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Effects of nimesulide and its reduced metabolite on mitochondria

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1 We investigated the effects of nimesulide, a recently developed non-steroidal anti-inflammatory drug, and of a metabolite resulting from reduction of the nitro group to an amine derivative, on succinate-energized isolated rat liver mitochondria incubated in the absence or presence of 20 μM Ca^{2+}, 1 μM cyclosporin A (CsA) or 5 μM ruthenium red.

2 Nimesulide uncoupled mitochondria through a protonophoretic mechanism and oxidized mitochondrial NAD(P)H, both effects presenting an EC_{50} of approximately 5 μM.

3 Within the same concentration range nimesulide induced mitochondrial Ca^{2+} efflux in a partly ruthenium red-sensitive manner, and induced mitochondrial permeability transition (MPT) when ruthenium red was added after Ca^{2+} uptake by mitochondria. Nimesulide induced MPT even in de-energized mitochondria incubated with 0.5 mM Ca^{2+}.

4 Both Ca^{2+} efflux and MPT were prevented to a similar extent by CsA, Mg^{2+}, ADP, ATP and butylhydroxytoluene, whereas dithiothreitol and N-ethylmaleimide, which markedly prevented MPT, had only a partial or no effect on Ca^{2+} efflux, respectively.

5 The reduction of the nitro group of nimesulide to an amine derivative completely suppressed the above mitochondrial responses, indicating that the nitro group determines both the protonophoretic and NAD(P)H oxidant properties of the drug.

6 The nimesulide reduction product demonstrated a partial protective effect against accumulation of reactive oxygen species derived from mitochondria under conditions of oxidative stress like those resulting from the presence of *t*-butyl hydroperoxide.

7 The main conclusion is that nimesulide, on account of its nitro group, acts as a potent protonophoretic uncoupler and NAD(P)H oxidant on isolated rat liver mitochondria, inducing Ca^{2+} efflux or MPT within a concentration range which can be reached *in vivo*, thus presenting the potential ability to interfere with the energy and Ca^{2+} homeostasis in the liver cell.
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Keywords: Nimesulide; mitochondria; uncoupling; NAD(P)H oxidation; mitochondrial permeability transition (MPT); mitochondrial Ca^{2+} efflux; reactive oxygen species; liver

Abbreviations: ANT, adenine nucleotide translocase; BHT, butylhydroxytoluene; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; c.i., confidence interval; CsA, cyclosporin A; DTT, dithiothreitol; EGTA, ethylene glycol bis(-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; HEPES, N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulphonic acid); MPT, mitochondrial permeability transition; NADH, nicotinamide adenine dinucleotide, reduced form; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; NEM, N-ethylmaleimide; ROS, reactive oxygen species; *t*-BHP, *tert*-butyl hydroperoxide; $\Delta\Psi$, electrical transmembrane potential difference

Introduction

Mitochondria carry out a variety of biochemical processes, including oxidative phosphorylation, which provide most of the energy for the cell. They are also the main source of reactive oxygen species (ROS). The oxidation of energy-linked substrates in mitochondria generates reducing equivalents, which are transferred *via* the pyridine nucleotides. The pyridine nucleotide NADPH is utilized primarily for reductive biosynthesis, while NADH is used for energy production. In addition, the pyridine nucleotides are a critical source of reducing equivalents needed to remove endogenous and exogenous ROS. Mitochondrial oxidative phosphorylation is dependent upon a proton electrochemical gradient generated by respiration and maintained by the impermeability of the inner mitochondrial membrane to protons (Kehrer & Lund, 1994; Pessayre *et al*., 1999). If the mitochondrial membrane is rendered permeable to protons (e.g. by the action of protonophores, which translocate protons across bilayers) the membrane potential dissipates, increasing the respiratory rate; under this condition (uncoupling) the organelle is no longer capable of sustaining ATP synthesis (Mitchell, 1961; Nicholls, 1982). It is, therefore, to be expected that mitochondrial impairment could be a relevant mechanism of drug-induced toxicity, in particular in liver, which is the major site of uptake and metabolism of drugs.

