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4-hydroxy nimesulide effects on mitochondria and HepG2 cells. A comparison with nimesulide

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Abstract

We previously reported that the nonsteroidal anti-inflammatory drug, nimesulide (*N*-[4-nitro-2-phenoxyphenyl]-methanesulfonamide), is an uncoupler and oxidizes NAD(P)H in isolated rat liver mitochondria, triggering mitochondrial Ca^{2+} efflux or, if this effect is inhibited, eliciting mitochondrial permeability transition (Mingatto et al., Br. J. Pharmacol. 131:1154–1160, 2000). We presently demonstrated that nimesulide's hydroxylated metabolite (4-hydroxy nimesulide) lacks the uncoupling property of the parent drug, while keeping its ability to oxidize mitochondrial NAD(P)H. In the presence of 10 μM Ca^{2+}, low (5–50 μM) concentrations of 4-hydroxy nimesulide elicited mitochondrial permeability transition, as assessed by cyclosporin A-sensitive mitochondrial swelling, associated with mitochondrial Ca^{2+} efflux/membrane potential dissipation ($\Delta\psi$), apparently occurring on account of the oxidation of mitochondrial protein thiols; no involvement of reactive oxygen species was observed. While nimesulide (0.5 or 1 mM, 30 h incubation) did not lead to significant HepG2 cell death, 4-hydroxy nimesulide caused a low extent (~15%) of cell necrosis, partly prevented by cyclosporine A, suggesting the involvement of mitochondrial permeability transition. Both nimesulide and 4-hydroxy nimesulide caused NADPH oxidation and $\Delta\psi$ dissipation in HepG2 cells. Because such $\Delta\psi$ dissipation induced by the metabolite was almost completely inhibited by cyclosporine A, it probably results from the mitochondrial permeability transition. Therefore, mitochondrial permeability transition, in apparent association with NAD(P)H oxidation, constitutes the most probable cause of HepG2 cell death elicited by 4-hydroxy nimesulide.

Keywords: Nonsteroidal anti-inflammatory drugs; Nimesulide; 4-hydroxy nimesulide; Liver mitochondria; Mitochondrial permeability transition; Permeability transition pores

1. Introduction

We have previously reported that nimesulide (*N*-[4-nitro-2-phenoxyphenyl]-methanesulfonamide), a nonsteroidal anti-inflammatory drug, is an uncoupler and oxidizes NAD(P)H in isolated rat liver mitochondria, triggering mitochondrial Ca^{2+} efflux or, if Ca^{2+} efflux is inhibited by ruthenium red, eliciting mitochondrial permeability transition; the nitro group of nimesulide was proposed to determine both properties (Mingatto et al., 2000). We also reported that nimesulide causes injury to isolated rat liver cells and that this effect is mediated by mitochondrial uncoupling (Mingatto et al., 2002). Earlier reports had demonstrated this uncoupling property of nimesulide (Caparroz-Assef et al., 1998; Moreno-Sanchez et al., 1999) and more recently, the issue of mitochondrial permeability transition involvement in nimesulide effects on mitochondria, has been raised (Ong et al., 2005; Tay et al., 2005; Berson et al., 2006). Concerning nimesulide cytotoxicity, different effects according to the type of cell and/or experimental procedures employed, have been reported (Rainsford et al., 2001; Mingatto et al., 2002; Ong et al., 2005; Berson et al., 2006).

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From the five nimesulide metabolites identified in humans, only the hydroxylated derivative (4-hydroxy nimesulide) retains its nitro group (Carini et al., 1998). In the present study we demonstrate that this metabolite lacks the uncoupling property of nimesulide, but elicits mitochondrial permeability transition even in the absence of ruthenium red, maintaining the ability to oxidize NADPH. Also, 4-hydroxy nimesulide, but not nimesulide, causes significant HepG2 cell death by necrosis, in apparent association with mitochondrial permeability transition.

2. Materials and methods

This study and protocols were approved by the Ethics Committee of Animal Research of the University of São Paulo — Ribeirão Preto "campus", Brazil.

