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THE MECHANISM OF INHIBITION OF MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION BY THE NON-STEROIDAL ANTI-INFLAMMATORY AGENT DIFLUNISAL

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Abstract—The anti-inflammatory agent diflunisal was found to induce a progressive loss of respiratory control in tightly coupled rat liver mitochondria, starting at low concentrations (3.3 μM). This loss of control was accompanied by a stimulation of state 4 respiration in the presence of either succinate or glutamate plus malate as the respiratory substrate. The inhibition of state 3 respiration by oligomycin was released by diflunisal. Mitochondrial ATP hydrolysis was stimulated by diflunisal over the same concentration range that affected state 4 respiration: the stimulation was inhibited by oligomycin. It was concluded that diflunisal was acting as an uncoupler of mitochondrial oxidative phosphorylation. An identical action was found in mitochondria isolated from the livers of mice, rabbits and guinea-pigs. Potencies similar to diflunisal were found with flufenamic acid and mefenamic acid, but other anti-inflammatory agents were either less potent or inactive.

Acetylsalicylic acid and related salicylates have been shown to interfere with mitochondrial ATP synthesis by an uncoupling action on oxidative phosphorylation [1–3]. The uncoupling effect is only observed at relatively high concentrations, and therefore is unlikely to be related to the anti-inflammatory or analgesic properties of these compounds. The introduction in recent years of other non-steroidal anti-inflammatory agents with greater pharmacological potencies than aspirin [4–6] has prompted us to investigate their effects on mitochondrial energy transfer reactions. The aim of the study was to answer the following questions. (1) Do diflunisal and related compounds affect mitochondrial energy transfer reactions? (2) How do the potencies of diflunisal and related compounds against the sensitive reactions compare with the potency of acetylsalicylic acid? (3) By what mechanism does diflunisal affect mitochondrial energy transfer reactions? (4) Is there any species variation in the effects? A preliminary account of the actions of diflunisal has been presented to the British Pharmacological Society [7].

MATERIALS AND METHODS

Mitochondria. Tightly coupled mitochondria were obtained from the livers of rats, mice, rabbits and guinea-pigs by the method of Chappell and Hansford [8].

Enzyme activities. Oxygen consumption was measured polarographically using a Clark-type oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.). ATPase activity was measured as described by Beechey [9], the inorganic phosphate released being determined by the method of Fiske and Subbarow [10]. EC_{50} values (concentrations required to stimulate reactions by 50%) were derived from experiments using a full range of concentrations of the anti-inflammatory agent. The specific conditions employed in the measurement of reactions are described in the legends to the appropriate figures and tables.

Protein. Protein was determined by the method of Gornall *et al.* [11], after solubilization of the mitochondrial pellet with sodium deoxycholate (0.16% w/v); bovine serum albumin was used as standard.

Chemicals. Analytical grade laboratory chemicals and biochemicals were purchased from British Drug Houses Ltd. (Poole, U.K.) and Sigma Chemical Co. (St. Louis, MO). Diflunisal was provided by Merck Sharp & Dohme Ltd. (Hoddleston, Herts., U.K.) and flufenamic and mefenamic acids by Parke Davis Ltd. (Pontepoole, U.K.). Diflunisal and other insoluble anti-inflammatory agents were added to the reaction media as solutions in dimethylformamide; controls carried out with equivalent amounts of solvent showed that it had no effect on the reactions under consideration.

RESULTS

Respiratory control

In tightly coupled mitochondrial preparations, measurement of the respiratory control index provides a means of determining the extent of coupling of electron transport to phosphorylation of ADP. In the absence of ADP, but with substrate and oxygen in excess, the rate of respiration is relatively low (state 4). Addition of a limiting amount of ADP (0.2 μmole) results in a stimulation of respiration

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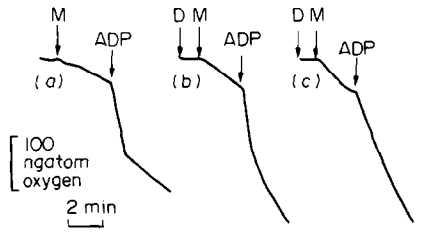

