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Diphenylamine as an Important Structure of Nonsteroidal Anti-inflammatory Drugs to Uncouple Mitochondrial Oxidative Phosphorylation

*Yasuhiro Masubuchi, Shoko Yamada and Toshiharu Horie**

LABORATORY OF BIOPHARMACEUTICS, FACULTY OF PHARMACEUTICAL SCIENCES, CHIBA UNIVERSITY, CHIBA 263-8522, JAPAN

ABSTRACT. A marked difference has been observed in the inhibitory effects of nonsteroidal anti-inflammatory drugs (NSAIDs) on oxidative phosphorylation of rat liver mitochondria. It should be noted that some of the potent inhibitors, *N*-phenylanthranilic acids and diclofenac, have a similar "skeleton" structure, diphenylamine. Diphenylamine itself was found to inhibit oxidative phosphorylation significantly, although its inhibition potency was weaker than that of NSAIDs with a diphenylamine structure. In addition to decreases in the respiration control index (ratio of state 3 to state 4 respiration), these compounds released oligomycin-inhibited state 3 respiration. These results demonstrated that diphenylamine, as well as *N*-phenylanthranilic acids and diclofenac, was an uncoupler of oxidative phosphorylation of rat liver mitochondria. Thus, diphenylamine was suggested to play an important role in the uncoupling effects of NSAIDs with a diphenylamine skeleton. BIOCHEM PHARMACOL **58**;5:861–865, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. oxidative phosphorylation; uncoupler; mitochondria; nonsteroidal anti-inflammatory drugs; diphenylamine

Some NSAIDs† such as mefenamic acid, flufenamic acid, and diflunisal have an uncoupling effect on oxidative phosphorylation in isolated rat mitochondria [1–3]. Salicylic acid and acetylsalicylic acid also have it [4, 5], but the effect occurs only at a higher concentration than for the other NSAIDs mentioned above [2, 3]. It has been demonstrated recently that diclofenac has an uncoupling effect on mitochondrial oxidative phosphorylation [6–8]. The uncoupling effect seems to be a common property of the NSAIDs, although its potency differs among the compounds.

Among structurally similar NSAIDs, lipophilicity, acidity, and some substituted groups play important roles in the uncoupling effect, as shown in analogues of *N*-phenylanthranilic acids [9, 10]. However, it is difficult to explain the difference in uncoupling potency among the different types of NSAIDs by physico-chemical properties alone, since the classified NSAIDs are in some cases structurally unrelated. The present paper describes one of the specific structures of the NSAIDs, which behaves as a potent uncoupler of mitochondrial oxidative phosphorylation.

* Corresponding author: Toshiharu Horie, Ph.D., Laboratory of Biopharmaceutics, Faculty of Pharmaceutical Sciences, Chiba University, 1–33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan. Tel. (81) 43-290-2934; FAX (81) 43-290-3021; E-mail: horieto@p.chiba-u.ac.jp

† *Abbreviations*: NSAIDs, nonsteroidal anti-inflammatory drugs; and MPT, mitochondrial permeability transition.

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MATERIALS AND METHODS

Chemicals

Salicylic acid, mefenamic acid, diclofenac sodium, indomethacin, diphenylamine, and cyclosporin A were purchased from Wako Pure Chemical Industries. Diflunisal, tolfenamic acid, flufenamic acid, naproxen sodium, fenoprofen, oligomycin, and BSA (fraction V) were obtained from the Sigma Chemical Co. ADP sodium salt was purchased from the Oriental Yeast Co., Ltd. All other chemicals and solvents were of analytical grade.

Preparation of Liver Mitochondria

Male Wistar rats (2 months old) were obtained from Takasugi Experimental Animals. The animals were housed in air-conditioned rooms (25°) under a 12-hr light–dark cycle for 1 week prior to use. Food (commercially available pellets from the Oriental Yeast Co., Ltd.) and water were given *ad lib*. Liver mitochondrial fraction was prepared according to the method described by Schneider and Hogeboom [11] in a medium containing 0.25 M sucrose, 10 mM Tris–HCl buffer (pH 7.4), and 0.5 mM EDTA. In some experiments, the medium contained 5 mg/mL of BSA or 0.5 mM EGTA.

