The Critical Role of Mitochondrial Energetic Impairment in the Toxicity of Nimesulide to Hepatocytes

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Received May 8, 2002; accepted July 8, 2002

ABSTRACT

We described the effects of nimesulide (N-[4-nitro-2-phenoxypyphenyl]-methanesulfonamide) and its reduced metabolite in isolated rat hepatocytes. Nimesulide stimulated the succinate-supported state 4 respiration of mitochondria, indicating an uncoupling effect of the drug. Incubation of hepatocytes with nimesulide (0.1–1 mM) elicited a concentration- and time-dependent decrease in cell viability as assessed by lactate dehydrogenase leakage, a decrease of mitochondrial membrane potential as assessed by rhodamine 123 retention, and cell ATP depression. Nimesulide also decreased the levels of NAD(P)H and glutathione in hepatocytes, but the extent of the effects was less pronounced in relation to the energetic parameters; in addition, these effects did not imply the peroxidation of membrane lipids. The decrease in the viability of hepatocytes was prevented by fructose and, to a larger extent, by fructose plus oligomycin; it was stimulated by proadifen, a cytochrome P450 inhibitor. In contrast, the reduced metabolite of nimesulide did not present any of the effects observed for the parent drug. These results indicate that: 1) nimesulide causes injury to the isolated rat liver cells, 2) this effect is mainly mediated by impairment of ATP production by mitochondria due to uncoupling, and 3) on account of the activity of its nitro group, the parent drug by itself is the main factor responsible for its toxicity to the hepatocytes.

Nimesulide (N-[4-nitro-2-phenoxypyphenyl]-methanesulfonamide) is a nonsteroidal anti-inflammatory drug with a relative selectivity for cyclooxygenase-2 (Roberts and Morrow, 2001), the use of which is increasing in clinical practice, although concerns have been recently raised regarding its hepatotoxicity (see Fig. 1 for structure). The drug can cause several types of liver damage, ranging from mild abnormal function to severe organ injuries; these effects are usually reversible upon discontinuation of the drug but occasionally can progress to fatal hepatic failure (Ferreiro et al., 2000; Merlani et al., 2001, Montesinos et al., 2001; Sbeit et al., 2001). We previously demonstrated that nimesulide is a powerful protonophoretic uncoupler and NAD(P)H oxidant in isolated rat liver mitochondria, inducing Ca2+ efflux, or mitochondrial permeability transition (MPT) in the presence of ruthenium red, whereas its reduced metabolite lacks these effects (Mingatto et al., 2000). The uncoupling effect of nimesulide on isolated mitochondria was also demonstrated by others (Caparroz-Assef et al., 2001).

Cytotoxic agents in general, including drugs/metabolites, may influence the energetic balance of cells by increasing ATP consumption and/or reducing ATP production. In this regard, ATP depletion is an early event in the course of drug-induced toxicity preceding the irreversible stages of cell injury. It results from electron transport/oxidative phosphorylation inhibition, mitochondrial membrane potential dissipation, and/or Ca2+ homeostasis disruption (Wallace and Starkov, 2000; Szewczyk and Wojtczak, 2002), as well as reactive oxygen species (ROS) generation (Kowaltowski et al., 2001, Qu et al., 2001). ROS are scavenged by the antioxidant defense of cells involving reduced glutathione (GSH), which generates GSSG, whose reduction back to GSH occurs at the expense of NADPH oxidation (Kowaltowski et al., 2001). Cytotoxic agents affecting mitochondria are of particular interest because of the key role of these organelles concerning cell death by necrotic or apoptotic pathways (Kroemer et al., 1998; Wallace and Starkov, 2000; Ferri and Kroemer, 2001; Plas and Thompson, 2002).

