The Anti-Inflammatory Drug, Nimesulide (4-Nitro-2-phenoxymethane-sulfoanilide), Uncouples Mitochondria and Induces Mitochondrial Permeability Transition in Human Hepatoma Cells: Protection by Albumin

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ABSTRACT

Like other nonsteroidal anti-inflammatory drugs, nimesulide (4-nitro-2-phenoxymethane-sulfoanilide) triggers hepatitis in a few recipients. Although nimesulide has been shown to uncouple mitochondrial respiration and cause hepatocyte necrosis in the absence of albumin, mechanisms for cell death are incompletely understood, and comparisons with human concentrations are difficult because 99% of nimesulide is albumin-bound. We studied the effects of nimesulide, with or without a physiological concentration of albumin, in isolated rat liver mitochondria or microsomes and in human hepatoma cells. Nimesulide did not undergo monoelectronic nitro reduction in microsomes. In mitochondria incubated without albumin, nimesulide (50 \mu M) decreased the mitochondrial membrane potential (\Delta \psi_m), increased basal respiration, and potentiated the mitochondrial permeability transition (MPT) triggered by calcium preloading. In HUH-7 cells incubated for 24 h without albumin, nimesulide (1 mM) decreased the \Delta \psi_m and cell NAD(P)H and increased the glutathione disulfide/reduced glutathione ratio and cell peroxides; nimesulide triggered MPT, ATP depletion, high cell calcium, and caused mostly necrosis, with rare apoptotic cells. Coincubation with either cyclosporin A (an MPT inhibitor) or the combination of fructose-1,6-diphosphate (a glycolysis substrate) and oligomycin (an ATPase inhibitor) prevented the decrease in \Delta \psi_m, ATP depletion, and cell death. A physiological concentration of albumin abolished the effects of nimesulide on isolated mitochondria or HUH-7 cells. In conclusion, the weak acid, nimesulide, uncouples mitochondria and triggers MPT and ATP depletion in isolated mitochondria or hepatoma cells incubated without albumin. However, in the presence of albumin, only a fraction of the drug enters cells or organelles, and uncoupling and toxicity are not observed.

Nimesulide (4-nitro-2-phenoxymethane-sulfoanilide) is a nonsteroidal anti-inflammatory drug (NSAID) with a preferential cyclooxygenase-2 inhibitory activity (Warner et al., 1999) and a multifactorial mode of action (Bennett and Villa, 2000), characterized by a rapid onset of pain relief (Bianchi and Broggi, 2003).

Like all other NSAIDs, nimesulide has been associated with cases of adverse hepatic reactions in a few recipients (Van Steenbergen et al., 1998). Although cholestatic hepatitis has occurred in a few patients, nimesulide most usually induces hepatocellular liver injury, with liver cell necrosis as the main liver lesion. Although a few patients with nimesu-
lidade-associated liver injury have exhibited features possibly consistent with an immunologic mechanism, hypersensitivity manifestations were absent in most patients, suggesting metabolic idiosyncrasy rather than immunologic (Van Steenbergen et al., 1998).

Mitochondria are important targets for drug-induced hepatotoxicity (Pessayre et al., 2002). In particular, most acidic NSAIDs can transude protons across the inner mitochondrial membrane (Moreno-Sanchez et al., 1999). The re-entry of protons into the mitochondrial matrix decreases the mitochondrial membrane potential (ΔΨ_m) and unleashes the flow of electrons in the respiratory chain, thus stimulating basal respiration (Pessayre et al., 2002). However, this increased respiration is wasted, to produce heat instead of ATP, because protons re-enter the matrix directly through the inner membrane, instead of going through ATP synthase and generating ATP (Pessayre et al., 2002).

In addition to uncoupling mitochondrial respiration from ATP formation, some NSAIDs like salicylic acid or diclofenac can trigger mitochondrial permeability transition (MPT) (Masubuchi et al., 2002). MPT pore opening triggers a massive influx of protons from the intermembrane space into the mitochondrial matrix, thus dissipating the ΔΨ_m and preventing ATP formation by the affected mitochondrion (Pessayre et al., 2002). If the pores open in all mitochondria, major ATP depletion prevents apoptosis (an energy-requiring process) and instead causes necrosis (Pessayre et al., 2002). MPT pore opening also causes mitochondrial swelling and outer membrane rupture, which releases proapoptotic substances from the intermembrane space into the cytosol (Pessayre et al., 2002). If the pore only opens in some mitochondria, then unaffected organelles still generate ATP (thus preventing necrosis), whereas pore opening in other mitochondria releases proapoptotic substances, which activate caspases, to cause apoptosis (Pessayre et al., 2002).

