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EFFECT OF OXIDATIVE PHOSPHORYLATION INHIBITORS ON CYCLIC ADENOSINE MONOPHOSPHATE SYNTHESIS IN RAT ADIPOSE TISSUE

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Abstract—The concentration of cyclic AMP in rat epididymal fat and in its incubation medium, was determined by the use of cyclic AMP-dependent protein kinases. The noradrenaline plus theophylline stimulation effect on cyclic AMP synthesis, was studied in the absence and in the presence of some oxidative phosphorylation inhibitors, rotenone, 2,4-dinitrophenol and oligomycin, drugs whose strong inhibitory effect on hormone-induced lipolysis is well known.

The concentration of cyclic AMP was reduced by all three drugs. The changes induced by oligomycin and dinitrophenol on the cyclic AMP level, were quantitatively quite similar to those induced on the rate of lipolysis. Rotenone, in contrast, decreased the cyclic AMP level to a lower extent than lipolysis. This could be correlated with the different site and mechanism of action of rotenone on oxidative phosphorylation.

The evidence indicates that the alteration of energy equilibrium (or the decreased availability of ATP) interferes with the lipolytic process at different levels, both before and after the synthesis of cyclic AMP.

VARIOUS inhibitors of oxidative phosphorylation were shown to antagonize hormone-induced lipolysis in adipose tissue.[1–8] The effect of these inhibitors suggested that the lipolytic process requires a continuous supply of energy. The inhibition exerted by the same drugs not only against noradrenaline, but also against theophylline and cyclic AMP,[2,6] indicated that ATP may be required in the lipolytic process at more than one level, that is, both before and, particularly, after the synthesis of cyclic AMP. This conclusion agreed with data obtained by Rizack[9] showing that in a cell-free lipolytic enzyme system from adipose tissue, the simultaneous presence of cyclic AMP and of ATP is necessary to stimulate the hormone-sensitive lipolytic activity. Finally, the same data are in complete accordance with the recent findings of Kuo and Greengard[10] demonstrating the presence in adipose tissue of a cyclic AMP-dependent protein kinase, which requires ATP to phosphorylate a still unknown substrate.

This correlation between energy metabolism and hormone-induced lipolysis, led us to investigate the effect of oxidative phosphorylation inhibitors on the level of cyclic AMP in adipose tissue alone, as well as in the presence of a lipolytic hormone (noradrenaline) together with an inhibitor of phosphodiesterase (theophylline).

We could, in this way, compare the levels of cyclic AMP with the rate of lipolysis in the presence of the tested inhibitors.

MATERIALS AND METHODS

Preparation and incubation of rat epididymal fat pads

Treatment of adipose tissue and determination of FFA and glycerol release for measurement of lipolysis, were the same as previously described.[3]

Fat pads removed from rats weighing 200–250 g and pooled to maximized random distribution, were weighed and placed (200 ± 5 mg) in 1·90 ml of Krebs–Ringer bicarbonate, pH 7·2, containing bovine albumin (2·5%) and, where indicated, the oxidative phosphorylation inhibitors. Rotenone, 2,4-dinitrophenol and oligomycin were dissolved in absolute ethanol; the same volume of ethanol (50 μl) was added to the control samples. After a preliminary incubation in a metabolic shaker at 37° for 30 min, noradrenaline and theophylline were added (each one dissolved in a volume of 50 μl of 0·9% NaCl) and the assays were further incubated for 15 min at 37°, in a shaking apparatus under air phase. At the end of the incubation period, tissue and medium were immediately separated by filtration and washed (1 ml of 0·9% NaCl) under vacuum. When the fat was not quickly separated from the medium (as in the case of centrifugation) cyclic AMP was prevalently found outside the tissue.

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 pmoles) was added to a control sample for the purpose of 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 50% TCA (0·15 ml) was added to the incubation medium. The precipitate was removed by centrifugation and the pH of the supernatant neutralized by the addition of 0·4 ml of 1 M tris. Before centrifugation, 100 pmoles 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.[11] 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:[12] 1-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 to 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[12] based upon the ability of a protein kinase from bovine heart to catalyze the transfer of ^{32}P to histone from γ ^{32}P-ATP in a reaction dependent on the presence of cyclic AMP. Protein kinase was prepared according to Kuo and Greengard[10] and to Kuo *et al.*[13] 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 pmoles. 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 pmoles, and the apparent K_m value for cyclic AMP was $1·2 \times 10^{-8}$ M. Overall recovery of synthetic cyclic AMP added to the control samples, was between 60 and 75 per cent. The data have been corrected in each experiment for the recovery.

Materials. DEAE-cellulose (medium mesh, 0·85 m-equiv./g), histone (Type II), cyclic AMP and bovine serum albumin (Fraction V) were purchased from Sigma. γ-^{32}P-ATP was obtained from the Radiochemical Centre, Amersham. AG 50W-X8 resin (200–400 mesh) purchased as analytical grade from BioRad Laboratories in the hydrogen form, was repeatedly washed in distilled water to remove the fine particles and kept as stock at 4° as a 50% (v/v) suspension in distilled water. Noradrenaline bitartrate monohydrate was from Recordati, theophylline from C. Erba, rotenone from S. B. Penick & Co., 2,4-dinitrophenol from B.D.H., oligomycin (a mixture of oligomycin A and B) from Upjohn. Other chemicals were reagent grade from standard suppliers.

