Journal of Steroid Biochemistry, 1974, Vol. 5, pp. 639–644. Pergamon Press. Printed in Great Britain

THE ACTIONS OF ANTI-INFLAMMATORY STEROIDS ON ISOLATED RAT LIVER MITOCHONDRIAL FUNCTION

A. M. SYMONS,* D. A. LEWIS† and R. J. ANCILL

Pharmacology Group, School of Pharmacy, University of Bath, Bath, Somerset, England

(*Received* 25 *January* 1974)

SUMMARY

The action of a number of natural and synthetic anti-inflammatory steroids on mitochondrial structure and function was found to be concentration dependent. Of the steroids tested most had little effect on mitochondrial respiration or oxidative phosphorylation at 10^{-5} M whereas at 10^{-3} M most had a stimulatory effect on State 4 respiration (substrate only, no ADP) and also inhibited oxidative phosphorylation as measured by a reduction in ADP/O ratios. This "uncoupling" action was shown to be due to steroid induced alterations in mitochondrial structure and was different from the action of the classic uncoupling agent 2,4-dinitrophenol (DNP). The significance of these results in relation to the mode of action of anti-inflammatory steroids is discussed.

INTRODUCTION

The uncoupling of oxidative phosphorylation is one of the many biochemical actions that has been considered to explain the anti-inflammatory effect of non-steroidal drugs. Adams and Cobb[1] were among the first to suggest a correlation between the anti-inflammatory anti-rheumatic activity of non-steroidal drugs and their capacity to uncouple oxidative phosphorylation. Much work on non-steroidal drugs has since been carried out by Whitehouse and associates [2–5]. Roche e Silva and Garcia Leme[6] review the situation with regard to non-steroidal drugs. Work with steroids and mitochondria includes that of Kerpolla[7], Kimberg, Loud and Weiner[8] and Smirnov, Plokhoj and Pushkina[9] who all report that steroids with anti-inflammatory properties uncoupled oxidative phosphorylation *in vivo*.

Goetsch and McDonald[10] showed a significant increase in oxygen uptake by rat liver homogenates from animals given a single injection of cortisol, prednisolone and 9α-fluoroprednisolone 5 h before sacrifice, whereas administration of prednisolone daily for 7 days caused a decrease in oxygen uptake in liver homogenates. Liljeroot and Hall[11] showed that cortisone increased P:O ratios in liver mitochondria isolated from adrenalectomized rats whereas triamcinolone had no apparent effect.

In vitro studies with mitochondria and steroids have been fewer than *in vivo* studies. Fluoromethylprednisolone has been reported [12, 13] not to affect respiration or oxidative phosphorylation in isolated rat liver mitochondria at a steroid concentration of 5×10^{-4} M. In contrast, cortisol at 6×10^{-4} M inhibited respiration but did not affect phosphorylation. Triamcinolone at 4×10^{-5} M was claimed to have increased respiration with NAD^+-dependent substrates and depressed oxidative phosphorylation. Its uncoupling action was equated with that of 2,4-dinitrophenol (DNP) although other workers have been unable to reproduce these results [14].

In view of these conflicting reports an investigation into the action of a range of natural and synthetic anti-inflammatory steroids on isolated rat liver mitochondria was made.

EXPERIMENTAL

Materials

The following steroids were gifts from pharmaceutical companies which we gratefully acknowledge. Betamethasone (Glaxo Laboratories Ltd.); dexamethasone (Organon Laboratories Ltd.); triamcinolone (Lederle Laboratories) and prednisolone stearoylglycolate (Carlo Erba, U.K. Ltd.). Other steroids were obtained from the Sigma Chemical Co. Ltd.

Isolation of rat liver mitochondria

Rat liver mitochondria were isolated using a modification of the method of Hogeboom, Schneider and

* Present address: Dept. of Biochemistry, University of Surrey, Guildford, Surrey, England.

† Present address: Dept. of Pharmacy, University of Aston, Birmingham, England.

Palade[15]. Male Sprague–Dawley rats (body weight 250–350 g), fed on Oxoid breeding diet and allowed water *ad libitum* were used. They were deprived of food 18 h before an experiment. The rats were killed by cervical dislocation and the livers removed, weighed and placed in ice cold "isolation medium" containing 0·25 M sucrose, 5 mM Tris chloride and mM ethylene glycol-bis-(beta-amino ethyl ether) N,N^1-tetra-acetic acid (EGTA) at pH 7·4. All subsequent procedures were at 4°C. Between 8–15 g liver was obtained from each animal.

