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FURTHER STUDIES OF THE UNCOUPLING ACTION OF TRIAMCINOLONE IN RAT LIVER MITOCHONDRIA

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Abstract—In fresh mitochondria triamcinolone stimulates respiration in media free of inorganic phosphate and phosphate acceptors and depresses the ADP–ATP* exchange reaction. The steroid does not affect these reactions in mitochondria aged for 24 to 72 hr at 0° to 2°. Upon aging of the mitochondria, the sensitivity of these reactions to triamcinolone is lost at the same time that the ^{32}P-ATP exchange disappears.

Azide, oligomycin and reduction of the respiratory chain inhibit the triamcinolone-stimulated adenosinetriphosphatase activity of fresh mitochondria.

These experiments are consistent with the previous hypothesis from this laboratory that triamcinolone uncouples oxidative phosphorylation in the same site as does 2,4-dinitrophenol. However, it was found that triamcinolone promotes mitochondrial swelling, whereas dinitrophenol in identical conditions does not effect this phenomenon. It is probable that the steroid causes a structural change in the mitochondria before functional changes become apparent.

IT HAS been reported that triamcinolone (9α-fluoro-11β,16α,17α,21 tetrahydroxy-1,4 pregnandiene-3,20 dione) uncouples oxidative phosphorylation in fresh mitochondria; this steroid increases respiration, depresses the ^{32}P-ATP exchange reaction and stimulates the latent adenosinetriphosphatase activity of fresh mitochondria.[1] These data suggested that triamcinolone and DNP uncouple oxidative phosphorylation in the same or at a very close site.

In this paper experiments are presented on the effect of triamcinolone on the ADP-ATP exchange reaction and on the respiration of fresh and aged mitochondria. The results agree with the previous hypothesis. Moreover, the effect of oligomycin and azide on the stimulation of the adenosinetriphosphatase activity of fresh mitochondria by triamcinolone is similar to the inhibiting effect of these two agents on the DNP-stimulated adenosinetriphosphatase activity of fresh mitochondria.[2, 3] Also the reduction of the respiratory chain by high concentrations of cyanide inhibits the triamcinolone-stimulated adenosinetriphosphatase activity.

MATERIAL AND METHODS

Animals. White male Wistar rats weighing 150 to 200 g were used in all experiments.

Mitochondria. Liver mitochondria were prepared in 0·25 M sucrose according to the method of Schneider and Hogeboom;[4] they were washed once after the first high-speed centrifugation. The following suspensions in 0·25 M sucrose were made: mitochondria

* The following abbreviations are used: ATP and ADP = adenosine- ,tri- and di-phosphate respectively; DNP = 2,4-dinitrophenol.

from 300 mg of liver in 1 ml of sucrose for the respiration experiments; mitochondria from 200 mg of liver in 0·5 ml for the studies of adenosinetriphosphatase activity; and mitochondria from 1 g of liver in 1 ml for measuring the exchange reactions and the swelling experiments.

Aging of mitochondria. Aging of mitochondria was accomplished by storing the mitochondrial suspension for variable periods of time at 0° to 2°.

Oxygen uptake. Oxygen uptake was measured by the conventional Warburg technique in a media free from inorganic phosphate and acceptors of phosphate. Measurement of oxygen uptake was started after an equilibration period of 10 min at a temperature of 25°.

ADP–ATP exchange reaction. The exchange using ADP-8-^{14}C was measured according to the technique described by Wadkins.[5] Mitochondria from 100 mg of rat liver were incubated with: 0·008 M ATP; 0·005 M ADP (18,000 counts/min); 0·05 M sucrose; 0·02 M Tris buffer, pH 7·4; and 0·05 ml of propylene glycol in a final volume of 0·5 ml. The reaction was stopped at the desired time by the addition of cold trichloroacetic acid to a final concentration of 6%.

