEN THE EFFECT OF GLYCOLYSIS INHIBITORS ON HORMONE-STIMULATED LIPOLYSIS AND ON CYCLIC AMP SYNTHESIS

Drugs in the medium M conc.	Inhibition (%)		
	Lipolysis*		Cyclic AMP† level
	Induced by NE	Induced by dibutyryl CAMP	
NaF 4×10^{-2}	82 ± 6	67 ± 2	96 ± 1
Iodoacetate 10^{-3}	73 ± 5	67 ± 2	66 ± 2

* Lipolysis induced by noradrenaline 2×10^{-5} M or by dibutyryl-cyclic AMP 5×10^{-3} M in rat epididymal fat incubated in Krebs–Ringer bicarbonate containing bovine albumin, at 37° for 150 min in a metabolic shaker. The fat was pre-incubated for 30 min at 37° in the presence of glycolysis inhibitors.

† Cyclic AMP synthesis, induced by noradrenaline 10^{-5} M in the presence of theophylline 3×10^{-3} M, was determined as described in Table 4. Cyclic AMP level was that of tissue plus medium. The data are the means (±S.E.) of four determinations.

DISCUSSION

The elucidation of the biochemical events that correlate hormone-induced lipolysis with the metabolic sources of ATP, is the main purpose of our researches.[2,3,5,7,9,17] The present results confirm and amplify the relationship between energy metabolism and lipolysis, and indicate that the alteration of energy equilibrium in adipose cell, interferes with the lipolytic process at different levels, both before and after the synthesis of cyclic AMP.

The increase induced by noradrenaline plus theophylline on the level of cyclic AMP in rat epididymal fat, was strongly inhibited by sodium fluoride and iodoacetate. The two inhibitors of glycolysis produced an antagonistic effect of a quite corresponding degree on hormone-stimulated lipolysis. These data indicate the importance of the glycolytic pathway on both cyclic AMP synthesis and lipolysis, and suggest that fluoride and iodoacetate act on lipolysis by interfering with cyclic AMP synthesis. The close correlation between the inhibitory effect of fluoride and iodoacetate on cyclic AMP synthesis and on lipolysis induced not only by noradrenaline but also by dibutyryl cyclic AMP, is clearly explained. In fact, it is glucose metabolism which normally supplies the larger amount of the metabolic substrate to the respiratory chain. Therefore, the inhibition of glycolysis produces also a decrease of aerobic-generated ATP, ATP which is involved in the lipolytic process after cyclic AMP synthesis too.[3,5,7]

Thus, on the whole, the relative importance of the two metabolic pathways (glycolysis and oxidative phosphorylation) in successive stages of the lipolytic process, before and after cyclic AMP synthesis, is as yet unclear, even if the present results, when compared with those obtained with oxidative phosphorylation inhibitors on cyclic AMP level increased by hormones[17] could suggest that glycolysis is prominent for the synthesis of cyclic AMP, whereas oxidative phosphorylation is probably more important in successive stages of the process. Research is in progress to confirm this initial observation.

Finally, the mechanism by which iodoacetate increases cyclic AMP level as well as lipolysis in basal conditions, is interesting but difficult to explain. Further investigations are needed to establish whether inhibition of phosphodiesterase or other mechanism are involved.

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