The livers were chopped finely and 3·5 g portions homogenized with 25–30 ml isolation medium using a Tri-R homogenizer, model K41 (Tri-R Instruments Inc., Rockville Centre, New York). The pestle and mortar used were of Teflon and glass respectively with a clearance of 0·15–0·23 mm. It was found that two up and down strokes with the pestle at a speed setting of 10 (approx. 1000 rev./min) were sufficient to form an homogenous suspension. This initial suspension was made 10% (w/v) with the isolation medium and centrifuged at 750 $g_{av.}$ for 10 min. The supernatant was then centrifuged at 8500 $g_{av.}$ for 10 min to sediment a mitochondrial fraction, which was washed once and finally suspended in isolation medium (2 ml for every gram of liver) and used in the experimentation. The protein concentration of this suspension was determined [16] with bovine serum albumin as standard.

Effects of anti-inflammatory steroids on mitochondrial respiration and oxidative phosphorylation

Mitochondria respiration was measured polarographically using a modified Clark oxygen electrode (Rank Bros., Bottisham, Cambridge) maintained at 30°C and coupled to a Honeywell Brown Electronik recorder with a 10 mV full scale deflection. Each individual experiment was performed by injecting 2·5 ml of "respiratory medium" (pH 7·4) into the reaction vessel. This medium contained 0·25 M sucrose, 12 mM magnesium sulphate and 12 mM Sorensen's phosphate buffer. A portion (0·5 ml) of the mitochondrial suspension was then added and the contents allowed to equilibrate for 2 min. Additions of substrates as their sodium salts (pH 7·4) were then made, usually to a final concentration of 1·67 mM. After a steady respiratory rate had been established with one of the substrates for 1–2 min, additions of ADP, respiratory inhibitors, uncouplers and steroids were made in separate experiments and their effect on respiration recorded. Reference to different respiratory "states" and the calculation of ADP/O ratios is as defined by Chance and Williams[17].

Effect of anti-inflammatory steroids on mitochondrial volume

The effect of anti-inflammatory steroids on mitochondrial volume was examined by nephelometry. The decrease in extinction values at 520 nm was taken as a measure of mitochondrial swelling [18]. Suspensions of freshly prepared mitochondria were diluted with the "respiratory medium" in the same proportion as for the oxygen electrode experiments. Addition of succinate (final concentration 1·67 mM) and the respiratory inhibitor antimycin A (3·3 μg/ml) was made to a bulk stock of mitochondria. Addition of antimycin A was made to block respiratory activity ensuring that only "passive" changes in mitochondrial volume were being measured. 3 ml portions of the mitochondrial suspension were added to silica cuvettes maintained at 30°C in an SP 800 spectrophotometer (Pye Unicam Ltd.). After equilibration (2–3 min) additions of steroids, solvents or DNP were made with syringes, quickly mixed by stirring with a glass rod and the changes in $E_{520\,nm}$ recorded.

RESULTS

Effects of anti-inflammatory steroids on respiration and oxidative phosphorylation in intact rat liver mitochondria

The effect of various concentrations (10^{-5} to 10^{-3} M) of betamethasone, cortisone, cortisol, dexamethasone, prednisone, prednisolone, prednisolone stearoylglycolate and triamcinolone on State 4 succinate respiration and ADP/O ratios were determined. Table 1 shows the results. It can be seen that the steroids at 10^{-5} M had little or no effect on respiration or phosphorylation. As the concentration was increased to 10^{-3} M, however, most of the steroids tested increased the State 4 succinate rate to a greater extent than the control experiment with dimethyl sulphoxide (DMSO, 30 μl) which increased State 4 succinate respiration by 14·7 ± 2·3% in the series of experiments. Betamethasone and dexamethasone showed the most pronounced increase in respiration while prednisolone stearoylglycolate and triamcinolone showed very little, if any effect. ADP/O ratios were also unaffected by 10^{-5} M concentrations of the steroids, but decreased as the concentration was increased to 10^{-3} M (see Table 1). Again prednisolone stearoylglycolate and triamcinolone had no appreciable effect on these parameters.