Uncouplers and NAD(P)H oxidant agents incubated with isolated Ca^{2+}-loaded mitochondria induce release of the matrix-accumulated cation and/or a transition in mitochondrial permeability (MPT) (Lehninger *et al*., 1978; Frei *et al*., 1985; Petronilli *et al*., 1994; Costantini *et al*., 1996; Lemasters *et al*., 1998). MPT is a Ca^{2+}-dependent process linked to the opening of a non-specific channel into the inner membrane, the MPT pore, which is selectively blocked by the immune

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suppressor cyclosporin A (CsA) (Hunter & Haworth, 1979; Crompton *et al*., 1988; Zoratti & Szabò, 1995; Bernardi *et al*., 1998; Kowaltowski & Vercesi, 1999). Ca^{2+} is released from mitochondria either through a low-capacity porter, comprising the $Ca^{2+}/2H^{+}$ and the $Ca^{2+}/2Na^{+}$ exchange processes, or through a high-capacity porter including the pro-oxidant-induced pathway, the Ca^{2+} uniporter operating in reverse and the MPT pore itself (Gunter *et al*., 1994; Bernardi & Petronilli, 1996); the forward and reverse reactions of the Ca^{2+} uniporter are prevented by ruthenium red (Pffeifer *et al*., 1983). The disruption of cell Ca^{2+} homeostasis has been often linked to the onset of cytotoxicity caused by pathological states or toxic agents (Orrenius *et al*., 1989). MPT, in particular, is now recognized to be closely involved in cell death either by necrosis or by apoptosis (Kroemer *et al*., 1998; Lemasters *et al*., 1998; Pessayre *et al*., 1999).

Nimesulide (N-[4-nitro-2-phenoxyphenyl]-methanesulfonamide) is a recently developed, therapeutically relevant non-steroidal anti-inflammatory drug (NSAID) (Insel *et al*., 1996). This class of drugs has been widely used in clinical practice because of its anti-inflammatory, analgesic and antipyretic actions (Brooks & Day, 1991). Hepatotoxicity, one of the common side effects of NSAIDs, has been often linked to their uncoupling effects on mitochondria (Boelsterli *et al*., 1995; Masubuchi *et al*., 1998). In this regard, it has been previously reported that nimesulide uncouples mitochondria (Caparroz-Assef *et al*., 1998; Moreno-Sanchez *et al*., 1999) and, in a preliminary study from our laboratory (unpublished data), we have found that it is a direct and powerful oxidant of NADH and NADPH.

Within this context, we investigated the effects of nimesulide and of one of its metabolites – the product of reduction of the nitro group to an amine derivative (Carini *et al*., 1998) – on responses involving uncoupling and NAD(P)H oxidation in isolated rat liver mitochondria in an attempt to establish a structure-effect relationship and to assess their potential ability to interfere *in vivo* with the energy, Ca^{2+} homeostasis and oxidative status in the hepatocyte.

Methods

All animal procedures used in this study were in strict accordance with the 'Ethical principles and guidelines for experiments on animals' of the Swiss Academy of Medical Sciences and Swiss Academy of Sciences.

Chemicals

Nimesulide and reduced nimesulide were gifts from Aché Lab. Farmac. S.A. (São Paulo, Brazil) and from Dr Randy Leavitt, Maxxam Analytics Inc. (Mississauga, Canada), respectively. All other reagents were of the highest commercially available grade. The amounts of dimethyl sulfoxide required to solubilize nimesulide and reduced nimesulide had no effect on the assays. All stock solutions were prepared using glass-distilled deionized water.

Isolation of mitochondria

Rat liver mitochondria were isolated by conventional differential centrifugation (Pedersen *et al*., 1978). Male Wistar rats weighing approximately 200 g were sacrificed by cervical dislocation; livers (10 – 15 g) were immediately removed, sliced in 50 ml of medium containing 250 mM sucrose, 1 mM EGTA and 10 mM HEPES – KOH, pH 7.2, and homogenized three times for 15 s at 1 min intervals in a Potter-Elvehjem homogenizer. Homogenates were centrifuged at 770 *g* for 5 min and the resulting supernatant was further centrifuged at 9800 *g* for 10 min. Pellets were suspended in 10 ml of medium containing 250 mM sucrose, 0.3 mM EGTA and 10 mM HEPES – KOH, pH 7.2, and centrifuged at 4500 *g* for 15 min. The final mitochondrial pellet was suspended in 1 ml of medium containing 250 mM sucrose and 10 mM HEPES – KOH, pH 7.2, and used within 3 h. All procedures were conducted at 4°C. Mitochondrial protein content was determined by the biuret reaction (Cain & Skilleter, 1987).