Measurement of Respiration Rates

The rates of oxygen consumption were measured polarographically with a Clark-type oxygen electrode (model

TABLE 1. Effects of NSAIDs on respiratory control in rat liver mitochondria

Additions	Oxygen consumption (nmol/min/mg protein) State 3	State 4	RC
Control	63.3 ± 2.28	11.0 ± 0.35	5.8
Salicylic acid	61.5 ± 2.07	11.5 ± 0.65	5.4
Diflunisal	40.9 ± 7.20*	29.7 ± 6.26	1.4
Mefenamic acid	44.6 ± 8.85	17.9 ± 0.86*	2.5
Tolfenamic acid	21.7 ± 1.12*	15.5 ± 1.33	1.6
Flufenamic acid	23.0 ± 3.61*	18.5 ± 0.77*	1.3
Diclofenac	42.0 ± 4.51	12.9 ± 1.39	3.3
Indomethacin	41.2 ± 2.85*	15.3 ± 1.06*	3.7
Naproxen	40.2 ± 2.84*	8.4 ± 0.36*	4.9
Fenoprofen	46.5 ± 5.14	10.4 ± 1.04	4.9

Mitochondria (1 mg protein/mL) were incubated in 1.6 mL of respiration buffer, containing succinate (5 mM) as a substrate at 30°. State 3 and state 4 respirations were measured after the addition of each NSAID (10 μM), as described in Materials and Methods. The respiration control index (RC) was calculated as the ratio of state 3/state 4 respiration. Results are means ± SEM of 3 different experiments.

*Significantly different from control ($P < 0.05$).

GU-BMP; Iijima Electronics Mfg. Co., Ltd.). Respiration buffer (pH 7.4) contained 225 mM sucrose, 10 mM KCl, 5 mM $MgCl_2$, 5 mM potassium phosphate, 0.5 mM EDTA, and 20 mM Tris–HCl. In some experiments, 0.5 mg/mL of BSA, 700 nM cyclosporin A, or 0.5 mM EGTA was added to the respiration buffer. Mitochondria (1 mg protein/mL) were preincubated at 30° in 1.6 mL of respiration buffer, containing succinate (5 mM) or glutamate (4 mM) plus malate (4 mM) as respiration substrates. State 3 and state 4 respiration rates were measured after the addition of each test compound in the presence (state 3) and after exhaustion (state 4) of ADP (87.5 μM). The respiratory control index was calculated as the ratio of state 3 to state 4 respiration [12].

Other Methods

Protein concentrations were assayed by the method of Lowry *et al.* [13]. Statistical significance was calculated by Student's *t*-test.

RESULTS

Table 1 shows the effects of various NSAIDs on oxygen consumption by rat liver mitochondria with succinate as a substrate. As previously reported [3], diflunisal stimulated basal oxygen consumption (state 4). In contrast, when mitochondrial respiration was stimulated with ADP (state 3), the response was inhibited by diflunisal. Stimulation of state 4 respiration and suppression of state 3 respiration are typical characteristics of uncouplers of mitochondrial oxidative phosphorylation. The addition of BSA to remove free fatty acids, which uncouple oxidative phosphorylation at low concentrations [14], from the reaction medium had no significant effect on oxygen consumption (control, state 3, 61.4 ± 3.33 nmol/min/mg protein, state 4, 14.9 ± 0.46 nmol/min/mg protein; BSA, state 3, 59.7 ± 2.43 nmol/min/mg protein, state 4, 14.1 ± 0.59 nmol/min/mg protein, mean ± SEM of 3 different experiments), indicating that the amounts of free fatty acids contained in the medium were not sufficient to influence the respiration rate. Stimulation of state 4 respiration and suppression of state 3 respiration also were induced by the three *N*-phenylanthranilic acids used: mefenamic acid, tolfenamic acid, and flufenamic acid. Based on the respiration control index, a sensitive parameter of mitochondrial dysfunction, these compounds were demonstrated to be potent inhibitors of mitochondrial function. Diclofenac and indomethacin also caused both inhibition of state 3 and stimulation of state 4, but the changes were ambiguous under the conditions used, as judged from the respiration control index.