2.1. Chemicals

Nimesulide was purchased from Sigma-Aldrich (St. Louis, MO), and hydroxy-nimesulide was a gift from Helsinn Healthcare SA (Pambio-Noranco, Switzerland). All other reagents were of the highest commercially available grade. The amounts of dimethyl-sulfoxide required to solubilize nimesulide or 4-hydroxy nimesulide had no effect on the assays. All stock solutions were prepared using glass-distilled deionized water (2 μM of contaminant Ca^{2+}).

2.2. Isolation of rat liver mitochondria

Mitochondria were isolated by standard 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 with a Potter–Elvehjem homogenizer. Homogenates were centrifuged at 580 ×*g* for 5 min and the resulting supernatant further centrifuged at 10300 ×*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 3400 ×*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. Mitochondrial protein content was determined by the biuret reaction. Respiratory Control Ratio for succinate-energized mitochondria was always around 5.

2.3. Standard incubation procedure

Mitochondria energized by 5 mM potassium succinate (+2.5 μM rotenone) in a standard incubation medium containing 125 mM sucrose, 65 mM KCl and 10 mM Hepes–KOH, pH 7.4, were incubated at 30 °C with different concentrations of compounds in the absence or presence of 0.5 mM EGTA or 10 μM $CaCl_2$ (mitochondrial suspension). For the assays with non-energized mitochondria, succinate/rotenone was replaced by 0.5 mM $CaCl_2$.

2.4. Continuous monitoring assays

Mitochondrial respiration was monitored polarographically with an oxygraph equipped with a Clark-type oxygen electrode (Gilson Medical Electronics, Middleton, WI, USA), and the mitochondrial membrane potential ($\Delta\psi$) was estimated spectrofluorimetrically using 0.4 μM rhodamine 123 as an indicator and a Model F-4500 Hitachi fluorescence spectrophotometer (Hitachi, Tokyo, Japan) at the 505/535 nm excitation/emission wavelength pair (Emaus et al., 1986). NAD(P)H oxidation was assessed spectrofluorimetrically in a Model F-4500 Hitachi fluorescence spectrophotometer at the 366/450 nm excitation/emission wavelength pair. Mitochondrial swelling was estimated from the decrease in apparent absorbance at 540 nm. Ca^{2+} efflux was followed spectrophotometrically using the colour change of arsenazo III (Scarpa, 1979) as monitored by difference in absorbance between 685 and 675 nm. Mitochondria-generated H_2O_2 was monitored using the Amplex Red (Molecular Probes, Eugene, OR) assay (Votyakova and Reynolds, 2001): mitochondria (0.5 mg/ml) was incubated in the standard medium with 50 μM Amplex Red and 0.025 U/ml horseradish peroxidase, and fluorescence of oxidized probe was measured in a Model F-4500 Hitachi fluorescence spectrophotometer at the 563/587 nm excitation/emission wavelength pair.

2.5. Determination of mitochondrial protein thiols

The mitochondrial suspension (0.4 mg protein) was treated with perchloric acid (7% final concentration) and centrifuged at 4500 ×*g* for 5 min. The pellet was suspended with 100 μl of 7% perchloric acid, supplemented with 1 ml of water, and centrifuged at 4500 ×*g* for 5 min. The final pellet was suspended with 0.2 ml of 10% Triton X-100, and supplemented with 0.8 ml of water. An aliquot of 0.2 ml of 500 mM potassium phosphate, pH 7.6, was added to 0.8 ml of the suspension. The amount of thiol groups was determined from $\varepsilon = 13600\ M^{-1}cm^{-1}$, using the difference in absorbance at 412 nm, prior to and 5 min after the addition of DTNB (5,5′-dithiobis (2-nitrobenzoic acid)) (0.2 mM final concentration) (Jocelyn, 1987).