Standard incubation procedure

Assays were performed at 30°C using 5 mM potassium succinate as oxidizable substrate, in order to transfer reducing equivalents to the FAD in the succinate-dehydrogenase of the respiratory chain (i.e. to energize mitochondria), in the presence of sufficient rotenone to prevent substrate oxidation by NAD-requiring dehydrogenases. The standard incubation medium contained 125 mM sucrose, 65 mM KCl and 10 mM HEPES – KOH, pH 7.4, in the presence or absence of 0.5 mM EGTA plus 10 μM $CaCl_2$. The residual Ca^{2+} concentration in the assay medium, estimated from a standard curve prepared by spectrofluorometric analysis using arsenazo III as indicator, was approximately 10 μM. Therefore, in assays where Ca^{2+} was added, its final concentration was approximately 20 μM. CsA (1 μM) and the other MPT modulators were incubated with mitochondria from the beginning of the experiments in which they were used; ruthenium red (5 μM) was added to the medium after mitochondrial energization, immediately prior to the addition of nimesulide or reduced nimesulide.

Mitochondrial assays

Mitochondrial respiration was monitored polarographically with an oxygraph equipped with a Clark-type oxygen electrode (Gilson Medical Electronics, Middleton, WI, U.S.A.). The electrical transmembrane potential ($\Delta\Psi$) was monitored spectrofluorometrically using 0.4 μM rhodamine 123 as an indicator and a Model F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) at the 505/535 nm excitation/emission wavelength pair (Emaus *et al*., 1986). NAD(P)H oxidation was monitored spectrofluorometrically at the 366/450 nm excitation/emission wavelength pair. Ca^{2+} efflux was followed spectrophotometrically using the colour change of the dye arsenazo III (50 μM) as monitored by difference in absorbance between the wavelengths 685 nm and 675 nm. (Scarpa, 1979). Mitochondrial swelling was estimated from the decrease in absorbance at 540 nm using a Model DU-70 spectrophotometer (Beckman Coulter Inc., Fullerton, CA, U.S.A.). Hydrogen peroxide (H_2O_2) production was monitored spectrofluorometrically using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) at the 503/529 nm excitation/emission wavelength pair (Cathcart *et al*., 1983).

Mitochondrial swelling in hyposmotic potassium acetate medium

Mitochondria (0.45 mg protein) were incubated in a medium containing (mM) potassium acetate 54, HEPES – NaOH 5, pH 7.1, EGTA 0.1, EDTA 0.2, sodium azide 0.1, 0.1% bovine serum albumin, 15 μM atractyloside, 1 μM antimycin

A, and 0.3 mM propranolol in order to inhibit the inner membrane anion channel, followed by 1 μM valinomycin and nimesulide, in a final volume of 1.5 ml. Swelling was estimated from the decrease in absorbance at 540 nm (Nicholls, 1982).

Analysis of data

Per cent prevention data in Table 1 are presented as arithmetic mean $\pm$ s.e.mean. Data in Figure 1 are presented as mean $\pm$ s.e.mean with EC_{50} values given as geometric mean with 95% confidence interval (c.i.). EC_{50} was estimated using the GraphPad Prism software, version 3.00 for Windows, GraphPad Software, San Diego, CA, U.S.A.

Results

Figure 1 shows the release of succinate-supported resting (state 4) respiration (A), the decrease of $\Delta\Psi$ (B) and the oxidation of mitochondrial NAD(P)H (C) induced by nimesulide in Ca^{2+}-free (0.5 mM EGTA) and Ca^{2+}-loaded (20 μM Ca^{2+}) isolated rat liver mitochondria. A close parallel between these curves was evident under both assay conditions. Responses were concentration-dependent, tending to saturation above 10 μM nimesulide. The EC_{50} values for Ca^{2+}-free and Ca^{2+}-loaded mitochondria were 4.29 μM (geometric mean, c.i. 2.30–7.99 μM) and 5.88 μM (4.26–8.12 μM; $n=5$; Figure 1A), 5.28 μM (3.65–7.63 μM) and 6.16 μM (3.49–7.99 μM; $n=5$; Figure 1B) and 5.90 μM (3.24–10.7 μM) and 6.29 μM (4.27–8.14 μM; $n=5$; Figure 1C), respectively. It is worth mentioning that these curves were closely similar, regardless of the presence or absence of Ca^{2+}, and that CsA and ruthenium red had only an approximately 10% preventive and stimulating effect, respectively, on the maximum value obtained in the curve for the Ca^{2+}-loaded mitochondria (results not shown). Figure 2 shows that within the uncoupling concentration range nimesulide promoted a concentration-dependent swelling in mitochondria incubated in hyposmotic potassium acetate medium.