ABBREVIATIONS: MPT, mitochondrial permeability transition; ROS, reactive oxygen species; GSH, reduced glutathione; GSSG, glutathione disulfide; Nim, nimesulide; NimH, reduced nimesulide; DTT, dithiothreitol; LDH, lactate dehydrogenase; OPT, o-phthalaldehyde; MDA, malondialdehyde; DPH, 1,6-diphenyl-3,5-hexatriene.
C. In all experiments initial hepa-
0.1% bovine serum albumin, at 4 °C in Krebs-Henseleit buffer, pH 7.4, containing 12.5 mM HEPES and perfusion of the liver (Guguen-Guillouzo, 1992) and suspended in isolated hepatocytes from male Wistar rats weighing 200 to 250 g by collagenase were prepared using glass-distilled deionized water.

reduced nimesulide had no effect on the assays. All stock solutions of dimethyl sulfoxide required to solubilize nimesulide and other reagents were of the highest commercially available grade. The structure of nimesulide (Nim) and its reduced metabolite (NimH).

Fig. 1. Structure of nimesulide (Nim) and its reduced metabolite (NimH).

The liver is the major site for the uptake and metabolism of drugs and, therefore, an important target for their untoward effects (Jaeschke et al., 2002). Within this context, in the present work we evaluated the effects of the nimesulide/reduced metabolite on isolated hepatocytes to establish both its toxicologic potential in vivo and its mechanism involving the mitochondria.

Materials and Methods

Chemicals. Nimesulide was purchased from Sigma-Aldrich (St. Louis, MO), and reduced nimesulide was a gift from Dr. Randy Leavitt, Maxxam Analytics Inc. (Mississauga, ON, Canada). 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 and Incubation of Hepatocytes. Hepatocytes were isolated from male Wistar rats weighing 200 to 250 g by collagenase perfusion of the liver (Guguen-Guillouzo, 1992) and suspended in Krebs-Henseleit buffer, pH 7.4, containing 12.5 mM HEPES and 0.1% bovine serum albumin, at 4 °C. In all experiments initial hepatocyte viability, determined by trypan blue (0.16%) uptake, was more than 85%. For the assays, cells (1 × 10⁶/ml) were incubated in 25-ml Erlenmeyer flasks and kept under constant shaking (30 rpm) at 37 °C. Reactions were started by the addition of Nim or NimH dissolved in dimethyl sulfoxide (final concentration, <1%). Controls were performed with an equivalent volume of dimethyl sulfoxide alone for each condition at each time of evaluation. Aliquots (1 ml) were removed from the suspension at appropriate periods for evaluation of cell viability and biochemical parameters. In some experiments, cells were incubated with 20 mM fructose, 5 μM cyclosporin A, 0.1 mM probiduran, or 4 mM DTT, 15 min before the addition of Nim. Oligomycin (10 μg/ml), when used, was added at the same time as Nim.

Oxygen Uptake. Oxygen uptake by the isolated hepatocytes was monitored polarographically with an oxygraph equipped with a Clark-type oxygen electrode (Gilson Medical Electronics, Middleton, WI), at 37 °C. Respiration buffer contained 250 mM sucrose, 2 mM KH₂PO₄, 10 mM HEPES, pH 7.2, 0.5 mM EGTA, 0.5% bovine serum albumin, and 5 mM MgCl₂. Cells were treated with 0.002% digitonin, and state 4 and state 3 mitochondrial respiration rates were measured in the presence of 1 μg/ml oligomycin and 2 mM ADP, respectively (Moreadith and Fisckum, 1984).

Cell Viability. After centrifugation of the cell suspension at 50g for 5 min at 4 °C, hepatocyte viability was assessed in the supernatant on the basis of the leakage of lactate dehydrogenase (LDH) determined at 340 nm with a DU-70 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA), according to the manufacturer’s kit instructions (Labtest Diagnóstica S.A.; Lagoa Santa, MG, Brasil). LDH leakage is expressed as a percentage of the total leakage as obtained in cells treated with 0.5% Triton X-100.