2.6. Determination of GSH/GSSG ratio

The mitochondrial suspension (1 ml) was treated with 0.5 ml of 13% trichloroacetic acid and centrifuged at 900 ×*g* for 3 min. For determination of GSH, aliquots (100 μl) of the supernatant were mixed with 2 ml of 100 mM NaH_2PO_4 buffer, pH 8.0, containing 5 mM EGTA. One-hundred μl *o*-phthalaldialdehyde (1 mg/ml) was added and fluorescence was measured 15 min later at the 350/420 nm excitation/emission wavelength pair (Hissin and Hilf, 1976). GSSG was determined by the addition of aliquots (250 μl) of the supernatant to 250 μl of 0.04 M N-ethylmaleimide. After 20 min of incubation at room temperature, 500 μl of 1 M NaOH was added. One-hundred μl of the previous solution was added to 2 ml of 1 M NaOH and 100 μl *o*-phthalaldialdehyde (1 mg/ml) was added. Fluorescence was measured 15 min later in a model F-4500 Hitachi fluorescence spectrophotometer at the 350/420 nm excitation/emission wavelength pair (Hissin and Hilf, 1976).

2.7. Culture of HepG2 cells

HepG2 cells were cultured in Eagle's Minimum Essential Medium (with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate), and containing 10% fetal bovine serum. Cells were maintained in a humidified CO_2 incubator at 37 °C, and passed and harvested by detachment with 0.05% trypsin and 0.5 mM EDTA in PBS (phosphate-buffered saline); 70–80% confluent cells were used for experiments with nimesulide or 4-hydroxy nimesulide. The cells were incubated with the drugs in absence of fetal bovine serum; control cultures received an equivalent amount of vehicle (dimethyl-sulfoxide).

2.8. Assay of cell viability

Cell viability was determined by the propidium iodide/annexin V assay using flow cytometry (Zhivotovsky et al., 1999). After exposure for 30 h to nimesulide or 4-hydroxy nimesulide, cells were harvested by trypsinization, washed twice with PBS, resuspended in the working solution of 5 μg/ml propidium iodide and annexin V (0.25 μg/ml), incubated for 15 min at 37 °C, and analyzed with a FACSCanto flow cytometer (Becton Dickinson, San Jose, CA) using Diva software. Approximately 10^5 cells were analyzed after each treatment.

2.9. Assessment of NAD(P)H oxidation in cells

HepG2 cells were treated with nimesulide or 4-hydroxy nimesulide for 30 h, trypsinized and centrifuged at 100 ×*g* for 5 min. Pellets were washed with PBS solution, and suspended in 1 ml of 0.1% Triton X-100. NAD(P)H oxidation was assessed as described for isolated mitochondria.

2.10. Mitochondrial membrane potential (Δψ) assay in cells

Δψ was assessed with the fluorescent probe TMRM (tetramethyrodamine, methyl ester). Floating and adherent cells were washed in PBS, incubated for 15 min at 37 °C, and their fluorescence monitored with a FACSCanto flow cytometer (Becton Dickinson, San Jose, CA) using Diva software.

2.11. Analysis of data

EC_{50} values were estimated using the GraphPad Prism software, version 3.00 for Windows, GraphPad Software, San Diego, CA, USA. Statistical analysis was performed by analysis of variance (ANOVA) followed by the Bonferroni's test.

3. Results and discussion

3.1. 4-Hydroxy nimesulide does not present the uncoupling property of nimesulide on mitochondria; however, it maintains its capability to oxidize NAD(P)H

As already stated, nimesulide is a protonophoretic uncoupler and oxidizes NAD(P)H in isolated rat liver mitochondria (Mingatto et al., 2000). The lack of responses of the organelles to 4-hydroxy nimesulide concerning stimulation of state 4 respiration (Fig. 1A) and Δψ dissipation (Fig. 1B) indicates that this metabolite does not possess the uncoupling property of its parent drug. This suggests that the hydroxyl substitution in nimesulide affects either its hydrophobicity or its electron-withdrawing character, both properties closely associated with uncoupling activity. Yet, 4-hydroxy nimesulide maintained the ability of nimesulide to oxidize NAD(P)H, as was demonstrated by the fluorescence decrease reversed by isocitrate, which restores mitochondrial NADPH from $NADP^+$ (Fig. 2).