Within the same concentration range in which it caused uncoupling and oxidation of NAD(P)H, nimesulide induced mitochondrial Ca^{2+} efflux in a partly ruthenium red-sensitive manner (Figure 3A) and even MPT-associated mitochondrial swelling, provided that ruthenium red was incubated with mitochondria following Ca^{2+} uptake (Figure 3B). CsA not only caused an expected prevention of mitochondrial swelling but, remarkably, it almost completely prevented Ca^{2+} efflux in a condition in which swelling does not take place. Both mitochondrial swelling and Ca^{2+} efflux were also prevented to a similar extent by Mg^{2+}, ADP, ATP and BHT, whereas DTT and NEM, which markedly prevented swelling, had only a partial or no effect on Ca^{2+} efflux, respectively (Table 1). Nimesulide induced mitochondrial swelling even in de-energized mitochondria treated with antimycin A and incubated with 0.5 mM Ca^{2+} followed by ruthenium red. Swelling under these conditions was completely prevented by CsA and its extent was the same as for energized mitochondria (data not shown).

Table 1 Effects of MPT modulators on mitochondrial swelling and Ca^{2+} efflux induced by 25 μM nimesulide

Modulator	*Per cent prevention*	
	Swelling	*Ca^{2+} efflux*
0.2 mM ADP	100	100
0.2 mM ATP	100	100
1 mM Mg^{2+}	100	100
5 μM BHT	56$\pm$8	57$\pm$6
1 mM DTT	100	55$\pm$5
25 μM NEM	73$\pm$6	0

Assays were conducted as described in the legend to Figure 3. MPT modulators were added to the medium at the beginning of the experiment; RR (5 μM) was added after mitochondrial energization, immediately prior to the addition of nimesulide. Values are given as mean $\pm$ s.e.mean, $n=5$–10.