Mitochondrial Membrane Potential. Mitochondrial membrane potential was estimated on the basis of cell retention of the fluorescent cationic probe rhodamine 123 (Lemasters et al., 1993; Nakagawa and Moore, 1999). The cell suspension was incubated with 1 μM rhodamine 123 for 10 min before the addition of Nim or NimH, centrifuged at 50g for 5 min at 4 °C, washed with Krebs-Henseleit solution, and resuspended in 1 ml of 0.1% Triton X-100. After centrifugation at 2000g for 5 min, rhodamine 123 was determined in the supernatant with an F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) at the 505/535-nm excitation/emission wavelength pair. The results are expressed as percentage of the fluorescence values for control (untreated) hepatocytes.

Cell ATP Content. Cell ATP was determined by means of the firefly luciferin-luciferase assay system. The cell suspension was centrifuged at 50g for 5 min at 4 °C, and the pellet containing the hepatocytes was treated with 1 ml of ice-cold 1 M HClO₄. After centrifugation at 2000g for 10 min at 4 °C, aliquots (100 μl) of the supernatants were neutralized with 70 μl of 2 M KOH, suspended in 100 mM Tris-HCl, pH 7.8 (1 ml final volume), and centrifuged again. Bioluminescence was measured in the supernatant with a Sigma-Aldrich assay kit according to the manufacturer’s instructions using an AutoLumat LB 953 luminescence photometer (PerkinElmer Life Sciences, Wildbad, Germany).

NAD(P)H Levels. The cell suspension was centrifuged at 50g for 5 min at 4 °C, and the pellet was washed with Krebs-Henseleit solution and resuspended in 1 ml of 0.1% Triton X-100. NAD(P)H was determined with an F-4500 fluorescence spectrophotometer (Hitachi) at the 366/450-nm excitation/emission wavelength pair. The results are expressed as percentage of the fluorescence values for control (untreated) hepatocytes.

GSH and GSSG Levels. The levels of GSH and GSSG were determined by fluorometric reaction with o-phthalaldehyde (OPT) (Hissin and Hilf, 1976). The cell suspension was treated with 0.2 ml of 30% trichloroacetic acid and centrifuged at 2000g for 6 min. For GSH levels, aliquots (100 μl) of the supernatant were mixed with 1 ml of 100 mM NaH₂PO₄ buffer, pH 9.0, containing 5 μM EDTA. OPT (100 μl, 1 mg/ml) was added, and fluorescence was measured 15 min later using the 350/420-nm excitation/emission wavelength pair.
with an F-4500 fluorescence spectrophotometer (Hitachi). For GSSG levels, the supernatant was treated with 20 mM N-ethylmaleimide, which reacts with free thiol groups. Aliquots (100 µl) of samples were mixed with 1 ml of 1 M NaOH followed by OPT.

**Lipid Peroxidation.** Lipid peroxidation was assayed as malondialdehyde (MDA) generation. After centrifugation of the cell suspension at 50g for 5 min at 4°C, the pellet was treated with 1 ml 1% of TBA (dissolved in 50 mM NaOH), 0.1 ml of 10 M NaOH, and 0.5 ml of 20% H₃PO₄, followed by incubation at 85°C for 20 min. The reaction mixture was cooled, the MDA-TBA complex formed was extracted with 2 ml of n-butanol, and absorbance was measured at 535 nm with a DU-70 spectrophotometer (Beckman Coulter Inc.). The MDA concentration was calculated from $C = 150,000 \text{ M}^{-1} \text{ cm}^{-1}$.

**Fluorescence Response of DPH (1,6-Diphenyl-1,3,5-hexatriene)-Labeled Mitochondrial Membrane.** Membranes labeled with fluorophores produce a fluorescence response ($F$) whose static quenching can be described by the Stern-Volmer equation: $F/F_0 = 1 + K_{SV} [Q]$, where $F_0$ and $F$ are fluorescence intensities in the absence and presence of the quencher, respectively. Mitochondria were incubated for 30 min, at 37°C, with 0.5 µM DPH before Nim or NimH was added. Fluorescence was measured with an F-4500 spectrofluorometer (Hitachi) at excitation and emission wavelengths of 377 and 431 nm, respectively.