RESULTS

Effect of oxidative phosphorylation inhibitors on cyclic AMP levels in adipose tissue and in its incubation medium

Table 1 shows the levels of cyclic AMP in rat epididymal fat pad and in its incubation medium, in the absence and in presence of three inhibitors of oxidative phosphorylation: rotenone, oligomycin and 2,4-dinitrophenol. Oligomycin, 2,4-dinitrophenol

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

Drugs in the medium (M)	Cyclic AMP (pmoles/g fresh tissue)		
	Tissue	Medium	Tissue + medium
—	174·49 ± 47·16	520·13 ± 15·81	694·42
Oligomycin 10^{-5}	103·77 ± 28·35 ($P > 0·20$)	273·64 ± 49·76 ($P < 0·01$)	377·41
2,4-Dinitrophenol 10^{-3}	127·50 ± 19·32 ($P > 0·20$)	403·13 ± 31·20 ($P < 0·05$)	530·63
Rotenone 10^{-5}	229·31 ± 44·03 ($P > 0·20$)	287·66 ± 34·50 ($P < 0·005$)	516·97

Rat epididymal fat (200 ± 5 mg) was incubated in 2 ml of Krebs–Ringer bicarbonate containing bovine albumin, for 45 min at 37°, with oxidative phosphorylation inhibitors present where indicated. The experimental conditions applied for separating tissue from medium, and for extracting and purifying cyclic AMP, were as described in Methods. Reaction mixtures for the assay of cyclic AMP contained, in a final volume of 0·2 ml, 10 μmoles sodium acetate buffer pH 6·0; 2 μmoles magnesium acetate; 40 μg histone; 10–70 μl of tissue or medium extracts; 8 μg of heart protein kinase; 1 mμmole of γ ^{32}P-ATP, containing about $1·8 \times 10^{-6}$ counts/min. Incubation and the following steps were as described by Kuo and Greengard.[12] The data presented are the mean (± S.E.) of six determinations from two experiments.

and rotenone did not cause a significant variation in the level of cyclic AMP inside the tissue. A certain decrease of the cyclic nucleotide could be appreciated in the medium for all three inhibitors.

Intratissular and extratissular levels of cyclic AMP in adipose tissue incubated in the presence of noradrenaline and theophylline

The effect of noradrenaline 10^{-5} M on cyclic AMP levels of adipose tissue and medium, was determined in the presence of theophylline (3×10^{-3} M) for preserving cyclic AMP from hydrolysis. It was found (Table 2) that adipose tissue, after incubation with both these agents for 15 min, contained very high levels of cyclic AMP: an increase of more than 100 times in respect to the initial level was evident inside the tissue. A total increase of about 40 times was found in tissue plus medium. The increase of the nucleotide is therefore mostly evident inside the tissue.

Thus in epididymal fat pads *in vitro*, theophylline and noradrenaline acted sinergystically on cyclic AMP accumulation. When present alone, noradrenaline or theophylline were only mildly stimulatory on the levels of cyclic AMP (Table 2). Our results agree with those of Butcher *et al.*[14,15] and of Kuo and Greengard[12] in adipose tissue and in fat cells.

TABLE 2. 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)	Cyclic AMP (pmoles/g fresh tissue)		
	Tissue	Medium	Tissue + medium
—	174·49 ± 47·16	520·13 ± 15·81	694·42
Noradrenaline 10^{-5}	459·68 ± 69·50 (× 2·6)	1147·41 ± 46·25 (× 2·2)	1607·09 (× 2·3)
Theophylline 3×10^{-3}	151·63 ± 31·20 (no increase)	993·92 ± 87·50 (× 1·9)	1145·55 (× 1·6)
Noradrenaline 10^{-5} + Theophylline 3×10^{-3}	22,904·98 ± 1038·38 (× 131)	1942·23 ± 55·12 (× 3·7)	24,847·21 (× 36)

Rat epididymal fat (200 ± 5 mg) was pre-incubated 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 applied for separating tissue from medium and for extracting, purifying and titrating cyclic AMP, were as described under Table 1. The data presented are the mean (± S.E.) of 15 determinations from three experiments.

Effect of oxidative phosphorylation inhibitors on cyclic AMP level increase induced by noradrenaline and theophylline

In the presence of oligomycin 10^{-5} M, 2,4-dinitrophenol 10^{-3} M and rotenone 10^{-5} M (Table 3) the effect of noradrenaline plus theophylline in elevating cyclic AMP levels was decreased. The inhibition inside the tissue was of 77 per cent in the presence of oligomycin and of 94 per cent in the presence of dinitrophenol. Rotenone reduced the cyclic AMP level only to a lower extent (35 per cent). In contrast, looking on the effect in the medium, one can see that dinitrophenol did not change the level of cyclic AMP, while oligomycin and rotenone greatly decreased it.