The effect of 10^{-3} M dexamethasone on State 4 respiration and oxidative phosphorylation with other substrates was then determined. Table 2 shows the results obtained. With pyruvate + malate, α-ketoglutarate and succinate as respiratory substrates, dexa-

Table 1. Effect of anti-inflammatory steroids and 2,4-DNP on State 4 succinate respiration and ADP/O ratios in intact rat liver mitochondria

Steroid	Final Concn. (M)	Percentage increase in State 4 succinate respn.*	ADP/O ratio
Control (DMSO, 30 μl)	1·0%	14·7 ± 2·3 (19)	1·37 ± 0·13 (16)
Betamethasone	10^{-5}	16·3	1·20
	10^{-4}	27·5	1·03
	10^{-3}	52·1 ± 4·7 (6)	0·84 (3)
Cortisone	10^{-5}	15·4	1·24
	10^{-4}	19·7	1·04
	10^{-3}	21·3 ± 3·0 (6)	0·60 (3)
Cortisol	10^{-5}	16·9	1·53
	10^{-4}	20·4	1·32
	10^{-3}	25·1 ± 3·4 (6)	0·99 (3)
Dexamethasone	10^{-5}	18·3	1·30
	10^{-4}	30·3	1·09
	10^{-3}	58·3 ± 4·1 (6)	0·88 (3)
Prednisone	10^{-5}	13·4	1·04
	10^{-4}	21·0	0·83
	10^{-3}	26·3 ± 2·9 (6)	0·58 (3)
Prednisolone	10^{-5}	14·1	1·35
	10^{-4}	25·7	0·99
	10^{-3}	30·4 ± 2·8 (6)	0·70 (3)
Prednisolone stearoylglycolate	10^{-5}	14·9	1·09
	10^{-4}	14·7	1·09
	10^{-3}	17·6 ± 1·9 (6)	1·09 (3)
Triamcinolone	10^{-5}	15·3	1·36
	10^{-4}	15·0	1·25
	10^{-3}	16·3 ± 2·7 (6)	1·05 (3)
2,4-dinitrophenol	10^{-5}	149·8 (2)	0·46 (2)

Results are shown as means ± S.D. Number of experiments in parentheses.
*Control respiration was 44·6 ± 5·7 [19] nanogram atoms oxygen/min/mg mitochondrial protein.
All respiration results are expressed as nanogram atoms oxygen/min/mg mitochondrial protein.

Table 2. Effect of 10^{-3} M dexamethasone on respiration and oxidative phosphorylation with pyruvate and malate (both 1·67 mM), α-ketoglutarate, succinate and β-hydroxybutyrate (all 1·67 mM) as substrates

Substrate	State 4 respiration rate*	Rate with 10^{-3} M dexamethasone	Rate with ADP	ADP/O ratio
Pyruvate + malate	18·3	DMSO Control	62·7	1·20
		21·3		
Pyruvate + malate	18·1			
α-Ketoglutarate	12·5	27·6	40·2	0·45
		DMSO Control	55·2	2·17
		14·1		
α-Ketoglutarate	12·5	27·2	34·5	1·01
Succinate	41·2	DMSO Control	78·1	0·84
Succinate		44·3		
	37·0	59·6	59·6	00·0
β-Hydroxybutyrate	21·3	DMSO Control	42·6	1·79
		21·3		
β-Hydroxybutyrate	22·8	22·8	22·8	0·00

* All respiration rates are expressed as nanogram atoms oxygen consumed/min/mg mitochondrial protein.

Table 3. Effect of anti-inflammatory steroids on State 3 succinate respiration

Steroid	Final concn. of steroid (M)	Percentage inhibition of State 3 succinate respn.
None (DMSO control)	1%	4·5
Betamethasone	10^{-3}	35·3
Cortisone	10^{-3}	31·2
Cortisol	10^{-3}	41·2
Dexamethasone	10^{-3}	36·8
Prednisone	10^{-3}	37·0
Prednisolone	10^{-3}	40·0
Prednisolone stearoylglycolate	10^{-3}	21·8
Triamcinolone	10^{-3}	33·3

methasone (10^{-3} M) increased State 4 respiration, reduced the stimulatory response of added ADP and reduced the ADP/O ratios. With β-hydroxybutyrate as substrate, however, respiration was unaffected but phosphorylation was reduced to zero.