The mitochondrial effects of nimesulide have been previously assessed in isolated liver mitochondria and isolated rat hepatocytes (Moreno-Sanchez et al., 1999; Mingatto et al., 2000, 2002). Nimesulide behaves like a protonophoric weak acid and uncouples mitochondrial respiration (Moreno-Sanchez et al., 1999). Although this effect may be mediated by the sulfonamide moiety of nimesulide (see Discussion), it has been ascribed instead to the nitro group on the basis that the amine derivative formed by the nitro reduction of nimesulide did not uncouple mitochondrial respiration (Mingatto et al., 2000, 2002). Nimesulide was also shown to induce ATP depletion and cell death in isolated hepatocytes incubated for 90 min with a low concentration of albumin (1 mg/ml) (Mingatto et al., 2002). Because cell death was not prevented by the MPT inhibitor, cyclosporin A, it was concluded that the toxicity of nimesulide only involved uncoupling-mediated ATP depletion, but not MPT (Mingatto et al., 2002).

However, the relevance of these previous models in assessing possible mechanisms for nimesulide-induced hepatitis in humans is not certain. If one assumes that sustained mitochondrial uncoupling is required to progressively cause redox changes that eventually trigger MPT, then the use of isolated hepatocytes, which can only withstand short incubation times, may overlook MPT as a possible mechanism of cell toxicity. This hypothesis may be tested using longer incubation times with cultured cell lines. Moreover, previous experiments were performed with a low concentration of albumin. Because nimesulide is highly (99%) bound to proteins (Bernareggi, 1998), the concentrations having an effect in these in vitro systems cannot be directly compared with the total human plasma concentrations of nimesulide.

In the present study, the mitochondrial and cellular effects of nimesulide were tested with or without a physiological albumin concentration in isolated rat mitochondria and human hepatocyte-derived HUH-7 cells cultured for 24 h with nimesulide. In this model, nimesulide-induced cell death was caused by MPT and was completely reversed by physiological concentrations of albumin.

### Materials and Methods

**Chemicals and Animals.** Nimesulide (batch, 22001015; manufacturing date, April 2002; analysis, 2002040724) was provided by Helsinn Healthcare SA (Lugano, Switzerland). Male Sprague-Dawley Crl:CD(SD)BR rats (200–250 g) were from Charles River (Lasbresle, France). Animals were fed ad libitum an equilibrated standard diet (Autoclave 113; UAR, Villemoisson-sur-Orge, France) until sacrifice.

**Preparation of Rat Liver Mitochondria.** Mitochondria were prepared as described previously (Berson et al., 1994). For studying respiration and membrane potential, the isolation buffer was 0.3 M sucrose, 1 mM EGTA, 5 mM MOPS, 5 mM KH PO 4 and 0.1% bovine serum albumin (fatty acid free), pH 7.4 (adjusted with KOH). For studying mitochondrial permeability transition, the isolation buffer was 0.3 M sucrose, 5 mM TES, and 0.2 mM EGTA, pH 7.4 (adjusted with KOH). Rats were killed by cervical dislocation. Livers were removed, placed in ice-cold isolation buffer, minced, and homogenized using three up-and-down strokes of the homogenizer. The suspension was centrifuged for 10 min at 1000g at 4°C. The supernatant was decanted and centrifuged for 10 min at 10,000g at 4°C. The mitochondrial pellet was washed twice and finally resuspended in the same isolation medium at a mitochondrial protein content of approximately 20 mg/ml and kept at 4°C for quick use.

**Mitochondrial Respiration.** Isolated mitochondria (1.4 mg of proteins) were diluted in respiratory medium (1.2 ml) containing 1 mM EDTA, 5 mM MgCl 2, 10 mM KCl, 225 mM sucrose, 10 mM Tris, and 10 mM KH PO 4, pH 7.4, with or without 36 g/l bovine serum albumin (fatty acid free). Mitochondria were incubated with either glutamate and malate (5 mM each) or with succinate (10 mM) and with or without nimesulide. ADP (0.2 mM) was added, and the consumption of oxygen was monitored at 30°C with a Gilson K-IC oxygraph (Middletown, WI), first during the period of ADP consumption (state 3 respiration) and then after ADP exhaustion (state 4 respiration). Oxygen consumption was expressed in nanomoles of oxygen consumed per minute and per milligram of mitochondrial protein.

**ΔΨ_m in Isolated Rat Liver Mitochondria.** The fluorescent dye safranine was used to monitor changes in the ΔΨ_m (Feldmann et al., 2000). Isolated mitochondria (1.4 mg of proteins) were diluted in the respiratory medium (1.2 ml) described above in the presence or in the absence of 36 g/l bovine serum albumin (fatty acid-free) and were preincubated at 30°C with safranine (10 μM). Mitochondria were first energized by succinate (10 mM), causing the entry of safranine into the mitochondrial matrix and fluorescence quenching. We then added nimesulide (50 μM). In the absence of albumin, nimesulide decreased the ΔΨ_m, which increased safranine fluorescence. We compared these effects with those of 160 μM 2,4-dinitrophenol, which totally collapses the ΔΨ_m. The difference in safranine fluorescence before and after the addition of nimesulide (or 2,4-dinitrophenol) was recorded, with excitation at 510 nm and emission at 570 nm.