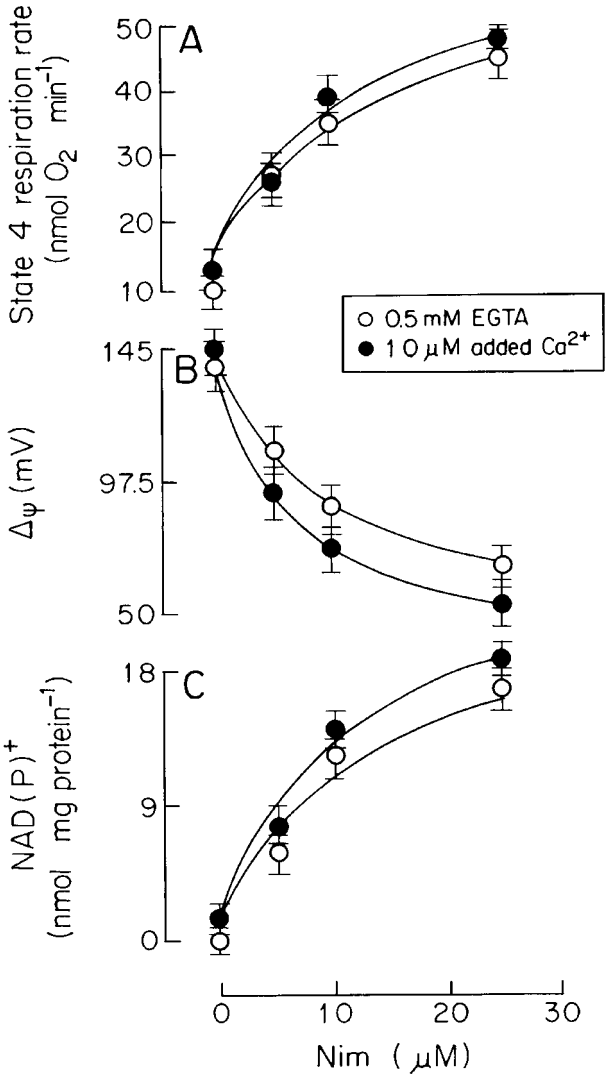

Figure 1 Concentration-response curves for the effects of nimesulide on (A) state 4 respiration rate, (B) $\Delta\Psi$ and (C) NAD(P)H oxidation in isolated rat liver mitochondria in the presence of 0.5 mM EGTA or 10 μM added Ca^{2+}. In (A), mitochondria (1.5 mg protein) were incubated at 30°C with 5 mM succinate and rotenone (2.5 nmol mg protein^{-1}) in a final volume of 1.5 ml. In (B), mitochondria (2 mg protein), incubated in the standard medium plus rotenone (2.5 nmol mg protein^{-1}) and 0.4 μM rhodamine 123, in a final volume of 2 ml, were energized by the addition of 5 mM succinate. In (C), mitochondria (2 mg protein), incubated in the standard medium plus rotenone (2.5 nmol mg protein^{-1}), in a final volume of 2 ml, were energized by the addition of 5 mM succinate. $\Delta\Psi$ and NAD(P)H oxidation responses of mitochondria were immediate and remained constant for at least 5 min; the extent of oxidation was estimated 30 s after drug addition. Data are presented as the mean $\pm$ s.e.mean of five experiments using different mitochondrial preparations.

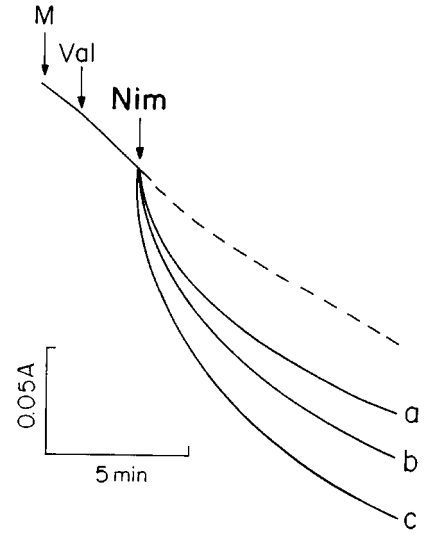

Figure 2 Mitochondrial swelling induced by nimesulide (Nim) on valinomycin-treated mitochondria incubated in hyposmotic potassium acetate medium. Mitochondria (0.45 mg protein) were incubated in the medium described in Methods, in a final volume of 1.5 ml. Valinomycin (Val, 1 μM) was added where indicated. Nimesulide was added to lines a, b and c at the concentrations of 5, 10 and 25 μM, respectively. The dotted line represents a control experiment. The figure is representative of five experiments using different mitochondrial preparations.