The effect of anti-inflammatory steroids on State 3 (ADP stimulated) succinate respiration was determined. Experimental procedure was as described, except in this series mitochondria were raised to the State 3 level of respiration (by the addition of 2 μmol ADP) before steroids were added. Table 3 shows the results. The respiration rates in the presence of the steroids have been expressed as a percentage of the State 3 respiration rate. It can be seen that all the steroids tested had a significant inhibitory effect on State 3 succinate respiration at concentrations of 10^{-4} M and above. At lower concentrations (10^{-5} M) the anti-inflammatory steroids tested had no effect. This State 3 respiration rate was not affected by additions of 10^{-5} M DNP, a concentration which stimulated State 4 respiration. This showed that the steroids had not merely reduced State 3 to State 4 respiration. It was also established that 10^{-5} M DNP had no effect on the ADP stimulated State 3 rate.

Effect of anti-inflammatory steroids on mitochondrial volume

The results shown in Table 4 have been expressed as the change in extinction at 520 nm/min/10 mg protein. The change in extinction over the 5 min experimental period was asymptotic and after 5 min no further significant change was noticed. The absolute values for the extinction at 520 nm of freshly prepared mitochondrial samples used for these studies was between 2·474 and 2·762 against distilled water as zero. It can be seen from Table 4 that the steroids tested (at 10^{-3} M), with the exception of triamcinolone, all caused a significant decrease (compared to the control) in the extinction at 520 nm. Lower concentrations (10^{-5} M) had very little effect. Additions of DNP to a final concentration of

Table 4. The effect of steroids and 2,4-DNP on mitochondrial swelling in the presence of 1·67 mM succinate and 3·3 μg/ml antimycin A.

Steroid	Final concn. (M)	Δ O.D. 520_{nm} min^{-1}/10 mg protein
None (DMSO control)	1%	−0·0015 (13)
None (H_2O control)	1%	No change (3)
Betamethasone	10^{-5}	−0·0021 (3)
Betamethasone	10^{-4}	−0·0035 (3)
Betamethasone	10^{-3}	−0·0067 (3)
Cortisol	10^{-5}	−0·0022 (2)
Cortisol	10^{-4}	−0·0028 (2)
Cortisol	10^{-3}	−0·0037 (2)
Dexamethasone	10^{-5}	−0·0022 (3)
Dexamethasone	10^{-4}	−0·0051 (3)
Dexamethasone	10^{-3}	−0·0084 (3)
Prednisolone	10^{-3}	−0·0026 (2)
Triamcinolone	10^{-3}	−0·0014 (3)
2,4-DNP	10^{-5}	+0·0015 (3)

The results shown are the mean of 2 or 3 experiments. Number of experiments is shown in parentheses. For experimental details see text.

10^{-5} M caused a slight increase in extinction at 520 nm suggesting slight shrinkage of the mitochondria. Buffa, Guaniera-Bobyleva, Muscatello and Pasquali-Rouchetti[19] using electron microscopy reported slight shrinkage in mitochondrial size after treatment with 10^{-5} M DNP.

Further experiments were conducted with the omission of succinate and antimycin A to establish whether the changes observed were a direct result of steroid action, rather than an influx of substrate. Results obtained were almost identical to those shown in Table 4 where succinate were present. This suggested that the structural changes taking place in the mitochondria were a direct action of the steroids and not "swelling" induced by substrate movements.

DISCUSSION

The action of anti-inflammatory steroids on State 4 succinate respiration rates and oxidative phosphorylation in isolated rat liver mitochondria is concentration dependent. Concentrations of 10^{-5} M had very little effect whereas at 10^{-3} M most of the steroids tested had a slight stimulatory effect on State 4 respiration and an inhibitory effect on oxidative phosphorylation, as measured by a decrease in ADP/O ratios. Betamethasone and dexamethasone at 10^{-3} M caused the greatest increase in State 4 succinate respiration rates (52·1 and 58·3% respectively) but this increase was comparatively low compared to the 149·8% increase caused by a 10^{-5} M concentration of the classic uncoupler DNP.

Dexamethasone at 10^{-3} M was also tested with pyruvate + malate, α-ketoglutarate and β-hydroxybutyrate as substrates (see Table 2). State 3 respiration rates were again increased with the exception of β-hydroxybutyrate, and ADP/O ratios were reduced in all cases, in some instances to zero. It is of interest that evidence has been presented [20] which shows that β-hydroxybutyrate is stored in a mitochondrial compartment operationally distinct from the space containing other substrates, and that the changes in various substrate oxidation rates with freezing and thawing a mitochondrial preparation, closely parallel the effects of the anti-inflammatory steroids on State 4 respiration rates [21].