**MPT in Isolated Rat Liver Mitochondria.** MPT was studied by spectrometry at 550 nm. When mitochondria are incubated in the presence of succrose, MPT pore opening causes succrose plus water to enter inside the mitochondria, and the resulting mitochondrial swell-
ing decreases the absorption of the mitochondrial suspension at 550 nm. Isolated mitochondria (1.4 mg of proteins) were incubated at room temperature in swelling buffer (1 ml) containing 0.2 M sucrose, 10 mM Tris-MOPS, 50 mM EGTA, 5 mM succinate, and 1 mM Pi, pH 7.4 (adjusted with KOH), with or without various compounds as indicated under Results. The opening of the MPT pore was assessed at room temperature by recording the absorption of the mitochondrial suspension at 550 nm.

Preparation of Mouse Liver Microsomes and ESR Studies. Mice were killed by cervical dislocation, and the liver was removed, weighed, minced, and homogenized in 3 volumes of ice-cold 0.15 M KCl and 0.01 M sodium potassium phosphate buffer, pH 7.4. The homogenate was centrifuged at 10,000g for 60 min, and the microsomal pellet was stored at −80°C until used (within 8 days after preparation).

Free radical ESR spectra were recorded with a Bruker ER 200 X-band spectrometer (Bruker BioSpin, Lisses, France) at 22°C (Berson et al., 1990). A 1-ml solution of 0.1 M sodium potassium phosphate buffer containing microsomes (10 mg/ml) and either nimesulide or nifurtimox (5 mM each) was gassed with nitrogen for 10 min in stoppered flasks. The reaction was initiated by adding NADPH (5 mM). A sample (0.5 ml) was transferred anaerobically onto the ESR cell, and spectra were recorded in the microwave band × frequency at 20 mW (Berson et al., 1990).

Cell Culture and Treatments. Human hepatoma HUH-7 cells were maintained in Dulbecco’s modified Eagle’s medium (GlutaMAX I; Gibco-BRL, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum, penicillin G (100 U/ml), streptomycin (100 U/ml), and gentamicin (1 μg/ml) (Gibco-BRL) in a humidified atmosphere in PBS containing 180 mM KCl and 0.01 M sodium potassium phosphate buffer, pH 7.4. The homogenate was centrifuged at 10,000g for 60 min, and the microsomal pellet was stored at −80°C until used (within 8 days after preparation).

LDH Activity. After incubation, the medium was removed, and the remaining, adherent cells were scrapped, washed in phosphate-buffered saline (PBS), and finally disrupted using three cycles of freezing and thawing before centrifugation at 1500g for 3 min. LDH activity was measured both in the culture medium and in the supernatant of disrupted cells, using a commercial kit (bioMerieux, Marcy-l’Etoile, France) and the recommendations of the manufacturer. The percentage of LDH released into the medium was calculated as the ratio of the LDH activity in the medium over the total LDH activity.

Assessment of Cell Death, Δψm, Cells with a Decreased DNA Content, Cell Ca2+, and Cell Peroxides by Flow Cytometry. Cell death and the Δψm were assessed with the fluorescent probes propidium iodide (Molecular Probes, Eugene, OR) and 3,3′-dihexyl oxacarbocyanine (DiOC6, Molecular Probes), respectively. In brief, floating and adherent cells (106) were washed in PBS and incubated for 10 min at 37°C with DiOC6 (20 nM in PBS) and 5 min at 37°C with propidium iodide (5 μg/ml in PBS). The green fluorescence (DiOC6) and red fluorescence (propidium red) emitted by cells was monitored by flow cytometry using an Epics XL-MCL Beckman Coulter (Villepinte, France).

Apoptosis was assessed by determining the percentage of cells with a reduced DNA content (sub-G0 cells) ( Nicoletti et al., 1991). Floating and adherent cells were washed in PBS and fixed at 4°C for at least 12 h in 100 μl of ice-cold ethanol (70%, v/v). After centrifugation (1500g, 3 min), cells were incubated 30 min at room temperature in PBS containing 180 μg/ml RNase A (Roche Diagnostics, Basel, Switzerland) and then treated with propidium iodide (final concentration, 50 μg/ml) for 15 min and finally monitored for red fluorescence.

Cell calcium was measured using the cell-permeable acetoxymethyl ester derivative of the calcium indicator Fluo-3 (Fluo-3 AM; Molecular Probes). Harvested cells were washed in PBS and incubated with 2 μM Fluo-3 AM for 1 h at 37°C. After incubation with the probe, cells were loaded with propidium iodide (5 μg/ml in PBS) for 5 min at 37°C before monitoring green fluorescence (Fluo-3 AM) and red fluorescence (propidium red) by flow cytometry.

Cellular levels of H2O2 and other peroxides were determined using the cell-permeable tracer 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes) (Sidoti de Fraisse et al., 1998). DCFH-DA is deacetylated by intracellular esterases into the non-fluorescent compound, 2′,7′-dichlorodihydrofluorescein, which is then oxidized to the green fluorescent 2′,7′-dichlorofluorescein (DCF) by cell peroxides including hydrogen peroxide. Harvested cells were washed in PBS and were loaded with 10 μM DCFH-DA for 30 min at 37°C. After incubation with DCFH-DA, cells were loaded with propidium iodide (5 μg/ml in PBS) for 5 min at 37°C before monitoring green fluorescence (DCF) and red fluorescence (propidium red) by flow cytometry.