Fig. 1. Effect of diflunisal on respiratory control in rat liver mitochondria. The oxygen electrode chamber contained 675 μmole sucrose, 9.2 μmole Tris–HCl buffer, pH 7.4, 10 μmole potassium phosphate buffer, pH 7.4, and 15 μmole sodium succinate. Additions were made as follows: (M) rat liver mitochondria (10 mg protein); (ADP) 0.2 μmole ADP; (D) diflunisal. The temperature was 30° and the reaction volume was 3 ml. (a) Control response to ADP; (b) effect of 3.3 μM diflunisal; (c) effect of 17 μM diflunisal.

(state 3), which returns to a level close to its original value (state 4) when all the ADP has been converted to ATP. The respiratory control index is the ratio of state 3 to state 4 (after exhaustion of ADP). In control rat liver mitochondrial preparations the respiratory control index was 4.5 ± 0.2 (n = 25) when glutamate plus malate was used as substrate, indicating that the mitochondria were tightly coupled. Increasing concentrations of diflunisal caused a progressive loss of respiratory control; for example, 3.3 μM diflunisal gave a respiratory control index of 2.24 ± 0.2 (n = 5), and 17 μM diflunisal an index of 1.4 ± 0.1 (n = 5) (see Fig. 1). Since this effect is indicative of an uncoupling action, further experiments were designed to investigate the possibility that diflunisal was uncoupling mitochondrial oxidative phosphorylation.

Substrate oxidation

Table 1 shows the effect of diflunisal on the oxidation of two substrates, succinate and glutamate plus malate, by mitochondrial preparations derived from rat liver. There was a progressive stimulation of state 4 respiration, with the maximal effect occurring at diflunisal concentrations between 33 and 70 μM. Above these concentrations, both state 3 and state 4 respiration rates were inhibited by diflunisal. EC_{50} values for stimulation of respiration were: 14.1 ± 3.5 μM (succinate) and 5.9 ± 1.2 μM (glutamate plus malate) (n = 5 in both cases). Similar results were obtained with the uncoupling agent 2,4-dinitrophenol (EC_{50} = 10.5 ± 1.2 μM for succinate oxidation; n = 5).

Diflunisal released oligomycin-inhibited state 3 respiration (Table 2), an effect that is characteristic of uncoupling agents [12].

ATP hydrolysis

Diflunisal stimulated rat liver mitochondrial ATPase (EC3.6.1.4) over the same concentration range that produced stimulation of state 4 respiration (Fig. 2). The EC_{50} for the stimulatory action on the ATPase was 10.5 ± 3.1 μM. At concentrations of diflunisal above 50 μM a concentration-dependent inhibition of the stimulated rate was observed. The diflunisal-stimulated ATPase was sensitive to oligomycin (Fig. 2).

Anti-inflammatory agents

A survey was made of the action of a number of anti-inflammatory compounds on state 4 respiration and ATP hydrolysis in mitocondria isolated from rat liver. On the basis of the results obtained, the compounds could be assigned to three distinct groups. (1) A number of derivatives had no activity at concentrations up to 5 mM. These included indomethacin, itaprophenic acid, propoxyphene hydrochloride, propoxyphene napsylate and sulindac. (2) An intermediate group, active over the concentration range 0.5–5 mM, comprised the salicylates: acetylsalicylic acid, methylsalicylic acid, sodium salicylate and salicylic acid. (3) The most active group affected the enzyme activities over the concentration range 1–100 μM, and consisted of mefenamic acid, flufenamic acid and diflunisal. The EC_{50} values for these three compounds are given in Table 3, which includes