Determination of ATP. ATP was chromatographed, eluted, determined, and counted according to the technique previously described.[1]

Adenosinetriphosphatase activity. This was determined as previously described at conditions for maximum stimulation of adenosinetriphosphatase activity by triamcinolone.[1] The reduction of the respiratory chain was carried out according to Chefurka.[6] The cyanide in these experiments was freshly prepared.

Inorganic phosphorus. This was determined according to Lowry and López.[7] However, in the adenosinetriphosphatase experiments in which the effect of KCN was assayed, the method of Fiske and SubbaRow as modified by Sumner[8] was used since KCN was found to interfere with the colorimetric determination of phosphorus in the method of Lowry and López.

Mitochondrial swelling. The decrease in optical density at 520 mμ was taken as a measure of mitochondrial swelling.[9, 10] This was determined in 0·125 M KCl, 0·02 M Tris (pH 7·4), and 0·1 ml of propylene glycol in a final volume of 3·0 ml; to this 0·04 ml of mitochondrial suspension was added. With triamcinolone the initial reading had to be extrapolated owing to the very rapid fall in optical density.

Reagents. All reagents were commercially obtained. Triamcinolone was kindly donated by Squibb & Sons de México, S.A.

Number of experiments. Each set of data reported was repeated at least three times, and all determinations were carried out in duplicate or triplicate.

RESULTS

Oxygen uptake

The effect of triamcinolone on the oxygen uptake by fresh and aged mitochondria was studied. In fresh mitochondria, triamcinolone produced a clear-cut increase in oxygen consumption with glutamate, α-ketoglutarate, and β-hydroxybutyrate as substrates (Fig. 1).

Triamcinolone under identical conditions but with aged mitochondria did not stimulate respiration with β-hydroxybutyrate as substrate (Fig. 2A). Mitochondria lost the sensitivity to triamcinolone after being stored for 24 hr at 0° to 2°.

Aging by itself conditioned an increase in mitochondrial respiration. It is to be noted that the increase in oxygen uptake produced by triamcinolone in fresh mitochondria is quantitatively the same as the respiration of aged mitochondria with or without triamcinolone (Fig. 2B).

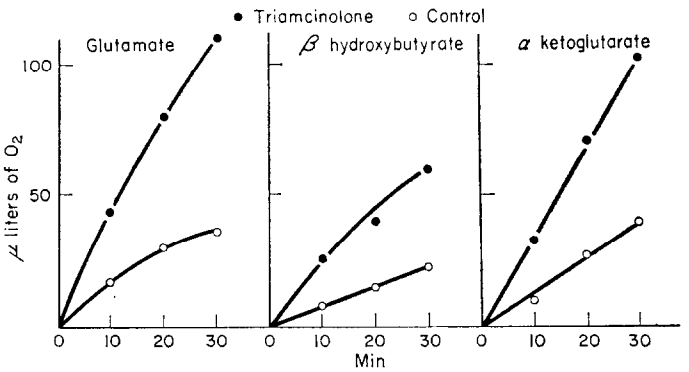

FIG. 1. Effect of triamcinolone on oxygen uptake by fresh mitochondria. Experimental conditions: 0·05 M KCl; 0·025 M Tris-HCl, pH 7·4; 0·008 M $MgCl_2$; 0·08 M sucrose; 0·01 M substrate; 0·1 ml propylene glycol; 4×10^{-4} M triamcinolone; mitochondria from 300 mg rat liver; final volume 3·0 ml; 0·2 ml of 50% KOH in center well; temperature 25°.