Measurement of Caspase-3 Activity. Floating and adherent cells (3 × 10⁷) were suspended in an isolation buffer (200 μl) containing 5 mM dithiothreitol, 1 mM EDTA, 0.1% 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate, 1% (w/v) pepstatin, 1% (w/v) leupeptin, and 50 mM HEPES, pH 7.4. Cells were homogenized (with 20 up-and-down strokes using a loosely fitted homogenizer, at 4°C) and centrifuged 10 min at 13,000g. Caspase-3 activity was measured in the supernatant using a commercial kit (Biomol, Plymouth Meeting, PA), according to the recommendations of the manufacturer.

Western Blot Analysis of Cytochrome c and Bax. Mitochondria and cytosol from cultured cells were isolated as described previously (Robin et al., 2003). In brief, floating and adherent cells (3 × 10⁶) were suspended in an isolation buffer (1 ml) containing 70 mM sucrose, 210 mM mannitol, 2 mM EDTA, pH 7.4, and a protease inhibitor cocktail (Complete, Roche Diagnostics GmbH, Mannheim, Germany). The cell suspension was homogenized (20 up-and-down strokes using a loosely fitted homogenizer, at 4°C) and centrifuged 10 min at 1000g. The 1000g supernatant was centrifuged at 13,000g for 20 min, and the resulting mitochondrial pellet was washed two times in isolation buffer. The 13,000g supernatant was further centrifuged 60 min at 100,000g to obtain the cytosolic (supernatant) fraction. After sampling for protein measurement, mitochondrial or cytosolic proteins (100 μg) were subject to SDS-polyacrylamide (15%) gel electrophoresis, transferred to nitrocellulose, and exposed to mouse monoclonal (IgG1) antibodies against cytochrome c (6H2B4; Becton Dickinson, Franklin Lakes, NJ) or Bax (6A7; Becton Dickinson). Blots were exposed to a peroxidase-conjugated goat anti-mouse IgG1 antibody (Biomol-Rockland, GmbH, Hamburg, Germany) and revealed by an enhanced chemiluminescence detection system (Amersham, Lea Ulis, France). After transfer to nitrocellulose membranes, gels were stained with Coomassie Blue (0.025% in acetate 10% for 4 h) to check for total protein extraction.

Cell ATP Contents. To measure cell ATP, the medium was removed and replaced by 1 ml of ice-cold 1 N perchloric acid. After centrifugation at 4°C, aliquots of the supernatant (400 μl) were neutralized with 5 mM K2CO3 (45 μl) and centrifuged again at 4°C. The pellet was suspended in PBS and used to determine protein contents, whereas the supernatant was used to measure ATP by a luciferin-luciferase assay (Roche Diagnostics).

NAD(P)H Levels. NAD(P)H cell contents were measured as described previously (Mingatto et al., 2002). After trypsinization, cells were washed in PBS and suspended in 1% Triton X-100. The autofluorescence of the cell mixture was determined with a Hitachi 2000 fluorescence spectrophotometer (Hitachi High Technologies, San Jose, CA), at the 366-480 nm excitation/emission wavelength pair.

Cell Glutathione. Reduced glutathione (GSH) contents were assessed by measuring nonprotein sulphydryls. In brief, 10⁶ cells were harvested in 0.1 M sodium potassium phosphate buffer, pH 8, supplemented with 10% (final) trichloroacetic acid to precipitate pro-
teins. After centrifugation, the supernatants were neutralized with NaOH, and thiol contents were determined by spectrometry at 412 nm in the presence of 600 μM 5,5′-dithiobis(2-nitrobenzoic acid). GSH disulfide (GSSG) was measured with the Bioxytech GSH/GSSG-412 kit (Tebu bio, Le Perray en Yvelines, France), according to the manufacturer’s recommendations.

**Cell Ultrastructure.** Harvested cells were washed in PBS and fixed for 20 min at 4°C in 0.1 M phosphate buffer, pH 7.4, containing 2.5% glutaraldehyde. After washing, cells were postfixed in a solution of 1% osmium tetroxide buffered with 0.1 M phosphate buffer, for 30 min at room temperature. Pellets were dehydrated by graded ethanol solutions and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Jeol 1010 electron microscope (JEOL, Tokyo, Japan).