Figure 1 shows the concentration-dependent effects of mefenamic acid, diclofenac, and diphenylamine on mitochondrial oxygen consumption energized with succinate. These compounds stimulated basal oxygen consumption (state 4) in a concentration-dependent manner (Fig. 1B). The maximal effects of all the compounds were about 2-fold of the control value. The drug concentrations required for the maximal stimulatory effects ranged within 10–100 μM and ranked as mefenamic acid < diclofenac < diphenylamine. In contrast, mitochondrial respiration stimulated with ADP (state 3) was inhibited by the compounds in a concentration-dependent manner (Fig. 1A). The drug con-

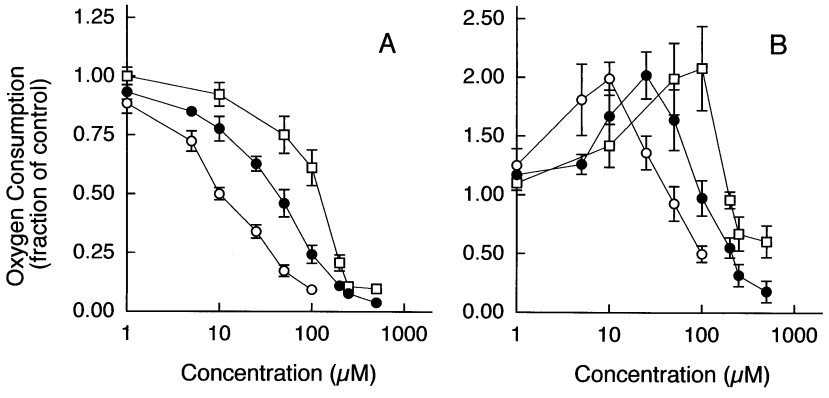

FIG. 1. Concentration dependency for the effects of mefenamic acid, diclofenac, and diphenylamine on respiratory control in rat liver mitochondria using succinate as a substrate. The conditions of the incubation were described in Table 1. State 3 (A) and state 4 (B) respirations were measured in the presence of various concentrations of mefenamic acid (○), diclofenac (●), and diphenylamine (□). Results are presented as fractions of the control and are means ± SEM of 3 different experiments. Control values obtained without drug were state 3, 58.9 ± 2.51; and state 4, 11.6 ± 1.38 nmol/min/mg protein.

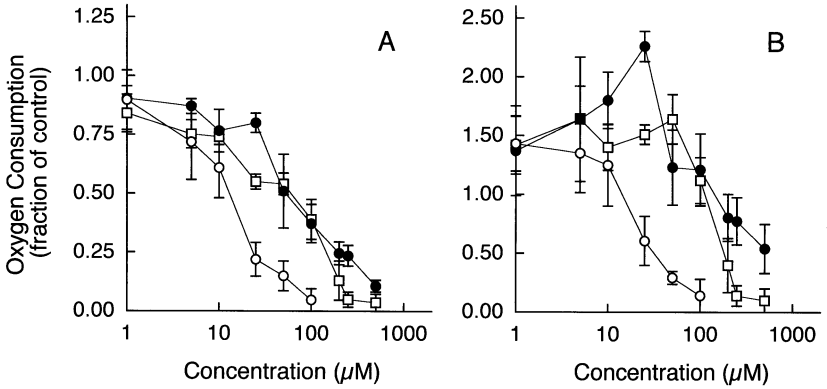

FIG. 2. Concentration dependency of the effects of mefenamic acid, diclofenac, and diphenylamine on respiratory control in rat liver mitochondria using glutamate plus malate as substrates. The conditions of the incubation were described in Table 1, except that succinate as a substrate was replaced with glutamate (4 mM) and malate (4 mM). Symbols correspond to those in Fig. 1. Results are presented as fractions of control and are means ± SEM of 3 different experiments. Control values obtained without drug were state 3, 20.4 ± 1.62; and state 4, 6.53 ± 0.43 nmol/min/mg protein.

centrations required for the half-maximal inhibitory effects also ranged within 10–100 μM and also ranked as mefenamic acid < diclofenac < diphenylamine. Figure 2 shows the effects of these compounds on mitochondrial oxygen consumption energized with glutamate and malate. Results similar to those with succinate were obtained, although the difference between diclofenac and diphenylamine was unclear on both state 3 and state 4 respiration with these substrates.