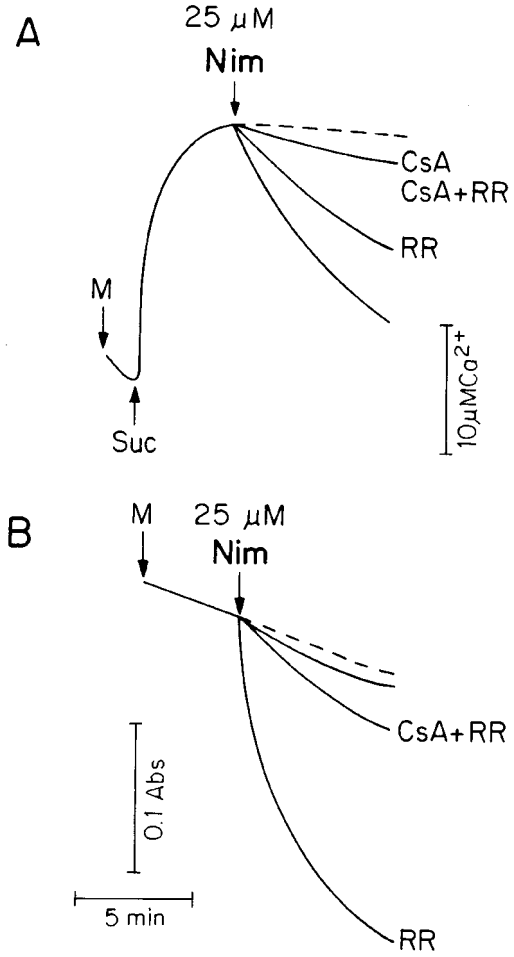

Figure 3 Mitochondrial Ca^{2+} efflux (A) and swelling (B) induced by nimesulide (Nim). In (A), mitochondria (M, 1 mg protein) incubated in the standard medium plus rotenone (2.5 nmol mg protein^{-1}), 10 μM added $CaCl_2$ and 50 μM arsenazo III, in a final volume of 1 ml, were energized by the addition of 5 mM succinate (Suc). In (B), mitochondria (0.4 mg protein) were incubated with 5 mM succinate + rotenone (2.5 nmol mg protein^{-1}) in the standard medium plus 10 μM added $CaCl_2$. Additions are indicated by arrows. CsA (1 μM), was incubated with mitochondria from the beginning of the experiment; ruthenium red (RR, 5 μM) was added after mitochondrial energization, immediately prior to nimesulide. The dotted lines represent control experiments. Figures are representative of five experiments using different mitochondrial preparations.

The effects of the reduced metabolite of nimesulide on the mitochondria, compared with the effects of nimesulide itself are shown in Figure 4. The reduction of the nitro group of nimesulide to an amine derivative completely suppressed the uncoupling and NAD(P)H oxidant properties of the drug, as well as the mitochondrial responses in relation to Ca^{2+} efflux and MPT. In addition, this nimesulide reduction product demonstrated the ability to partly protect against accumulation of H_2O_2 derived from mitochondria in the presence of *tert*-butyl hydroperoxide (*t*-BHP) (Figure 5).

Discussion

Nimesulide has been reported previously to uncouple mitochondria (Caparroz-Assef *et al*., 1998; Moreno-Sanchez *et al*., 1999). Our results show a close parallel between the concentration-response curves for release of state 4 respiration and $\Delta\Psi$ dissipation induced by nimesulide, stressing that the drug uncouples mitochondria through a protonophoretic mechanism. This evidence is in line with the electron-withdrawing property of its nitro group (Terada, 1981; Canton *et al*., 1996), and even with the ability of nimesulide to induce a concentration-dependent swelling in mitochondria incubated in a potassium acetate hyposmotic medium (Nicholls, 1982). It is noteworthy that, within the same concentration range in which it uncoupled mitochondria, nimesulide oxidized mitochondrial NAD(P)H, an effect predicted on account of the oxidant potential of this same nitro group. The above effects were paralleled by inhibition of the rate of ATP synthesis by mitochondria, as evaluated by using the glucose/hexokinase trap system (Cain & Skilleter, 1987) and the firefly luciferin-luciferase system (Lemasters & Hackenbrock, 1976) (results not shown).

Both protonophoresis and NAD(P)H oxidation are properties potentially implicated in mitochondrial responses such as release of mitochondrial matrix-accumulated Ca^{2+} and MPT, as based on the following literature data: (i) protonophore-induced dissipation of $\Delta\Psi$ and NAD(P)H oxidation are well established events inducing mitochondrial Ca^{2+} efflux (Nicholls & Crompton, 1980; Richter *et al*., 1990; Zoratti & Szabò, 1995); (ii)MPT is modulated by the redox state of mitochondrial NAD(P)H (Gunter *et al*., 1994; Zoratti & Szabò, 1995); (iii) MPT induction by protonophores is regulated by $\Delta\Psi$ (Petronilli *et al*., 1994) or by ROS accumulation due to exhaustion of reduced glutathione and NAD(P)H (Kowaltowski *et al*., 1996). Within this context, it is not easy to establish which of the properties of nimesulide – namely, the protonophoretic or NAD(P)H oxidant – is directly or indirectly implicated in Ca^{2+} efflux and MPT. However, it is worth observing that the effect of nimesulide as an inducer of mitochondrial Ca^{2+} efflux prevails over its effect as an MPT inducer, so that the drug causes MPT only if the former effect is prevented by treatment of mitochondria with ruthenium red, which inhibits the uncoupling-linked uniporter reversal-mediated Ca^{2+} efflux, a condition favouring MPT by keeping elevated the