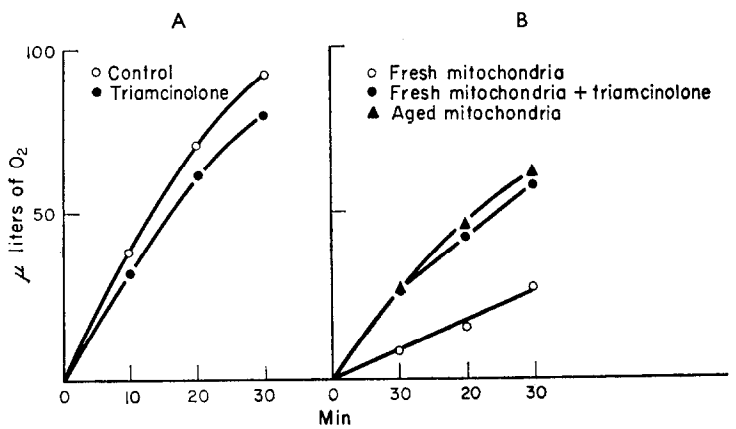

FIG. 2. Effect of triamcinolone on oxygen uptake by aged mitochondria. Experimental conditions: 0·05 M KCl; 0·025 M Tris-HCl, pH 7·4; 0·008 M $MgCl_2$; 0·08 M sucrose; 0·01 M β-hydroxybutyrate; 0·0005 M DPN; 0·1 ml propylene glycol; 4×10^{-4} M triamcinolone; mitochondria from 300 mg rat liver aged for 72 hr at 0° to 2°; final volume 3·0 ml; 0·2 ml of 50% KOH in center well; temperature 25°.

A: Respiration of aged mitchondria with and without triamcinolone. B: Respiration of aged mitochondria without triamcinolone and fresh mitochondria with and without 4×10^{-4} M triamcinolone. A and B are from different pools of mitochondria.

ADP–ATP exchange reaction

Triamcinolone depressed considerably the normal ADP–ATP exchange of fresh mitochondria (Fig. 3). There is a certain variability in the degree of inhibition, however (Table 1). The results obtained cannot be considered a reflection of a diminished amount of ATP because of the high activity of adenosinetriphosphatase under the

influence of triamcinolone;[1] more than 50% of the original ATP remained in the incubation mixture. Moreover, the results are expressed as counts per micromole of ATP remaining.

Upon aging of the mitochondria for 24 hr or more the inhibitory effect of the steroid on this reaction disappears, but the reaction itself diminishes to about 50% in 72 hr (Table 2). The resistance of the reaction to aging has been reported.[3]

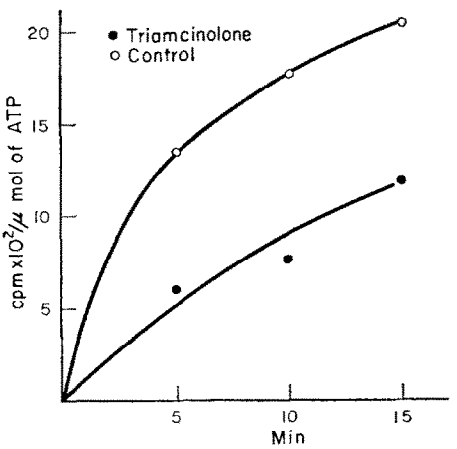

FIG. 3. Effect of triamcinolone on the ADP–ATP exchange by fresh mitochondria. Experimental conditions: 0·008 M ATP; 0·005 M ADP 8-^{14}C (18,000 counts/min); 0·05 M sucrose; 0·02 M Tris-HCl, pH 7·4; 0·1 ml propylene glycol; 4 × 10^{-4} M triamcinolone; mitochondria from 400 mg rat liver; final volume 2·0 ml; temperature 25°.

TABLE 1. EFFECT OF TRIAMCINOLONE ON THE ADP–ATP EXCHANGE BY FRESH MITOCHONDRIA

Control (counts × 10^2/μmole ATP)	Triamcinolone	Inhibition (%)
22·8	20·7	9
20·4	11·9	41
26·8	19·8	26
27·9	20·1	27
15·8	7·5	53
18·6	3·2	82
30·8	14·8	51

Experimental conditions: 0·008 M ATP; 0·005 M ADP-8-^{14}C (18,000 counts/min); 0·05 M sucrose; 0·02 M Tris-HCl, pH 7·4; 0·05 ml propylene glycol; 4 × 10^{-4} M triamcinolone; mitochondria from 100 mg rat liver; final volume 0·5 ml; temperature 25°; incubation time 15 min.