**Statistical Analysis.** Comparisons of the several treated groups and the relative controls were made by analysis of variance followed by the Dunnett’s test. Comparison between one control group and a single treated group was made by Student’s t test.

**Results**

**Effects of Nimesulide and Its Reduced Metabolite on Respiration of Mitochondria in Isolated Rat Hepatocytes.** Figure 2 shows the stimulation of succinate-supported state 4 (basal) respiration of mitochondria in digitonin-permeabilized hepatocytes by nimesulide in the presence of oligomycin. The effect was immediate and concentration-dependent, beginning already at a 0.025 mM concentration of the drug; at 0.15 mM its extent was comparable with state 3 (ADP-stimulated) respiration. The reduced metabolite did not stimulate state 4 respiration (Fig. 2), and neither nimesulide nor the reduced metabolite inhibited state 3 respiration (results not shown). These results indicate that neither the drug nor the reduced metabolite inhibits the respiratory chain, whereas the parent drug effectively uncouples the oxidative phosphorylation of mitochondria as assessed in isolated hepatocytes. The nimesulide-induced stimulation of state 4 respiration was completely inhibited by the respiratory chain inhibitors antimycin A and KCN (results not shown), indicating that the observed stimulation of oxygen consumption was indeed due to the uncoupling.

**Nimesulide-Induced Injury of Isolated Rat Hepatocytes.** Addition of increasing concentrations of nimesulide to hepatocytes resulted in decreased cell viability as assessed by LDH leakage into the incubation medium (Fig. 3A). LDH leakage was concentration- and time-dependent, with a significant increase being observed at the nimesulide concentration of 0.5 mM at 60 min incubation. A close correlation is evident between this response and the decrease in cell viability (Fig. 3A), suggesting a cause-effect relationship. Indeed, a decrease in ATP levels occurred already at 30 min incubation of cells with a 0.25 mM concentration of the drug (Fig. 3C). The rapid ATP depression preceded decrease of cell viability, which began to be noticeable only 60 min after the drug. At 90 min incubation with 0.5 mM or higher concentrations of drug, when almost all cells lost viability (Fig. 3A), ATP was practically depleted.

**Effects of Nimesulide on the Antioxidant Defense of Isolated Rat Hepatocytes.** The antioxidant defense of hepatocytes in the presence of nimesulide was evaluated by measuring the levels of NAD(P)H and GSH, as well as MDA, as an index of lipid peroxidation. Time course curves closely similar to those for the energetic parameters (Fig. 3, B and C) were obtained for the nimesulide-induced decrease of NAD(P)H (Fig. 4A) and GSH (Fig. 4B), but the extent of the effects was, in general, less pronounced. In addition, GSSG hepatocytes also resulted in a decrease in mitochondrial membrane potential, as estimated by retention of rhodamine 123 by the cells (Fig. 3B). The effect was concentration- and time-dependent, with a significant decrease being observed at the nimesulide concentration of 0.5 mM at 60 min incubation. A close correlation is evident between this response and the decrease in cell viability (Fig. 3A), suggesting a cause-effect relationship. Indeed, a decrease in ATP levels occurred already at 30 min incubation of cells with a 0.25 mM concentration of the drug (Fig. 3C). The rapid ATP depression preceded decrease of cell viability, which began to be noticeable only 60 min after the drug. At 90 min incubation with 0.5 mM or higher concentrations of drug, when almost all cells lost viability (Fig. 3A), ATP was practically depleted.

**Effects of Nimesulide on Mitochondrial Membrane Potential and ATP Levels in Isolated Rat Hepatocytes.** Addition of increasing concentrations of nimesulide to the
levels did not significantly increase and the thiol group protector, DTT, did not prevent the GSH level decrease (results not shown). Accordingly, this decrease in the antioxidant defense of the cells, NAD(P)H and GSH, did not imply peroxidation of membrane lipids (Fig. 4C).