Oligomycin, which inhibits the utilization of ADP/ATP, was added to the incubation mixture in the presence of ADP (state 3). The respiration rate was decreased markedly (ADP + oligomycin in Table 2). Consecutive addition of mefenamic acid, diclofenac, and diphenylamine at the concentrations that exhibit an approximately half-maximal effect on state 3 respiration (Fig. 1) partially released the oligomycin-inhibited state 3 respiration (Table 2). This phenomenon is general and characteristic for uncouplers of mitochondrial oxidative phosphorylation.

Mitochondria were pretreated with cyclosporin A, a potent inhibitor of MPT [15], to determine the involvement of MPT in the observed effects of mefenamic acid, diclofenac, and diphenylamine on respiratory control. Pretreatment with cyclosporin A affected neither the stimulation of state 4 respiration nor the suppression of state 3 respiration induced by any compound used (Table 3). Pretreatment of mitochondria with EGTA, which depletes Ca^{2+} contents and then suppresses MPT, did not affect either state 3 or state 4 respiration (data not shown).

TABLE 2. Effects of mefenamic acid, diclofenac, and diphenylamine on ADP-stimulated respiration in the presence of oligomycin

Additions	Oxygen consumption (nmol/min/mg protein)
Control	8.6 ± 0.99
ADP	53.7 ± 1.58
Mefenamic acid	23.8 ± 1.17
Diclofenac	23.6 ± 1.34
Diphenylamine	16.9 ± 1.61
ADP + oligomycin	6.1 ± 0.31
ADP + oligomycin + mefenamic acid	23.3 ± 2.12
ADP + oligomycin + diclofenac	22.0 ± 2.51
ADP + oligomycin + diphenylamine	15.0 ± 2.47

Incubation conditions were described in Table 1. Drug concentrations used were: mefenamic acid, 10 μM; diclofenac, 25 μM; diphenylamine, 100 μM; and oligomycin, 2 μg/mL. Results are means ± SEM of 3 different experiments.

DISCUSSION

During our recent study on the cytotoxicity of acidic NSAIDs to freshly isolated rat hepatocytes, diclofenac and the *N*-phenylanthranilic acids mefenamic acid, tolfenamic acid, and flufenamic acid were found to decrease hepatocellular ATP content extensively [16]. All of these NSAIDs have a common "skeleton" structure, diphenylamine (Fig. 3). Furthermore, diphenylamine itself also caused a decrease in hepatocellular ATP content. This suggested inhibitory effects on mitochondrial function. On the other hand, *N*-phenylanthranilic acids and diclofenac are reported to be potent uncouplers [2, 3, 6–7]. Thus, we have noted that a diphenylamine structure, which constitutes these NSAIDs, should affect mitochondrial oxidative phosphorylation. In conformity with these findings, the oxygen consumption study in Table 1 indicated that all of the NSAIDs with a diphenylamine structure (mefenamic acid, tolfenamic acid, flufenamic acid, and diclofenac) affected oxygen consumption by rat liver mitochondria (inhibition of state 3 respiration, stimulation of state 4 respiration), whereas the effects of the other NSAIDs except for diflunisal (salicylic acid, indomethacin, naproxen, and fenoprofen) were relatively weak.