Adenosinetriphosphatase

Azide and oligomycin do not affect the latent adenosinetriphosphatase activity of mitochondria, but they are inhibitors of the DNP-stimulated adenosinetriphosphatase activity.[2, 3] If the hypothesis that triamcinolone and DNP have the same site of action were true, azide and oligomycin would block the stimulation of the adenosinetri-

phosphatase activity produced by triamcinolone. It was found that the increase in activity produced by triamcinolone was checked by azide and oligomycin (Table 3).

With these results, it can be supposed that the steroid could cause the rupture of two hypothetical high-energy intermediates. In order to determine which of the two intermediates was degraded, the effect of the reduction of the respiratory chain on the triamcinolone-stimulated adenosinetriphosphatase activity was studied (see Discussion).

TABLE 2. EFFECT OF TRIAMCINOLONE ON THE ADP–ATP EXCHANGE BY AGED MITOCHONDRIA

Aging (hr)	Control (counts × 10^2/μmole ATP)	Triamcinolone
0	26·8	19·8
24	22·2	22·0
48	16·7	17·4
72	13·0	14·5

Experimental conditions the same as for Table 1. Mitochondria were aged by storing in 0·25 M sucrose at 0° to 2° for the time indicated.

TABLE 3. EFFECT OF AZIDE AND OLIGOMYCIN ON THE TRIAMCINOLONE-STIMULATED ADENOSINETRIPHOSPHATASE ACTIVITY OF FRESH MITOCHONDRIA

Additions	Control (μmoles P formed in 20 min)	Triamcinolone
	1·7	7·8
10^{-3} M azide	2·3	1·3
	1·0	7·2
Oligomycin (μg/ml)	1·0	2·6

Azide and oligomycin were assayed with different pools of mitochondria. Experimental conditions: 0·05 M KCl; 0·02 M Tris-HCl, pH 7·4; 11 μmoles ATP; 0·08 M sucrose; 10^{-4} M triamcinolone; 0·1 ml propylene glycol; mitochondria from 200 mg rat liver; final volume 1·5 ml; temperature 25°

The first attempt to reduce the chain was made by incubating mitochondria with succinate, or β-hydroxybutyrate, or both; these substrates, alone or combined, did not affect the triamcinolone-stimulated adenosinetriphosphatase activity of mitochondria (Table 4).

However, addition of cyanide at high concentration inhibited the triamcinolone-stimulated adenosinetriphosphatase (Table 4). The combination of cyanide with succinate or β-hydroxybutyrate did not alter the pattern of inhibition obtained with cyanide alone (Table 4).

Mitochondrial swelling

It has been reported that triamcinolone inhibits mitochondrial swelling in a medium made of 0·1 M sucrose and 0·02 M Tris, pH 7·4;[1] similar effects in a sucrose medium

have been reported for DNP.[9] However, in the studies designed to measure any of the enzyme systems mentioned above, the mitochondrial suspensions incubated with triamcinolone became less turbid.

This observation suggested that triamcinolone produced alterations in the mitochondria. Thus, mitochondrial swelling was measured in a medium of KCl and Tris. Triamcinolone promoted swelling in a rather dramatic form (Fig. 4). Within the first minute the steroid caused a drastic fall of optical density, later the optical density stabilized. DNP in this respect differed from triamcinolone; the mitochondrial suspensions incubated with DNP behaved like the controls.