Effects of Fructose, Fructose plus Oligomycin, and Cyclosporin A on Nimesulide-Induced Injury of Isolated Rat Hepatocytes and ATP Levels. Fructose, an efficient substrate for glycolytic ATP formation in hepatocytes, protects against loss of cell viability by mitochondrial impairment, and such protection implies that cytotoxicity involves the inhibition of nonglycolytic mitochondrial ATP formation. Also, because uncouplers stimulate ATPase, increasing the rate of ATP hydrolysis, its inhibition by oligomycin compensates the decrease of cell ATP levels (Nieminen et al., 1994, Masubuchi et al., 2000). In this regard, the decrease of both cell viability and ATP levels induced by nimesulide was prevented by the incubation of hepatocytes with fructose and, to a larger extent, by fructose plus oligomycin (Fig. 5), indicating that a mitochondrial energetic impairment indeed has a critical role in nimesulide-induced hepatotoxicity and that uncoupling is involved. On the other hand, no significant effect of cyclosporin A was observed on cell viability (result not shown), indicating that MPT, which is a Ca²⁺-dependent, cyclosporin A-sensitive permeability transition of the mitochondrial membrane (Zoratti and Szabó, 1995), was not significantly involved.

Comparison of the Effects of Nim and NimH on Isolated Rat Hepatocytes. Figure 6 shows the comparative effects of nimesulide and its reduced metabolite on the above parameters, namely cell viability, mitochondrial membrane potential, and levels of NAD(P)H, GSH, and ATP. The reduced metabolite of nimesulide did not present any of the effects observed for the parent drug in isolated hepatocytes.
Effect of Inhibition of Nimesulide Metabolism on Injury of Isolated Rat Hepatocytes.

Nimesulide-induced LDH leakage was evaluated in the presence or absence of proadifen, a cytochrome P450 inhibitor (Fig. 7). Proadifen stimulated the nimesulide-induced cell injury, suggesting that the parent drug by itself is the main factor responsible for the toxic effect on isolated hepatocytes. The greatest effect was observed at low drug concentrations, namely at 0.1 and 0.25 mM.

Discussion

Previously, we demonstrated that nimesulide is a potent protonophoretic uncoupler and NAD(P)H oxidant in isolated rat liver mitochondria. As for the low concentration range in which the effects were observed, we proposed that nimesulide has a potential ability to interfere in vivo with the liver cells (Mingatto et al., 2000). Indeed, the present results on isolated hepatocytes show that nimesulide is an effective uncoupling agent also in situ, as demonstrated by its ability to stimulate the state 4 (basal) respiration of mitochondria when the respiratory substrate, succinate, was added to the digitonin-permeabilized cells. All the other assays were performed in nonpermeabilized cells incubated in Krebs-Henseleit medium, which includes glucose as an energy source. The results show, in general, that nimesulide, but not its reduced metabolite, is toxic to the isolated hepatocytes: almost all the cells lost viability as early as 90 min after a 0.5 mM concentration of drug. In parallel, as potential causes for the cell injury, nimesulide induced concentration- and time-dependent decrease of the mitochondrial membrane potential and depression of the intracellular levels of ATP, NAD(P)H, and GSH.

It is well established that depression of the intracellular ATP levels is a critical event for development of cell damage by necrosis (Wallace and Starkov, 2000; Szewczyk and Wojtczak, 2002). In this regard, from the biochemical parameters evaluated in this study, the cell ATP levels were the first to be affected by nimesulide, preceding loss of cell viability, suggesting that their depression is, at least, the main cause...
of it. As for the evidence that nimesulide stimulates state 4 respiration and decreases membrane potential of mitochondria it seems likely that ATP depletion results mainly from the uncoupling of oxidative phosphorylation. Accordingly, when fructose, a substrate for the glycolytic pathway in liver that prevents ATP depletion and cell damage induced by toxic compounds (Wu et al., 1990; Nieminen et al., 1994), was added to the cell suspension, a significant protection against both the nimesulide-induced injury of hepatocytes and ATP level decrease occurred, and the additional presence of oligomycin stressed this effect. On the other hand, the lack of a significant protection of cyclosporin A against cell injury indicates that MPT onset (Zoratti and Szabó, 1995), as observed in isolated mitochondria, is not significantly involved. In this regard, in isolated mitochondria, MPT only was triggered by nimesulide in the presence of ruthenium red, an inhibitor of the Ca\(^{2+}\) transport by the uniporter, a condition not available in the incubation medium used in this study and also in vivo (Mingatto et al., 2000).