TABLE 3. EFFECT OF OXIDATIVE PHOSPHORYLATION INHIBITORS ON THE CYCLIC AMP LEVEL INCREASE INDUCED BY NORADRENALINE AND THEOPHYLLINE IN ADIPOSE TISSUE

Drugs in the medium (M)	Cyclic AMP (pmoles/g fresh tissue)		
	Tissue	Medium	Tissue + medium
Noradrenaline 10^{-5} + Theophylline 3×10^{-3}	22,904·98 ± 1038·38	1942·23 ± 55·12	24,847·21
Noradrenaline 10^{-5} + Theophylline 3×10^{-3} + *Oligomycin* 10^{-5}	5290·15 ± 262·36* (− 77%)	438·31 ± 61·74* (− 78%)	5728·46 (− 77%)
Noradrenaline 10^{-5} + Theophylline 3×10^{-3} + *2,4-Dinitrophenol* 10^{-3}	1297·87 ± 109·82* (− 94%)	1813·33 ± 109·90* (n.s.)	3111·20 (− 88%)
Noradrenaline 10^{-5} + Theophylline 3×10^{-3} + *Rotenone* 10^{-5}	14,799·19 ± 1683·33* (− 35%)	335·12 ± 14·29* (− 83%)	15,134·31 (− 39%)

Rat epididymal fat (200 ± 5 mg) was introduced in 2 ml of Krebs–Ringer bicarbonate containing bovine albumin and, where indicated, the oxidative phosphorylation 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 presented are the mean (± S.E.) of 9–15 determinations from three experiments.

* $P < 0{\cdot}001$.

The last column of Table 3 reports the total amount of cyclic AMP accumulated in tissue plus medium and the whole inhibitory effect induced by the three drugs tested. It is evident how the effect of rotenone is, on the whole, much lower than that of oligomycin and dinitrophenol.

Finally, in Table 4 one can observe the comparison between the inhibition exerted on hormone-stimulated lipolysis and on cyclic AMP synthesis, by the same concen-

TABLE 4. COMPARISON BETWEEN THE EFFECT OF OXIDATIVE PHOSPHORYLATION INHIBITORS ON HORMONE-STIMULATED LIPOLYSIS AND CYCLIC AMP SYNTHESIS

Drugs (M)	Lipolysis* (% inhibition)	Cyclic AMP level (% inhibition)
Oligomycin 10^{-5}	63 ± 6	77 ± 6
2,4-DNP 10^{-3}	68 ± 1	88 ± 1
Rotenone 10^{-5}	66 ± 4	39 ± 6

* Lipolysis induced by noradrenaline 2×10^{-5} M in rat epididymal fat incubated in Krebs–Ringer bicarbonate buffer pH 7·2 containing bovine albumin, at 37° for 150 min in a metabolic shaker. Free fatty acids and glycerol were titrated according to Dole[16] and Korn.[17]

[Partially reproduced from G. FASSINA, *Life Sci.* **6**, 825 (1967).]

trations of the three drugs. The changes induced by oligomycin and dinitrophenol on the rate of lipolysis, as well as on cyclic AMP levels, are of a quite correspondent degree, whilst the same is not true in the case of rotenone.

DISCUSSION

The concentration of cyclic AMP in rat epididymal fat and in its incubation medium, was greatly increased by noradrenaline in the presence of theophylline. The increase was mostly evident inside the tissue (more than 100 times). The noradrenaline plus theophylline stimulant effect was inhibited by some oxidative phosphorylation inhibitors: oligomycin, 2,4-dinitrophenol and rotenone.

It is of interest to remark that the same drugs exerted a strong inhibitory effect on hormone-stimulated lipolysis.[1–3,6,7] This emphasizes the relationship between lipolysis and energy metabolism and particularly, with oxidative phosphorylation process. Moreover, the same results apparently support the conclusion of Butcher *et al.*[15] that the rate of lipolysis is related to changes in intracellular cyclic AMP levels in fat pads under a variety of experimental conditions.

The data obtained with rotenone deserve a further comment. The inhibitory effect of this drug on cyclic AMP synthesis was much lower than that of oligomycin and dinitrophenol. In contrast, all the three drugs, at the same concentrations tested on cyclic AMP, exerted on lipolysis an inhibitory effect of a quite correspondent degree (Table 4). Thus, while the changes induced on the rate of lipolysis and on cyclic AMP levels, are very similar in the case of oligomycin and dinitrophenol, this is not true in the case of rotenone. At the moment we cannot find any explanation. A strong possibility is that this effect may be correlated with the different site and mechanism of inhibition by rotenone on oxidative phosphorylation. It is known that rotenone, by acting on the respiratory chain at the level of NADH-cytochrome *b* reductase segment, permits oxidation of succinate with the coupled ATP synthesis. Thus a certain amount of ATP could be available for cyclic AMP formation. Research is in progress in our laboratory to investigate the relationships between ATP availability and cyclic AMP synthesis.

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