TABLE 4. THE EFFECT OF REDUCING THE RESPIRATORY CHAIN ON THE TRIAMCINOLONE-STIMULATED ADENOSINETRIPHOSPHATASE ACTIVITY OF FRESH MITOCHONDRIA

Experiment	Additions	Control	Triamcinolone
		(μmoles P formed in 20 min)	
		0·7	4·1
1	Succinate	1·1	3·7
	β-hydroxybutyrate	1·1	3·5
	Succinate + β-hydroxybutyrate	1·3	3·6
		0·5	6·1
	10^{-4} M KCN	0·6	6·4
2	10^{-3} M KCN	1·0	4·7
	10^{-2} M KCN	1·6	1·8
		0·5	5·2
3	10^{-2} M KCN	1·0	1·6
	10^{-2} M KCN + succinate	1·1	2·0
	10^{-2} M KCN + β-hydroxybutyrate	1·3	1·2

Experimental conditions: 0·05 M KCl; 0·02 M Tris-HCl, pH 7·4; 11 μmoles ATP; 0·08 M sucrose; 0·1 ml propylene glycol; 10^{-4} M triamcinolone; 10 μmoles succinate and/or β-hydroxybutyrate; mitochondria from 200 mg rat liver; final volume 1·5 ml; temperature 25°.

It was thought that the alterations produced by triamcinolone on the reactions related to oxidative phosphorylation could be secondary to changes in mitochondrial structure. Experiments in which swelling and adenosinetriphosphatase activity were measured simultaneously gave the results presented in Fig. 4. Swelling occurred almost instantaneously, while inorganic phosphate liberation appeared later on and continued when the optical density stabilized.

ATP at the concentrations used in these experiments did not protect or modify the response of the mitochondria to the swelling action of the steroid.

DISCUSSION

The purpose of the present work is to present certain characteristics on the uncoupling action of triamcinolone in rat liver mitochondria. It is probable that the pharmacological effects of triamcinolone are not related to the uncoupling action of this steroid; hydrocortisone and fluoromethyl-prednisolone administered to an animal

produce effects quite similar to those obtained with triamcinolone, yet the effects of these three steroids on mitochondrial respiration and oxidative phosphorylation are widely different.[1] Moreover, the concentration of steroid required to uncouple oxidative phosphorylation is much higher than the calculated physiological concentration in the whole animal.[11]

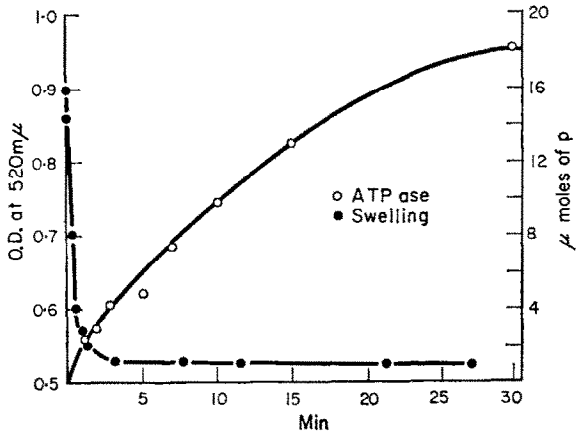

FIG. 4. Simultaneous measurement of mitochondrial swelling and adenosinetriphosphatase activity of fresh mitochondria under the influence of triamcinolone. Experimental conditions: The incubation media in the swelling experiments contained 0·125 M KCl; 0·02 M Tris–HCl, pH 7·4; 11μmoles ATP; 0·1 ml propylene glycol; 4×10^{-4} M triamcinolone; final volume 3·0 ml. For the measurement of adenosinetriphosphatase activity the incubating medium contained 0·125 M KCl; 0·02 M Tris, pH 7·4; 44 μmoles ATP; 4×10^{-4} M triamcinolone; 0·4 ml propylene glycol; 0·08 M sucrose; mitochondria from 800 mg rat liver; final volume 12 ml; temperature 25°.