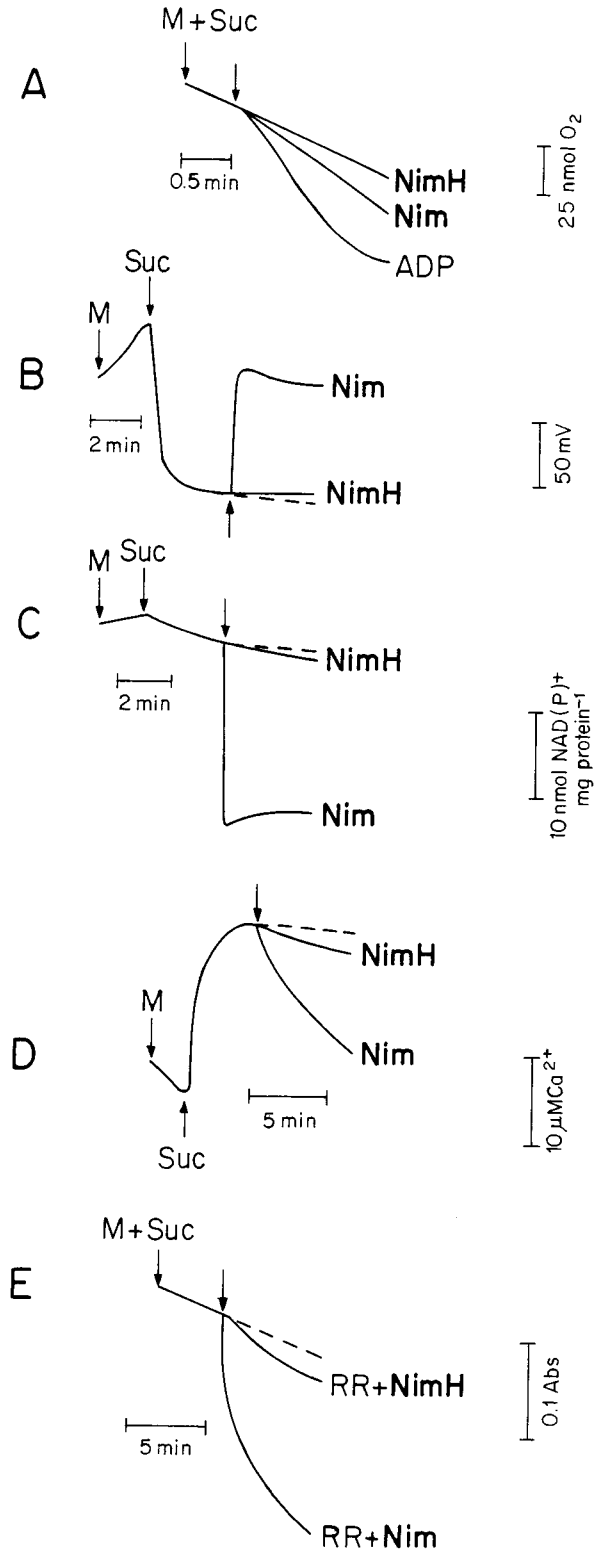

Figure 4 Responses of isolated rat liver mitochondria in the presence of 25 μM nimesulide (Nim) or 25 μM reduced nimesulide (NimH): (A) state 4 respiration, (B) $\Delta\Psi$, (C) NAD(P)H oxidation, (D) Ca^{2+} efflux and (E) swelling. The assays were performed as described in Figure 1, in the presence of 0.5 mM EGTA (A, B, C) or as described in Figure 3 (D, E). Additions are indicated by arrows. RR (5 μM) was added after mitochondrial energization, immediately prior to the drugs. The dotted lines represent control experiments. Figures are representative of five experiments using different mitochondrial preparations.

levels of matrix Ca^{2+} (Igbavboa & Pfeiffer, 1988; Petronilli *et al*., 1994).

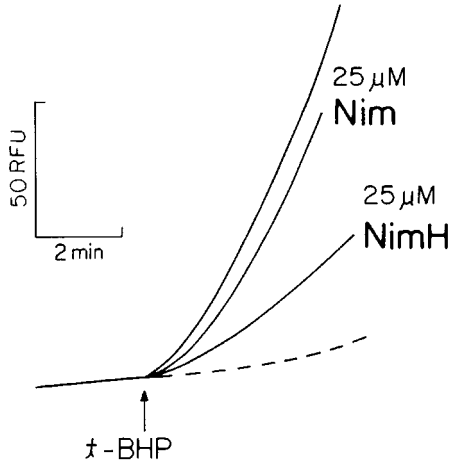

Figure 5 Effects of 25 μM nimesulide (Nim) and of 25 μM reduced nimesulide (NimH) on H_2O_2 derived from mitochondria in the presence of 0.5 mM *tert*-butyl hydroperoxide (*t*-BHP), under the standard incubation conditions described in Methods, in the presence of 0.5 mM EGTA. Additions are indicated by arrows. The dotted line represents a control experiment in the absence of *t*-BHP. The figure is representative of five experiments using different mitochondrial preparations. RFU: relative fluorescence units.