In addition to its uncoupling effect on mitochondria, nimesulide is a powerful NAD(P)H oxidant (Mingatto et al., 2000), and, therefore, the nimesulide-induced decrease of both NAD(P)H and GSH levels in the isolated hepatocytes might be due to oxidation of the former by the drug, in such a way that reduction of GSSG back to GSH is impaired. However, the evidence that the decrease in GSH levels was not accompanied by a significant increase in GSSG levels and that DTT does not protect against it suggests that GSH levels decrease mainly due to conjugation with nimesulide metabolites, as previously reported (Bernareggi, 1998; Carini et al., 1998). Within this context, since the respiratory chain of mitochondria is an important intracellular source of ROS, such a decrease of the antioxidant defense of cells would be expected to accumulate ROS and, consequently, to cause peroxidation of the membrane lipids. On the other hand, it has been well established that lowering the mitochondrial membrane potential by uncoupling inhibits the generation of ROS by the respiratory chain (Kowaltowski et al., 2001). Therefore, since nimesulide both oxidizes NAD(P)H and uncouples oxidative phosphorylation, we believe that the decrease of the cell antioxidant defense due to nimesulide is counterbalanced by the lower generation of ROS due to uncoupling, thus preventing membrane lipid peroxidation.

Five nimesulide metabolites have been identified in humans. Of these, only one, hydroxynimesulide, keeps the nitro group in its structure (Carini et al., 1998). As to the observed stimulation of the nimesulide-induced cell injury by proadifen, a known cytochrome P450 inhibitor, and the observed lack of toxic effects with regard to the evaluated parameters for the reduced metabolite, we believe that the toxicity of nimesulide to hepatocytes is mainly due to the parent drug and that the nitro group in its structure is responsible for it.

The nimesulide concentrations affecting the isolated hepatocytes in this study (0.25–1 mM) are higher than the concentrations affecting isolated mitochondria (5–25 μM) (Mingatto et al., 2000) and than the therapeutic plasma levels of drug (20–50 μM) (Gandini and Montalto, 1991). However, two points should be considered: first, since nimesulide is largely eliminated via metabolic transformation, its pharmacokinetics may be affected by hepatic insufficiency (Bernareggi, 1998); second, nimesulide can accumulate into hydrophobic regions such as the interior of membranes, as demonstrated for the isolated nonenergized mitochondria themselves by the Stern-Volmer constants for the drug-induced quenching of the membrane-incorporated DPH fluorescence. The values obtained, 37.35 ± 0.68 and 2.75 ± 0.44 nM\(^{-1}\) for Nim and NimH, respectively, indicate that the drug, but not its reduced metabolite, has the ability to penetrate deeply into the hydrophobic region of membranes.

In conclusion, the present results indicate that nimesulide causes injury to the isolated rat liver cells and that this toxic effect is mediated by impairment of ATP production by mitochondria due to uncoupling of oxidative phosphorylation. They also show that, on account of the activity of its nitro group, the parent drug by itself is the main factor responsible for its toxicity to the hepatocytes.

References


Fig. 7. Effects of proadifen (P) on the time course of nimesulide (Nm)-induced decrease of viability of isolated rat hepatocytes. C, control. Results are mean ± S.E.M. of three experiments with different cell preparations. *, **, significantly different from “without proadifen” for the corresponding time points (P < 0.05 and P < 0.01, respectively).
Toxicity of Nimesulide to Mitochondria of Hepatocytes