3.2. Unlike nimesulide, 4-hydroxy nimesulide induces mitochondrial permeability transition/associated Ca^{2+} efflux and Δψ dissipation, closely following protein thiol oxidation

Mitochondrial permeability transition is mediated by the opening of permeability transition pores, as demonstrated by cyclosporine A-sensitive swelling of the isolated organelles. It is induced in response to Ca^{2+} and different agents, a combination that triggers conformational changes in mitochondrial membrane proteins (Zoratti and Szabò, 1995; Kowaltowski et al., 2001; Kim et al., 2003; Halestrap, 2005). Mitochondrial permeability transition has been generally considered to play an important role in cell necrosis and apoptosis (Skulachev, 2000; Kim et al.,

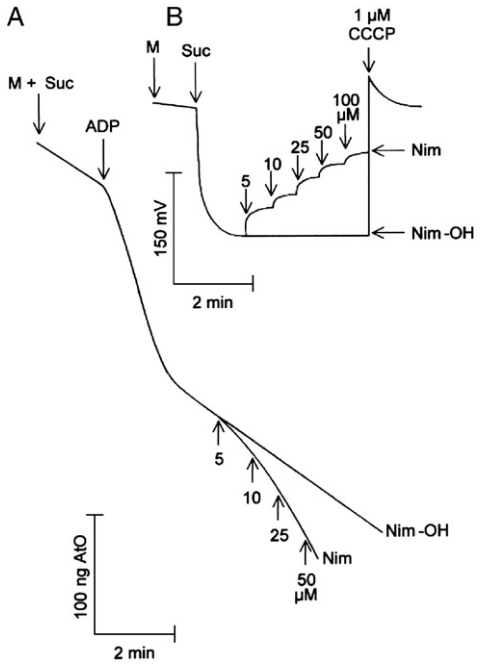

Fig. 1. Effects of nimesulide (Nim) and 4-hydroxy nimesulide (Nim-OH) on state 4 respiration (A) and Δψ (B) in succinate-energized isolated rat liver mitochondria (M). In the respiratory assays, mitochondria (1.5 mg protein) were incubated at 30 °C with 5 mM succinate+2.5 μM rotenone (Suc) in a standard medium containing 125 mM sucrose, 65 mM KCl and 10 mM HEPES–KOH, pH 7.4, in the presence of 0.5 mM EGTA and 10 mM K_2HPO_4, at a final volume of 1.5 ml; state 3 respiration was initiated with 0.4 μmol ADP. In the Δψ assay, mitochondria (2 mg protein) incubated in the standard medium plus 2.5 μM rotenone and 0.4 μM rhodamine 123, at a final volume of 2 ml, were energized by the addition of 5 mM succinate (Suc). Tracings are representative of five experiments with different mitochondrial preparations. CCCP: uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone.

2003; Kroemer, 2003). Both succinate-energized (Fig. 3A) and non-energized mitochondria (results not shown) underwent extensive swelling in the presence of 4-hydroxy nimesulide plus 10 μM Ca^{2+}. Maximal extent of this process at each concentration tested was attained after a 10 min incubation period (time-course tracings not shown); the EC_{50} value was 5.07 μM±0.08. The observed inhibition of swelling by cyclosporine A, as well as its strictly dependence on Ca^{2+} (no swelling being observed in the presence of EGTA), tends to characterize the process as mitochondrial permeability transition. Fig. 3B and C show respectively, 4-hydroxy nimesulide-induced release of Ca^{2+} from mitochondria and $\Delta\psi$ dissipation. The inhibition of these processes by cyclosporine A (Fig. 3B-1 and C-1, respectively), indicates that they result from mitochondrial permeability transition.

Oxidation of mitochondrial protein thiols elicited by 4-hydroxy nimesulide under swelling conditions, is shown in Fig. 3D. This process was not inhibited by cyclosporine A, an indication that it may not result from mitochondrial permeability transition. Nevertheless, the pattern of the concentration–response curves for this effect (EC_{50} value of 5.53 μM±0.33) that closely followed the curves describing mitochondrial swelling, suggests that protein thiol oxidation may indeed to be the cause of mitochondrial permeability transition elicited by 4-hydroxy nimesulide. This conclusion is in agreement with the concept that conformational change of mitochondrial proteins associated with the oxidation of structural thiol groups is an important mechanism involved in mitochondrial permeability transition (Zoratti and Szabò, 1995; Kowaltowski et al., 2001; Kim et al., 2003).