As shown for the inhibitory effect on state 3 respiration and the stimulatory effect on state 4 respiration (Figs. 1 and 2), diphenylamine, as well as *N*-phenylanthranilic acids and diclofenac, was seen to have an uncoupler-like effect on mitochondrial oxidative phosphorylation. In addition, oligomycin-inhibited state 3 respiration was released by these compounds, indicating that diphenylamine also is an uncoupler with a potency similar to the structurally related NSAIDs (Table 2). While the drug concentrations required for the maximal stimulatory effects on state 4 respiration were different for mefenamic acid, diclofenac, and diphenylamine, similar concentration versus efficiency profiles were obtained for the three compounds used in the present

TABLE 3. Effects of cyclosporin A on NSAID- and diphenylamine-induced alteration of respiratory control in rat liver mitochondria

	Oxygen consumption (nmol/min/mg protein)			
	Without cyclosporin A		With cyclosporin A	
Additions	State 3	State 4	State 3	State 4
Control	59.2 ± 1.83	15.0 ± 0.36 (3.9)	59.7 ± 2.90	15.2 ± 0.33 (3.9)
Mefenamic acid	35.4 ± 4.25*	25.5 ± 2.25* (1.4)	32.8 ± 5.42*	25.4 ± 3.07* (1.3)
Diclofenac	45.9 ± 2.36*	21.9 ± 1.04* (2.1)	51.0 ± 4.82	23.4 ± 2.01* (2.2)
Diphenylamine	36.3 ± 3.59*	28.3 ± 1.24* (1.3)	35.5 ± 2.30*	27.4 ± 0.12* (1.3)

Incubation conditions were described in Table 1. Drug concentrations used were: mefenamic acid, 10 μM; dicloferac, 25 μM; diphenylamine, 100 μM; and cyclosporin A, 700 nM. Values in parentheses are RC calculated as the ratio of state 3/state 4 respiration. Results are means ± SEM of 3 different experiments.

*Significantly different from control ($P < 0.05$).

study (Figs. 1 and 2). These observations indicated that a diphenylamine structure essentially contributed to the uncoupling effects of the NSAIDs with the diphenylamine skeleton.

It was found recently that some compounds induce MPT, resulting in uncoupling of oxidative phosphorylation [17]. Thus, the involvement of the "indirect" uncoupling action was determined by pretreatment of mitochondria with cyclosporin A, a potent inhibitor of MPT, and with EGTA, which depletes Ca^{2+} and suppresses MPT [15]. Neither treatment affected mefenamic acid-, diclofenac-, or diphenylamine-induced suppression of state 3 respiration or stimulation of state 4 respiration (Table 3), suggesting that the uncoupling effects of these compounds were not due to MPT.

Various compounds are known to behave as uncouplers. Although their mechanism has not been elucidated completely, uncouplers generally are considered to behave as protonophores to cause their effect [14, 18, 19]. It is widely accepted that it is essential for NSAIDs that behave as potent uncouplers of mitochondrial oxidative phosphorylation to have a carboxyl group in their structure, and to be weakly acidic (relatively large pK_a) and lipophilic to a certain extent [9, 10]. Diphenylamine itself is a basic compound and may not behave as a protonophore unless it protonates and/or forms lipophilic ion pairs with certain anions (e.g. SCN^-) as suggested for some amine local anesthetics [20]. Thus, protonophore activity is unlikely to explain the uncoupling potency of diphenylamine observed under the present conditions. To explain the similarity of the uncoupling ability of structurally related but physicochemically unrelated compounds, a mechanism other than protonophore activity may be required. The present observation would contribute to finding new characteristics of the compounds called "uncouplers."

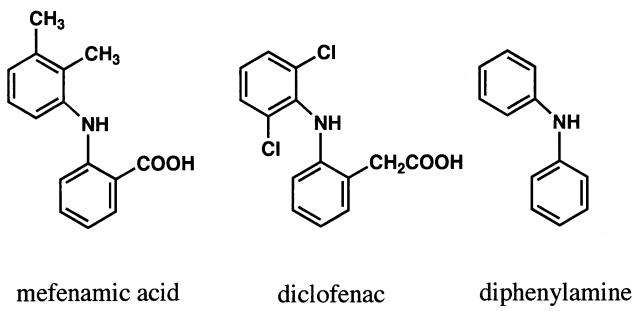

FIG. 3. Diphenylamine and its structurally related NSAIDs.

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