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

**Results**

**Effects of Nimesulide in Isolated Rat Liver Mitochondria.** Electron transfer in the respiratory chain is associated with the extrusion of protons from the mitochondrial matrix into the intermembrane space of the mitochondria, thus building up a large Δψm across the inner mitochondrial membrane (Pessayre et al., 2002). Once achieved, this high Δψm limits the flow of electrons in the respiratory chain, thus leading to a low basal (state 4) respiratory rate. However, protonophoric, uncoupling drugs allow the re-entry of protons into the matrix through the inner membrane. The resulting decrease in the Δψm unleashes the flow of electrons in the respiratory chain and, therefore, increases the respiratory rate (Pessayre et al., 2002). Like uncouplers, ADP stimulates the re-entry of protons, but through ATP synthase. The resulting decrease in the Δψm increases the flow of electrons and the respiratory rate. This high ADP-stimulated (state 3) respiratory rate is barely increased by uncouplers because the ADP-mediated decrease in the Δψm decreases the Δψm-mediated cycling of the protonophoric drug across the inner membrane, so that the re-entry of protons mediated by the protonophoric drug is relatively negligible compared with the reentry mediated by ADP (Pessayre et al., 2002). Because nimesulide is a weak acid that may behave as a protonophoric drug, we studied the effects of nimesulide on the Δψm (Table 1) and on the rates of states 4 and 3 respiration in isolated rat liver mitochondria (Table 2). In the absence of albumin, nimesulide decreased the Δψm (Table 1), increased basal (state 4) mitochondrial respiration (Table 2), did not change state 3 respiration (Table 2), and decreased the respiratory control ratio (Table 2). The addition of albumin (36 g/l) prevented these nimesulide-mediated effects (Tables 1 and 2), indicating that albumin, by sequestering nimesulide, prevents the uncoupling of mitochondrial respiration by nimesulide.

Anionic uncouplers can potentiate calcium-induced mitochondrial swelling under conditions ensuring that the uncoupler-mediated decrease in the Δψm does not prevent the Δψm-driven accumulation of calcium into the mitochondrial matrix. Nimesulide (50 μM) did not trigger mitochondrial swelling when mitochondria were preincubated with nimesulide before the addition of Ca2+ (data not shown), probably because the nimesulide-mediated decrease in the Δψm decreased the electrophoretic uptake of calcium by mitochondria. However, either nimesulide or the potent uncoupler, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) increased the rate of swelling (Table 3), when Ca2+ was added before the addition of the uncoupling drug, and when the reverse activity of the Ca2+ uniporter was prevented with Pi (Litsky and Pfeiffer, 1997), thus preventing secondary mitochondrial calcium efflux, whereas the reverse activity of ATP synthase was prevented with oligomycin, thus preventing partial restoration of the mitochondrial membrane potential. The potentiating effect of nimesulide on

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**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Increase in Safranine Fluorescence (Decrease in Δψm) after Addition of Various Substances</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer alone</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>2,4-Dinitrophenol (160 μM)</td>
<td>91.2 ± 6.1*</td>
</tr>
<tr>
<td>Nimesulide (50 μM)</td>
<td>17.5 ± 3.5*</td>
</tr>
<tr>
<td>Nimesulide (50 μM) + albumin (36 g/l)</td>
<td>1.0 ± 1.0</td>
</tr>
</tbody>
</table>

* Different from control, P < 0.05.

**Table 2**

<table>
<thead>
<tr>
<th>Respiratory Substrate(s)</th>
<th>Nimesulide</th>
<th>Albumin (36 g/l)</th>
<th>Respiratory Rate</th>
<th>RCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td></td>
<td>State 3</td>
<td>State 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>O/min/mg protein</td>
<td></td>
</tr>
<tr>
<td>Malate + glutamate</td>
<td>0</td>
<td>–</td>
<td>95 ± 6</td>
<td>10.2 ± 0.6</td>
</tr>
<tr>
<td>Malate + glutamate</td>
<td>25</td>
<td>–</td>
<td>103 ± 3</td>
<td>3.9 ± 0.7*</td>
</tr>
<tr>
<td>Malate + glutamate</td>
<td>50</td>
<td>–</td>
<td>119 ± 8</td>
<td>3.5 ± 0.6*</td>
</tr>
<tr>
<td>Malate + glutamate</td>
<td>50</td>
<td>+</td>
<td>102 ± 6</td>
<td>12.7 ± 4.7</td>
</tr>
<tr>
<td>Succinate</td>
<td>0</td>
<td>–</td>
<td>157 ± 25</td>
<td>7.1 ± 1.7</td>
</tr>
<tr>
<td>Succinate</td>
<td>25</td>
<td>–</td>
<td>167 ± 5</td>
<td>3.7 ± 0.7*</td>
</tr>
<tr>
<td>Succinate</td>
<td>50</td>
<td>–</td>
<td>171 ± 17</td>
<td>2.3 ± 0.3*</td>
</tr>
<tr>
<td>Succinate</td>
<td>50</td>
<td>+</td>
<td>215 ± 49</td>
<td>6.9 ± 0.9</td>
</tr>
</tbody>
</table>

* Different from the corresponding control, P < 0.05.
NADPH/NAD

mitochondrial swelling could be prevented either by the MPT inhibitor, cyclosporin A, or by sequestering nimesulide with albumin (Table 3).