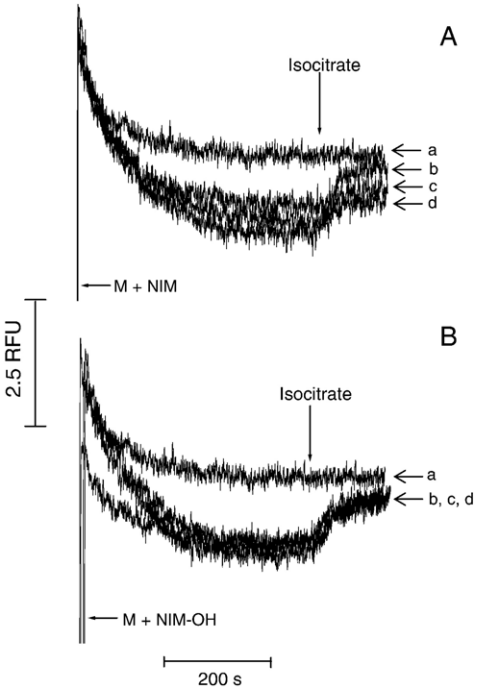

Fig. 2. Effects of nimesulide (Nim, A) and 4-hydroxy nimesulide (Nim-OH, B) on NADPH oxidation (reversed by 1 mM isocitrate) in succinate-energized isolated rat liver mitochondria. Mitochondria (M, 2.0 mg protein) were incubated at 30 °C with 5 mM succinate+2.5 μM rotenone in a standard medium containing 125 mM sucrose, 65 mM KCl and 10 mM HEPES–KOH, pH 7.4, in a final volume of 2 ml. Experimental conditions were: a) control; b) 0.5 μM Nim or Nim-OH; c) 1.0 μM Nim or Nim-OH; d) 2 μM Nim or Nim-OH. Isocitrate (1 mM) was added as indicated. Tracings are representative of three experiments with different mitochondrial preparations. RFU: relative fluorescence units.