It has been proposed that a pro-oxidant-induced Ca^{2+}/H^{+} exchange in mitochondria, inhibited by CsA, is closely associated with MPT. According to this hypothesis, more than one open state resulting in different selectivity is involved in the opening of the MPT pore (Novgorodov & Gudz, 1996; Ichas & Mazat, 1998), with a common mechanism accounting for both this process and the pro-oxidant-induced mitochondrial Ca^{2+} efflux (Novgorodov & Gudz, 1996). Within this context, in the present study we report a single compound, nimesulide, which is able to induce mitochondrial Ca^{2+} efflux or MPT through at least partly shared mechanisms. This suggests that nimesulide-induced MPT and Ca^{2+} efflux are events resulting from uncoupling-linked $\Delta\Psi$ dissipation and/or NAD(P)H oxidation, with the preference for manifestation depending simply on the ability of mitochondria to prevent the cation efflux through reversal of the uniporter. The main evidence indicating the participation of common mechanisms in these processes is their equivalent prevention by classical MPT inhibitors like CsA, ADP, ATP and Mg^{2+}. However, it is worth observing that the preventive effect of NEM and, to a lesser extent, of DTT, was far more pronounced with respect to MPT than with respect to Ca^{2+} efflux.

Since these agents favour a reduced state of mitochondrial membrane protein thiol groups (Zoratti & Szabò, 1995), it is believed that the nimesulide-induced condition inducing swelling did implicate a greater extent of oxidation of these groups, which is a well established event involved in the MPT mechanism. In agreement with previously proposed MPT mechanisms (Lê Quôc & Lê Quôc, 1988; Castilho *et al*., 1995; Halestrap *et al*., 1998), oxidation of membrane protein thiol groups of the adenine nucleotide translocase (ANT) induced by nimesulide, intermediated by ROS, would sensitize the MPT pore in the presence of Ca^{2+}. The involvement of ANT, ROS and protein thiol oxidation is supported by evidence showing preventive effects against nimesulide-induced swelling exerted by ADP and ATP, BHT, NEM and DTT, respectively.

From a biochemical viewpoint, the present study shows that nimesulide is a protonophore and mitochondrial NAD(P)H oxidant agent and that these properties are

implicated in mitochondrial responses such as Ca^{2+} efflux and MPT. From a toxicological/pharmacological viewpoint, it demonstrates that nimesulide has the potential ability to interfere with the energy and Ca^{2+} homeostasis in the hepatocyte. Supporting this conclusion, is the fact that the effects of nimesulide occurred at concentrations easily attainable *in vivo* (approximately 5 μM) (Maffei *et al*., 1993). On the other hand, the metabolite studied (i.e. the reduced nimesulide), in addition to having no deleterious effect on mitochondria, partly protects against accumulation of reactive oxygen species (ROS) derived from the organelle under conditions of oxidative stress. One such condition is that resulting from incubation of mitochondria with the pro-oxidant *t*-BHP, whose primary metabolic pathway involves the glutathione peroxidase-glutathione reductase system leading to oxidation of glutathione and NADPH (Lemasters *et al*., 1998). This protective effect may arise from an anti-oxidant activity inherent to the amine group of the metabolite, as in the case for other substituted aromatic amines such as benzocaine (Melentyeva & Antonova, 1988).

In summary, this study shows that nimesulide is a potent protonophoretic uncoupler and NAD(P)H oxidant, inducing, through at least partly shared mechanisms, mitochondrial Ca^{2+} efflux or MPT if Ca^{2+} efflux is prevented. The results obtained using reduced nimesulide indicate that the nitro group in the structure of the drug is a determinant of these properties and that the metabolite, in addition to having no deleterious effects on mitochondria, exhibits some antioxidant activity. The low concentration range in which the effects of the drug/metabolite take place on isolated mitochondria confers on them the potential ability to interfere *in vivo* with the energy, Ca^{2+} homeostasis and oxidative status in the liver cell.

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