Nimesulide Does Not Form a Nitro Anion-Free Radical with Rat Liver Microsomes and Does Not Cause Early GSH Depletion or Early Reactive Oxygen Species Formation in HUH-7 Cells Incubated without Albumin. Microsomal NADPH-cytochrome P-450 reductase catalyzes the mono-electronic reduction of some, but not all, nitroaromatic compounds into an ESR-detectable nitro anion free radical, which redox cycles with oxygen to form the superoxide anion radical (Sasame and Boyd, 1979; Berson et al., 1990, 1993).

Anaerobic incubation of nitrofurantoin (5 mM) with liver microsomes and NADPH generated a multilin ESR spectrum (Fig. 1), which computer simulation ascribed to the nitrofurantoin nitroanion free radical (data not shown). However, under the same conditions, nimesulide (5 mM) did not generate an ESR spectrum (Fig. 1), indicating that liver microsomes do not catalyze the mono-electronic reduction of nimesulide into a nitroanion free radical.

Cell GSH and cell peroxide levels also remained unchanged in HUH cells incubated for 5 h with nimesulide (1 mM) (Fig. 1), ruling out the extensive formation of reactive metabolites and/or ROS-forming redox cycling as early mechanism(s) for nimesulide-induced toxicity.

Nimesulide Progressively Triggers Uncoupling-Related Redox Changes in HUH-7 Cells Incubated without Albumin. Uncouplers of oxidative phosphorylation un-leash the flow of electrons in the respiratory chain and increase the reoxidation of NADH into NAD⁺ by the respiratory chain (Costantini et al., 1996). This decreases the NADH/NAD⁺ ratio, which in turn decreases the NADPH/ NAD⁺ ratio (Costantini et al., 1996) and the regeneration of GSH from GSSG by GSH reductase. The increased GSSG concentration increases the extrusion of GSSG from cells, which eventually depletes GSH, thus decreasing the activity of glutathione peroxidase and increasing cell peroxide levels (Brigelius et al., 1982; Eklöw et al., 1984; Lauterburg et al., 1984).

Therefore, we measured cell NAD(P)H levels, GSSG, GSH, and cell peroxides in HUH-7 cells incubated for 24 h with or without nimesulide (1 mM). Nimesulide decreased NAD(P)H contents by 44%, and this effect was reproduced by 30 μM FCCP, a potent uncoupler of mitochondrial respiration (Fig. 2). Concomitantly nimesulide increased cell GSSG by 109% and decreased cell GSH by 71% (Fig. 2), thus increasing 7.4-fold the GSSG/GSH ratio. The nimesulide-mediated decrease in cell GSH was reproduced by the uncoupler, FCCP, but was not prevented when both fructose-1,6-diphosphate and oligomycin were coadded with nimesulide (Fig. 2) to prevent ATP depletion and cell toxicity as discussed further on. Collectively, these data suggest that the late GSSG and GSH changes caused by nimesulide are related to the uncoupling effect of nimesulide and the resulting NAD(P)H depletion, which prevents GSSG reduction into GSH. The nimesulide-mediated thiol/disulfide redox change was associated with a 30% increase in cell peroxides. Indeed, in HUH-7 incubated for 24 h with or without 1 mM nimesulide, DCF fluorescence (expressed as the percentage of the mean value in control cells) was 100 ± 8% in living untreated cells, but 130 ± 8% in living nimesulide-treated cells (mean ± S.E.M. for three experiments; P < 0.05).

Toxic Effects of Nimesulide in HUH-7 Cells Incubated without Albumin. In HUH-7 cells incubated for 24 h without albumin, nimesulide (0.5 or 1 mM) dose-dependently decreased both the ΔΨm, and cell ATP content and dose-

TABLE 3
Potentiation of calcium-induced mitochondrial swelling by FCCP or nimesulide

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rate of Absorption Decrease at 540 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O.D. units/min</td>
</tr>
<tr>
<td>Control</td>
<td>0.041 ± 0.006</td>
</tr>
<tr>
<td>FCCP (0.2 μM)</td>
<td>0.423 ± 0.056*</td>
</tr>
<tr>
<td>Nimesulide (50 μM)</td>
<td>0.214 ± 0.062*</td>
</tr>
<tr>
<td>Nimesulide (50 μM) + cyclosporin A (2 μM)</td>
<td>0.011 ± 0.005</td>
</tr>
<tr>
<td>Nimesulide (50 μM) + albumin (36 g/l)</td>
<td>0.047 ± 0.005</td>
</tr>
</tbody>
</table>

* Different from control, P < 0.05.