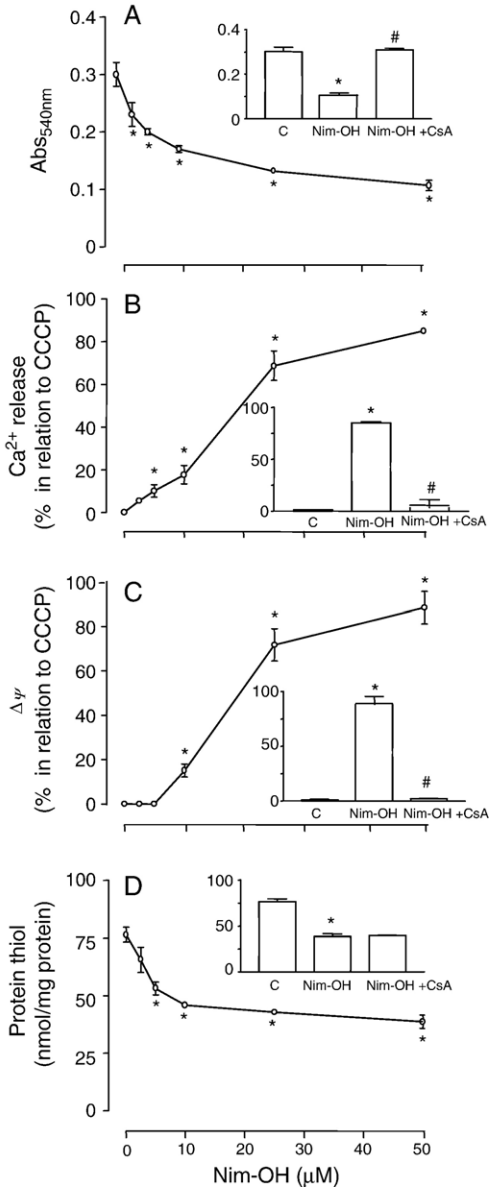

Fig. 3. Concentration–response curves for 4-hydroxy nimesulide (Nim-OH)-induced swelling (A), Ca^{2+} release (B) and $\Delta\psi$ dissipation (C) represented as percentage in relation to responses elicited by the uncoupler CCCP (100%), and protein thiol oxidation (D), in succinate-energized isolated rat liver mitochondria (1 mg protein/ml, except for swelling, 0.4 mg protein/ml), incubated in the standard medium described in legend of Fig. 1, in the presence of 10 μM $CaCl_2$. Inserts: effects of cyclosporine A (CsA) on respective responses induced by 25 μM Nim-OH, in relation to controls in absence of the metabolite (c). The extents of responses were estimated 10 min after Nim-OH addition, when the swelling tracings reached a plateau. Data are presented as the means±s.e.m. of a series of 5 experiments with different mitochondrial preparations. *,#Significantly different ($P<0.05$) from controls and from Nim-OH, respectively.

3.3. 4-Hydroxy nimesulide apparently does not cause GSH oxidation nor H_2O_2 accumulation

Oxidation of GSH and accumulation of respiratory chain-generated reactive oxygen species have often been implicated

in mitochondrial permeability transition. Yet, we were unable to detect in mitochondria exposed to 4-hydroxy-nimesulide over the swelling period, significant changes of GSH/GSSG ratio (Fig. 4A) or of H_2O_2 levels, the later being estimated with Amplex Red (Fig. 4B), or alternatively assayed by H_2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) oxidation or luminol-derived chemiluminescence (results not shown). In this connection, two classes of sites have been postulated as being involved in the mitochondrial permeability transition: one remaining in close equilibrium with glutathione and acting mostly as a H_2O_2-detoxifying agent, and another, in apparent redox equilibrium with pyridine nucleotides, the thioredoxin/thioredoxin reductase behaving as a redox system using NADPH, and showing potential capability to reduce protein thiols (Costantini et al., 1996). By considering this hypothesis, 4-hydroxy nimesulide-induced mitochondrial permeability transition may result from NADPH oxidation *via* the later class of these sites.

3.4. Relationships between NAD(P)H oxidation, protein thiol oxidation, and mitochondrial permeability transition/associated processes induced by 4-hydroxy nimesulide

In addition to GSH oxidation and accumulation of respiratory chain-generated reactive oxygen species, NAD(P)H oxidation has long been implicated in the mitochondrial permeability transition mechanism (Costantini et al., 1996; Nieminen et al., 1997; Kowaltowski et al., 2001). The ratios of mitochondrial GSSG/GSH and NAD(P)/NAD(P)H, substrates of the antioxidant enzymes glutathione peroxidase and glutathione reductase, respectively, are thought to induce the process when the shift of the redox state toward a more oxidized condition permits mitochondria to accumulate respiratory chain-generated reactive oxygen species. Agents allowing mitochondrial NAD(P)H oxidation, like acetoacetate, which is enzymatically reduced to β-hydroxybutirate by pyridine nucleotides, and *tert*-butyl hydroperoxide, a generator of alkoxyl or peroxyl radicals, have been well established. Both agents can elicit mitochondrial permeability transition in the presence of Ca^{2+} *via* membrane protein thiol cross linking subsequent to reactive oxygen species-mediated oxidation of these groups (Kowaltowski et al., 2001). Although we were unable to detect an increase of H_2O_2 levels in mitochondria exposed to 4-hydroxy nimesulide, an eventual involvement of respiratory chain-generated reactive oxygen species in mitochondrial permeability transition elicited by this metabolite should not be ruled out. In this case, the protein thiols undergoing oxidation would be located close to the site at which reactive oxygen species are generated, leading to lack of accumulation of these species and perhaps explaining the lack of H_2O_2 detection in our assays.