Table 1. Effect of diflunisal on substrate oxidation in rat liver mitochondria

Diflunisal (μM)	Oxygen consumption (ngatom/min per mg mitochondrial protein)			
	Succinate		Glutamate plus malate	
	State 3	State 4	State 3	State 4
Control	59.6 ± 4.2	20.4 ± 1.8	43.1 ± 6.2	11.3 ± 1.0
3.3	56.4 ± 4.6	24.0 ± 2.2	40.8 ± 4.2	18.2 ± 1.5
7	52.4 ± 5.2	26.5 ± 2.5	37.4 ± 4.0	22.5 ± 1.8
17	45.5 ± 4.2	30.0 ± 2.7	36.3 ± 5.0	26.2 ± 3.3
33.3	40.3 ± 4.8	35.1 ± 2.5	28.5 ± 4.0	30.6 ± 3.4
70	32.3 ± 4.9	42.0 ± 3.1	26.3 ± 3.9	22.3 ± 2.9
117	26.2 ± 3.6	38.2 ± 3.5	18.4 ± 4.0	15.4 ± 3.5
170	17.8 ± 3.2	24.1 ± 2.9	15.3 ± 3.7	10.7 ± 3.3

The reaction conditions were as described in the legend to Fig. 1, except that sodium succinate was replaced with 15 μmole sodium glutamate plus 15 μmole sodium malate where indicated. Diflunisal was added to the reaction medium at the start of each experiment, at the concentrations shown. Values are the mean ± S.E. mean of five different experiments.

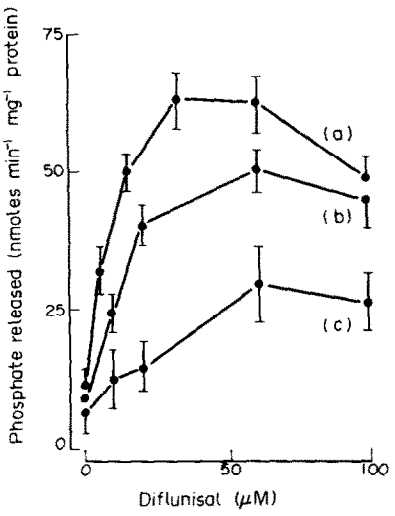

Fig. 2. Effect of diflunisal on rat liver mitochondrial adenosine triphosphatase in the presence and absence of oligomycin. At zero time, 0.1 ml mitochondrial suspension (2 mg protein) was added to 0.9 ml reaction medium containing 125 μmole sucrose, 63 μmole Tris–HCl buffer, pH 7.4, and 2.5 μmole ATP. Diflunisal was included in the reaction medium at the concentrations shown. After 10 min incubation at 30°, the reaction was stopped by the addition of 0.1 ml 20% (w/v) trichloroacetic acid, and the inorganic phosphate liberated was estimated by the method of Fiske and Subbarow [10]. (a) Effect of range of concentrations of diflunisal; (b) effect of range of concentrations of diflunisal in the presence of oligomycin (1 μg); (c) effect of range of concentrations of diflunisal in the presence of oligomycin (5 μg). Bars are the mean ± S.E. mean of five different experiments.

the values obtained for acetylsalicylic acid and 2,4-dinitrophenol for comparison.

Species comparison

The three most active compounds, diflunisal, flufenamic acid and mefenamic acid, were examined for their effects on state 4 respiration in mitochondria isolated from four mammalian species (rat, mouse, rabbit and guinea-pig). The pattern of activities found with each compound was essentially the same in all four species (Table 4).

DISCUSSION

Several lines of evidence suggest that diflunisal inhibits mitochondrial ATP synthesis by uncoupling oxidative phosphorylation. At low concentrations (<70 μM) diflunisal caused a loss of respiratory control, stimulated state 4 respiration and released the inhibition of state 3 respiration brought about by oligomycin. The mitochondrial ATPase was stimulated by diflunisal over the same concentration range that affected the respiratory activities, and the diflunisal-stimulated ATPase was sensitive to oligomycin. At high concentrations of diflunisal (>70 μM) both the stimulation of state 4 respiration and the stimulation of the ATPase were inhibited by diflunisal. Since all these actions are characteristic of uncoupling agents, it is proposed that diflunisal can be included in this category.