The experiments designed to study the changes in mitochondrial volume showed that the anti-inflammatory steroids tested significantly altered the membrane configuration at 10^{-3} M. It is therefore concluded that anti-inflammatory steroids are capable of uncoupling oxidative phosphorylation in isolated rat liver mitochondria. The mode of uncoupling action of steroids is different to that of DNP and could be described as "physical uncoupling" rather than "chemical uncoupling" as may be the case with the DNP [22].

The concentration of anti-inflammatory steroid needed to inhibit ATP production by a significant degree is above 10^{-4} M. This would seem higher than concentrations likely to exist in therapy although little is known of the local concentration of anti-inflammatory drugs or their metabolites which may exist in inflammatory conditions. In other work [23] we have shown that lysosomes concentrate steroids from solution. Although the mode of action of the steroids on mitochondrial function has been shown to involve structural changes, other actions are undoubtedly taking place as well, and it is difficult to evaluate these results to the action of steroids in therapy in inflammatory conditions.

Anti-inflammatory steroids have a biphasic action on lysosomal enzyme release [24]. When considered with the results of this paper, it appears that concentrations of anti-inflammatory steroids that uncoupled oxidative phosphorylation *in vitro* had a lytic effect on lysosomes and concentrations that stabilized lysosomes *in vitro* (a possible mode of action of the drugs) did not uncouple oxidative phosphorylation.

It would therefore seem that the uncoupling of oxidative phosphorylation is probably not significant in considering the mode of action of anti-inflammatory steroids. This conclusion is, however, from *in vitro* studies only and its extention to *in vivo* situations must remain tentative.

REFERENCES

1. Adams S. S. and Cobb R.: *Nature* **181** (1958) 773–774.
2. Whitehouse M. W.: *Biochem. Pharmac.* **13** (1964) 319–336.
3. Whitehouse M. W. and Dean P. D. G.: *Biochem. Pharmac.* **14** (1965) 557–567.
4. Skidmore I. F. and Whitehouse M. W.: *Biochem. Pharmac.* **14** (1965) 547–555.
5. Whitehouse M. W.: *Biochem. Pharmac.* Special supplement March (1968) 293–307.
6. Roche e Silva M. and Garcia Leme J.: *Chemical Mediators of the Acute Inflammatory Reaction.* Pergamon Press, Oxford (1972).
7. Kerpolla W.: *Endocrinology* **67** (1960) 252–263.
8. Kimberg D. V., Loud A. V. and Weiner J.: *J. Cell Biol.* **37** (1968) 63–79.
9. Smirnov M. I., Plokhoj V. I. and Pushkina L. A.: *Vop. med. Khim.* **14** (1968) 85–89.
10. Goetsch D. D. and McDonald L. E.: *Am. J. Physiol.* **202** (1962) 343–346.
11. Liljeroot B. S. and Hall J. C.: *J. biol. Chem.* **240** (1965) 1446–1452.
12. Gomez-Puyou A., Pena-Dias A., Guzman-Garcia J. and Laguna J.: *Biochem. Pharmac.* **12** (1963) 331–340.
13. Gomez-Puyou A., Tuena A., Guzman-Garcia J. and Pena J.: *Biochem. Pharmac.* **12** (1963) 669–677.
14. Ogata E., Aiba N., Suzuki H. and Yoshitoshi Y.: *Biochem. Pharmac.* **17** (1968) 835–838.
15. Hogeboom G. H., Schneider W. G. and Palade G. H.: *J. biol. Chem.* **172** (1948) 619–635.

16. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: *J. biol. Chem.* **193** (1951) 265–276.
17. Chance B. and Williams G. R.: *Adv. Enzymol.* **17** (1956) 65–134.
18. Chappell J. B. and Greville G. D.: *Nature* **183** (1959) 1737–1738.
19. Buffa P., Guaniera-Bobyleva V., Muscatello U. and Pasquali-Rouchetti I.: *Nature* **226** (1970) 272–274.
20. Harris E. J. and Manger J. R.: *Biochem. J.* **113** (1969) 617–628.
21. Symons A. M.: *Some sub-cellular actions of anti-inflammatory steroids.* Ph.D. Thesis (1971) University of Bath.
22. Griffiths D. E.: In *Essays in Biochemistry.* Vol. 1. (Eds. P. N. Campbell and G. D. Greville). Academic Press. London 91–120.
23. Symons A. M., Lewis D. A. and Ancill R. J.: *J. Pharm. Pharmac.* **22** (1970) 944–945.
24. Lewis D. A., Symons A. M. and Ancill R. J.: *J. Pharm. Pharmac.* **22** (1970) 902–908.