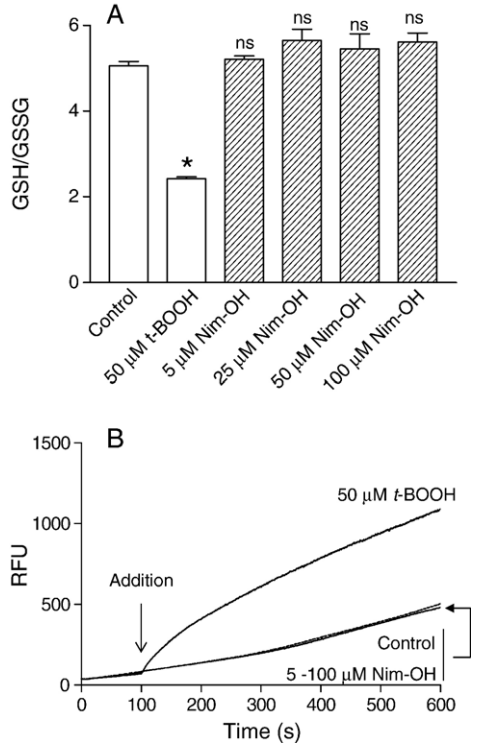

Fig. 4. Lack of effects of 4-hydroxy nimesulide on GSH oxidation (A) and H_2O_2 accumulation (B) in succinate-energized isolated rat liver mitochondria. Mitochondria (M, 2.0 mg protein) were incubated at 30 °C with 5 mM succinate +2.5 µM rotenone in a standard medium containing 125 mM sucrose, 65 mM KCl and 10 mM HEPES–KOH, pH 7.4, in a final volume of 2 ml. Experimental conditions are described in Materials and methods. Tracings are representative of three experiments with different mitochondrial preparations. RFU: relative fluorescence units. *t*-BHP: *t*-butyl hydroperoxide, a positive control. *,nsSignificantly and non-significantly different ($P<0.05$) from control, respectively ($n=3$).

3.5. The potential toxicity of nimesulide on liver mitochondria includes the induction of mitochondrial permeability transition by 4-hydroxy nimesulide

Nimesulide has a potential toxicity on liver, and mitochondria are probably involved in this effect. In our previous reports (Mingatto et al., 2000; Mingatto et al., 2002) we proposed that this potential toxicity involves mitochondrial uncoupling, but concluded that as an uncoupler, nimesulide only elicits mitochondrial permeability transition when Ca^{2+} efflux from mitochondria is inhibited by rutheniun red. Recent reports have, however, proposed that nimesulide causes mitochondrial permeability transition *per se* (Ong et al., 2005; Tay et al., 2005). By considering our present results together with those early reported (Mingatto et al., 2000), we suggest that nimesulide may induce mitochondrial permeability transition *in vivo via* its 4-hydroxy metabolite, and that NADPH oxidation is the most likely cause. The exact mechanism of NADPH oxidation by 4-hydroxy nimesulide is not clear, although recent studies from our laboratory employing electrochemical analysis (unpublished results), have demonstrated such a potential capability of nimesulide and its 4-hydroxy metabolite.

3.6. Nimesulide and 4-hydroxy nimesulide effects on mitochondria of HepG2 cells

The question of the cytotoxicity of nimesulide, *via* mechanisms involving or not mitochondria, still remains controversial. Different effects of nimesulide/metabolites have been reported according to the type of cell and/or experimental procedures employed. For example, Rainsford et al. (2001), did not find