Because some aspirin-like compounds have been shown to interfere with mitochondrial energy production [3, 13, 14], one aspect of the present study was to compare the activity of diflunisal with other

Table 2. Effect of diflunisal on substrate oxidation in the presence of oligomycin

Additions	Oxygen consumption (ngatom/min per mg mitochondrial protein)
Control	11.3 ± 1
ADP	43.1 ± 6.2
Diflunisal (17 μM)	26.2 ± 3.3
ADP plus oligomycin (1 μg/ml)	12.0 ± 1.8
ADP plus oligomycin (1 μg/ml) plus diflunisal (17 μM)	29.2 ± 2.4

The reaction conditions were as described in the legend to Fig. 1, except that sodium succinate was replaced with 15 μmole sodium glutamate plus 15 μmole sodium malate. Values are the mean ± S.E. mean of five different experiments.

Table 3. Effect of anti-inflammatory agents on mitochondrial enzyme activities

Drug	State 4 succinate oxidation (EC_{50}, μM)	ATP hydrolysis (EC_{50}, μM)
Diflunisal	14.1 ± 3.5	10.5 ± 3.1
Mefenamic acid	38.6 ± 4.2	31.4 ± 5.0
Flufenamic acid	8.4 ± 2.1	7.8 ± 3.8
Acetylsalicylic acid	2100 ± 800	1850 ± 300
2,4-Dinitrophenol	10.5 ± 1.2	7.5 ± 0.1

Succinate oxidation was measured as described in the legend to Fig. 1, and ATP hydrolysis as described in the legend to Fig. 2. Values are the mean ± S.E. mean of five different experiments.

Table 4. Effect of anti-inflammatory agents on substrate oxidation in mitochondria isolated from four species

Substrate and drug	EC_{50} values (μM)			
	Rat	Mouse	Rabbit	Guinea-pig
(a) Succinate				
Diflunisal	14.1 ± 3.5(5)	6.1 ± 1.9(5)	9.6 ± 2.1(5)	10.8 ± 1.7(5)
Flufenamic acid	8.4 ± 2.1(5)	6.3 ± 1.2(5)	5.6 ± 1.2(5)	7.3 ± 1.4(5)
Mefenamic acid	38.6 ± 4.2(5)	33.5 ± 5.6(3)	34.9 ± 6.1(3)	36.4 ± 5.2(3)
(b) Glutamate plus malate				
Diflunisal	5.9 ± 1.1(5)	6.1 ± 1.4(5)	6.6 ± 1.3(5)	7.5 ± 1.7(5)
Flufenamic acid	12.7 ± 3.1(5)	11.8 ± 2.2(5)	7.7 ± 1.9(5)	14.3 ± 3.2(5)
Mefenamic acid	20.7 ± 3.7(5)	21.4 ± 4.5(3)	23.1 ± 4.9(3)	26.3 ± 3.4(3)

Reaction conditions were as described in the legends to Fig. 1 and Table 1. Results are the mean ± S.E. mean with the number of experiments shown in parentheses.

anti-inflammatory agents. Many compounds, including indomethacin, had no uncoupling activity in our experiments, and the salicylates tested had only moderate activity. The most active compounds were mefenamic acid, flufenamic acid and diflunisal. These agents were between 50 and 100 times more potent than acetylsalicylic acid. Uncoupling activity was not, therefore, a common property amongst this series of compounds, indicating that this effect would be unlikely to contribute to the anti-inflammatory actions of the compounds. However, it remains to be established whether the uncoupling action of the most potent compounds is relevant to their *in vivo* actions, since extrapolation to anti-inflammatory effects cannot be based on experiments with liver alone.

As a first stage in determining the relevance of the uncoupling activity, a survey was made of the effect of the most potent agents on substrate oxidation by mitochondria isolated from four species. This showed that the compounds were almost equally active in all four species examined. Thus, the EC_{50} values for diflunisal ranged from 5.9 to 14.1 μM, for flufenamic acid from 5.6 to 14.3 μM, and for mefenamic acid from 21.4 to 38.6 μM, indicating that all four species would be susceptible to the uncoupling action of these compounds at relatively low doses.

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