Cooper and Lehninger have proposed the following scheme of oxidative phosphorylation.[12]

$$(1)\ AH_2 + C \rightleftarrows AH_2{-}C$$
$$(2)\ AH_2{-}C + B \rightleftarrows A \sim C + BH_2$$
$$(3)\ A \sim C + P \rightleftarrows P \sim C + A$$
$$(4)\ P \sim C + ADP \rightleftarrows ATP + C$$

A and B are two adjacent members of the respiratory chain. The energy liberated in the process of oxidation of A and reduction of B is conserved in the intermediate A ~ C. The adenosinetriphosphatase reaction stimulated by triamcinolone will be represented by reaction (5) preceded by reaction (4) and (3).

$$(4)\ ATP + C \rightleftarrows ADP + P \sim C$$
$$(3)\ P \sim C + A \rightleftarrows A \sim C + P$$
$$(5)\ P \sim C \xrightarrow{\text{triamcinolone}} P + C \text{ or}$$
$$A \sim C \xrightarrow{\text{triamcinolone}} A + C$$

Triamcinolone stimulates respiration in fresh mitochondria in media free of phosphate acceptors; it is thus logical to suppose that triamcinolone acts by rupture of an intermediate in the phosphorylation reactions. This could occur either before or after the entrance of inorganic phosphate. In a preceding study,[1] it was proposed that triamcinolone could cause the rupture of either P ~ C or A ~ C. The results presented in this paper suggest that the steroid uncouples oxidative phosphorylation by the degradation of A ~ C.

The ADP–ATP exchange is considered by many investigators in the field to be the terminal step of oxidative phosphorylation.[3, 5] This exchange is markedly inhibited by DNP, bis-hydroxycoumarin (Dicumarol), and gramicidin[5, 13] in intact mitochondria. The exchange itself is not affected to a large extent by aging the mitochondria for as long as 72 hr.[3] Triamcinolone in fresh mitochondria depresses this reaction, but the exchange itself is not affected by the steroid in aged mitochondria. This proves that triamcinolone does not act directly on this reaction, but rather on a reaction that takes place before the final transformation of ADP into ATP.

Triamcinolone, like DNP, inhibits the ^{32}P–ATP exchange reaction[1, 12]. This reaction, according to Cooper and Lehninger,[12] disappears upon aging of the mitochondria. In our hands, this reaction is inactivated by storing the mitochondria at 0° to 2° for 24 hr; simultaneously with this inactivation, the sensitivity of the ADP–ATP exchange and the respiration of mitochondria to triamcinolone disappear.

Azide and oligomycin, uncoupling agents of oxidative phosphorylation and inhibitors of DNP-stimulated adenosinetriphosphatase activity of mitochondria,[2, 3] prevent the stimulation by triamcinolone of the adenosinetriphosphatase activity of fresh mitochondria, although azide and oligomycin have no effect on this activity. The similarities in behavior of triamcinolone and DNP suggest that these two agents have a common site of action.

All the above data suggest that triamcinolone acts on either P ~ C or A ~ C as previously suggested.[1]

The reduction of the respiratory chain would not affect the triamcinolone-stimulated adenosinetriphosphatase reaction if P ~ C were the locus of action of triamcinolone, since the formation of this intermediate in the adenosinetriphosphatase reaction is independent of the redox state of the carriers; but this is not the case, since the results show that cyanide at high concentrations influences the high adenosinetriphosphatase activity produced by triamcinolone.

Upon reduction of the carriers, the formation of A ~ C is prevented, since A in the reduced form will not be available for the formation of A ~ C. These formulations would account for the resulting decrease in the stimulatory action of triamcinolone on the adenosinetriphosphatase reaction. Thus, A ~ C seems the most probable site of action of triamcinolone. On the same basis DNP would act on the same high-energy intermediate.[6]

Data presented in this paper together with results presented before[1] are consistent with the hypothesis that triamcinolone and DNP uncouple oxidative phosphorylation in the same place, but a definite proof cannot be offered. The different effects of triamcinolone and DNP on mitochondrial swelling could indicate either a different locus of activity or a different mechanism acting at the same site.

The chronological sequence of two events registered simultaneously—i.e. swelling and adenosinetriphosphatase activity—suggests that a structural alteration occurs

first and that this is followed by a modification of the activity of the adenosinetriphosphatase system. It cannot be concluded that the functional behavior of the mitochondria is secondary to the structural alteration, but this seems a very interesting hypothesis.

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