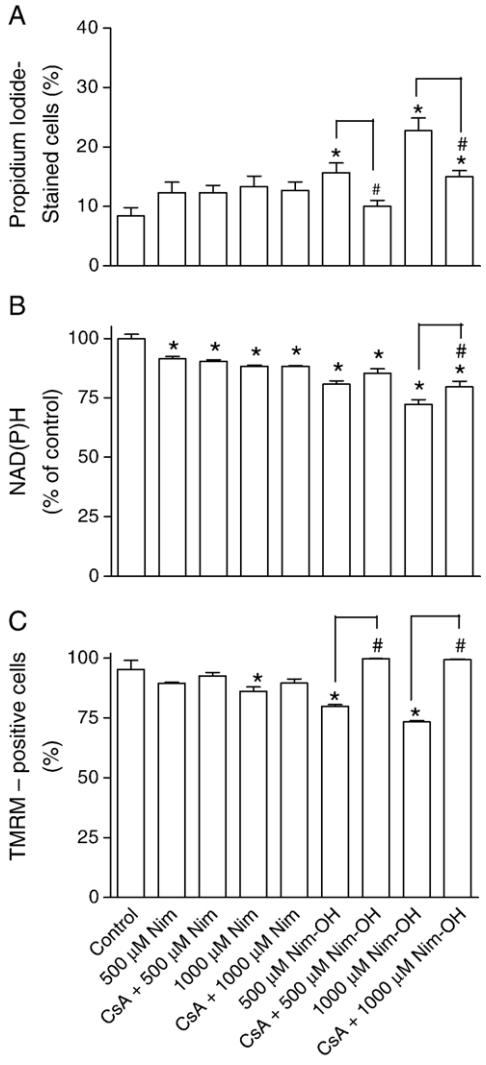

Fig. 5. Effects of nimesulide (Nim) and 4-hydroxy nimesulide (Nim-OH) on (A) cell viability, (B) NAD(P)H oxidation (reversed by isocitrate) and (C) mitochondrial membrane potential. HepG2 cells were treated for 30 h with Nim or Nim-OH (500 or 1000 μM), in the absence or presence of 5 μM cyclosporine A. Cell viability was assessed by propidium iodide/annexin assay, NAD(P)H by fluorescence decrease reversed by 1 mM isocitrate, and mitochondrial membrane potential by TMRM (tetramethyrodamine, methyl ester) positive fluorescence, using a flow cytometer, as described in Materials and methods. Data are presented as the means±s.e.m. of a series of 3 experiments. *,#Significantly different ($P<0.05$) from controls and from Nim-OH, respectively.

any appreciable cytotoxic effect of nimesulide or its major metabolites on HepG2 cells using the MTT dye technique, while Mingatto et al. (2002), by assessing cell LDH leakage, found a decrease of viability of isolated rat hepatocytes exposed to nimesulide. Ong et al. (2005) observed that oxidative damage to mitochondria in *Sod2*$^{+/-}$ mice is capable of aggravating the extent of liver cell's apoptosis. Berson et al. (2006), using propidium iodide, detected a nimesulide-induced hepatocyte-derived HUH-7 cells death by necrosis in the absence, but not in the presence of albumin. In the present study we employed the propidium iodide/annexin V assay to evaluate HepG2 cell death by necrosis or apoptosis, observing that while nimesulide did not cause significant death of cells cultured in the presence of albumin, but assayed in its absence, 4-hydroxy nimesulide causes a low extent (approximately 15%) of cell necrosis (Fig. 5A). This action was prevented to a significant extent by cyclosporine A, an indication of the involvement of the mitochondrial permeability transition. Both nimesulide and 4-hydroxy nimesulide caused NADPH oxidation (Fig. 5B) and dissipation of mitochondrial membrane potential (Fig. 5C) in HepC2 cells. In agreement with the results on isolated mitochondria, membrane potential dissipation by the metabolite was almost completely inhibited by cyclosporine A, and probably results from the mitochondrial permeability transition. Therefore, mitochondrial permeability transition, in apparent association with NAD(P)H oxidation, constitutes the most probable cause of HepG2 cell death by 4-hydroxy nimesulide. In fact, mitochondrial permeability transition has often been implicated in cell death by either necrosis or apoptosis (Kim et al., 2003). We therefore propose that mitochondrial permeability transition induced by 4-hydroxy nimesulide is an additional, potentially toxic mechanism of nimesulide on the liver. However, it should be considered that although 4-hydroxy nimesulide is nimesulide's major metabolite, it does only account for approximately 20% of the drug's metabolites (Carini et al., 1998), it is therefore possible that its effect is essentially manifested only under special circumstances such as idiosyncratic drug hepatotoxicity. In addition, the concentrations of nimesulide/metabolite in our study with cells are higher than the concentrations affecting isolated mitochondria or the therapeutic plasmatic levels (Gandini et al., 1991). While these concentrations are unlikely to find clinically, it should be considered that nimesulide is greatly metabolized and cleared by liver (Boelsterli, 2002), and therefore it is interesting to establish the critical concentrations required for its toxicity upon liver cells, as well as the potential mechanisms involved in susceptible conditions like long-term exposure of the organ to nimesulide or hepatic insufficiency interfering with its elimination (Bernareggi, 1998; Boelsterli, 2002).

Acknowledgments

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