Fig. 1. No evidence for mono-electronic nitro reduction, early GSH consumption, or early ROS formation in microsomes or hepatocytes incubated with nimesulide. A, ESR spectra generated during the anaerobic incubation of mice liver microsomes (10 mg proteins/ml), 5 mM NADPH, and either nitrofurantoin (5 mM) or nimesulide (5 mM). The microwave power (band X) was 20 mW, and the modulation amplitude was 0.32 G. B, cell GSH and peroxides in human hepatoma HUH-7 cells incubated for 5 h with or without nimesulide (1 mM). To detect peroxides, we incubated cells for 5 min at 37°C with 2′,7′-dichlorodihydrofluorescein (10 μM) and then loaded them with propidium iodide (5 μg/ml) before monitoring the green fluorescence of dichlorofluorescein (DCF) and the red fluorescence of propidium red by flow cytometry. DCF fluorescence was only measured in living (propidium iodine-negative) cells. Results are mean ± S.E.M. for 3 experiments.
dependently increased both LDH release into the medium and the percentage of propidium iodide-positive (dead) cells (Fig. 3).

**Protective Effects of Fructose-1,6-Diphosphate, Oligomycin, or Cyclosporin A.** The glycolysis substrate, fructose-1,6-diphosphate, or the ATPase inhibitor, oligomycin, partially prevented nimesulide-induced ATP depletion when added alone (Fig. 4). Used in combination, fructose-1,6-diphosphate and oligomycin totally prevented ATP depletion, as did the MPT inhibitor, cyclosporin A (Fig. 4). Concomitantly, cyclosporin A or the combination of fructose-1,6-diphosphate and oligomycin markedly decreased the percentage of propidium iodine-stained cells and partially improved the ΔΨm (Fig. 4).

**Effects of Nimesulide on Cell Calcium.** Cell calcium was measured only in living cells using both the cell-permeable, Fluo-3 AM, and propidium iodine to exclude dead cells. Cell calcium (expressed as the percentage of its mean value in control cells) was unchanged after 5 h of incubation with 1 mM nimesulide (mean ± S.E.M. for three experiments, 100 ± 5% in control cells and 108 ± 7% in treated cells). However, after 24 h of incubation with or without 1 mM nimesulide, cell calcium was 100 ± 2% in control cells but 358 ± 49% in nimesulide-treated cells (mean ± S.E.M. for three experiments; P < 0.01).

**Effects of Nimesulide on the Percentage of Sub-G1 Cells and the Localization of Cytochrome c and Bax in HUH-7 Cells Incubated without Albumin.** Apoptosis is associated with the activation of endonucleases and a loss of cell DNA. In ethanol-fixed cells, which are permeable to propidium iodide, the intensity of DNA staining by propidium iodide differentiates between apoptotic cells with a low DNA content (sub-G1 cells) and cells with a normal DNA content (cells in the G1 or G2 state of the cell cycle). As a positive control, we used the potent proapoptotic agent, LY294002 (20 μM), a phosphatidylinositol-3 kinase inhibitor (Yao and Cooper, 1995), which gave 69 ± 6% of sub-G1 cells after 48 h of culture (Fig. 5A). After 24 h of incubation with or without nimesulide (1 mM), the percentage of sub-G1 cells (mean ± S.E.M. for three experiments) was 3 ± 1% in control cultures but 12 ± 3% and 14 ± 2% with 0.5 and 1 mM.
nimesulide, respectively (Fig. 5A). In cells incubated with 1 mM nimesulide, coincubation with both fructose-1,6-diphosphate and oligomycin decreased the percentage of sub-G1 cells by only 14%, whereas coincubation with cyclosporin A decreased the percentage of sub-G1 cells by 43% (Fig. 5A). We also assessed cell apoptosis by measuring the cellular activity of caspase-3, which was increased 6.5-fold by 1 mM nimesulide (Fig. 5A).

After 24 h of incubation with or without 1 mM nimesulide, mitochondrial and cytosolic fractions were prepared, and equal amounts of proteins (100 μg) were loaded onto polyacrylamide gels (Fig. 5B). Protein bands of cytochrome c and Bax were quantified on Western blots and expressed as the percentage of their mean value in control cells. Although mitochondrial porin and cytosolic β-actin were also quantified, these proteins decreased by the nimesulide treatment, precluding their use as reference standard to assess equal protein loading (Fig. 5B). Considering absolute values, there was a significant decrease in mitochondrial cytochrome c, Bax, and porin (Fig. 5B), which may be possibly consistent with mitochondrial swelling, outer membrane rupture, and the recovery of mitoplasts rather than whole mitochondria during the isolation of mitochondria. In the cytoplasm, cytochrome c was significantly decreased (Fig. 5B), possibly due to the rupture of the plasma membrane and the egress of cytochrome c from cells.

**Fig. 5.** Effects of nimesulide on the percentage of apoptotic cells, caspase-3 activity, cytochrome c, Bax, porin, and β-actin. A, human hepatoma HUH-7 cells were cultured for 24 h with or without nimesulide (0.5 or 1 mM), cyclosporin A (CA) (3 μM), fructose-1,6-diphosphate (F-1,6-dP) (10 mM), and oligomycin (1 μg/ml). For comparison, some cells were cultured for 48 h with the potent antiapoptotic agent, LY294002 (20 μM). After fixation with ethanol and addition of propidium iodide, the percentage of cells with a low DNA content (apoptotic sub-G1) was assessed by flow cytometry. To assess caspase-3, nonfixed cells were homogenized and centrifuged, and caspase-3 activity was determined in the supernatant. Results are means ± S.E.M. for at least three experiments. B, mitochondrial and cytosolic fractions were prepared, and equal amounts of proteins (100 μg) were loaded onto the gels. Protein bands on Western blots were quantified and expressed as the percentage of their mean value in control cells. The figure shows the blots of one representative experiment and gives quantified results (mean ± S.E.M.) for at least five experiments. *, significantly different from control, P < 0.05. †, significantly different from nimesulide alone, P < 0.05.

**Effects of Nimesulide on the Ultrastructure of HUH-7 Cells Incubated without Albumin.** Cell morphology was studied by electron microscopy after 24 h of incubation with or without nimesulide. After the control incubation, most cells had a normal ultrastructural aspect, even though a few apoptotic cells were observed. In cells treated with nimesulide, a variegated pattern was observed (Fig. 6). Although some cells had no lesions or only discrete lesions, other cells were clearly necrotic, with cell swelling and organelle swelling, rupture of the plasma membrane, and formation of cell debris. Finally, the apoptosis of a few cells was attested by the presence of some apoptotic bodies. Collect-
tively, these observations indicated that although several cells were still intact, many other cells had died of necrosis.

**Coincubation with Albumin Prevents the Toxicity of Nimesulide in Human Hepatoma Cells.** In vivo, nimesulide is bound to plasma albumin, which considerably decreases its hepatic uptake because only a small fraction of the drug is not bound to albumin and can cross the hepatocyte plasma membrane. We therefore tested whether nimesulide would still be toxic toward hepatocytes when albumin (32 g/l) is coadded to the culture medium (Fig. 7).

Although nimesulide (0.5 or 1 mM) increased LDH release in the absence of albumin, no significant LDH release occurred in cells coincubated with nimesulide and 32 g/l albumin. After 24 h of incubation with albumin and 0, 0.5, or 1.0 mM nimesulide, the percentage of LDH release was 20 ± 5, 28 ± 5, and 31 ± 11%, respectively (no significant differences) (Fig. 7).

After 24 h of incubation with albumin and either 0 or 1 mM nimesulide, the percentage of propidium iodide-stained cells was 6 ± 1% in control cells and 5 ± 1% in nimesulide-treated cells (no significant difference) (Fig. 7). DiOC6 fluorescence (assessing the $\Delta\phi_m$) was 100 ± 5% in control cells and 112 ± 2% in cells coincubated with nimesulide and albumin (no significant difference) (Fig. 7).

**Discussion**

The present study confirms previous data showing that nimesulide uncouples respiration in mitochondria incubated without albumin (Moreno-Sanchez et al., 1999; Mingatto et al., 2000), and it shows that MPT plays a major role in the mechanism of ATP depletion and cell death in human hepatoma cells incubated for 24 h with nimesulide in the absence of albumin (Fig. 8A).

In mitochondria incubated without albumin, nimesulide (50 $\mu$M) dissipated the $\Delta\phi_m$ (Table 1) and increased basal respiration (Table 2), confirming its uncoupling effects. Nimesulide is a sulfonamide, and such compounds are weak acids (Remko and von der Lieth, 2004). Like carboxylic compounds (Wieckowski and Wojtczak, 1997), nimesulide could translocate protons into the mitochondrial matrix as follows. The uncharged nimesulide may cross the inner mitochondrial membrane to enter the matrix. In the alkaline matrix, nimesulide may dissociate into a proton and the nimesulide anion, which, being negatively charged, may be electrophoretically extruded from the matrix. As with fatty acid anions, this second crossing of the inner membrane might occur through membrane transporters (Wieckowski and Wojtczak, 1997). Once inside the acidic intermembrane space, the nimesulide anion may be protonated again to reform uncharged nimesulide. At each cycle of entry into the matrix, nimesulide may thus translocate a proton into the matrix, thus decreasing the $\Delta\phi_m$. The decreased $\Delta\phi_m$ allows electrons to rush along the respiratory chain, thus increasing state 4 respiration (Table 2). Failure of the amine derivative of nimesulide to uncouple respiration (Mingatto et al., 2000) could be due to the presence of a positive charge on the protonated amine, which might counteract the cycling described above across the inner membrane.

In HUH-7 cells incubated without albumin, nimesulide
caused NAD(P)H oxidation, and this effect was reproduced by the uncoupler, FCCP (Fig. 2). FCCP unleashes the flow of electrons in the respiratory chain and increases the reoxidation of NADH into NAD⁺ by complex I, thus depleting NADH (Costantini et al., 1996; Catisti and Vercesi, 1999). NADH depletion decreases the regeneration of NADPH from NAD⁺ by NAD⁺ transhydrogenase, thus also depleting NADPH (Costantini et al., 1996; Catisti and Vercesi, 1999).

In HUH-7 cells incubated for 24 h with nimesulide without albumin, NAD(P)H oxidation was associated with increased cell GSSG, decreased GSH (Fig. 2), and increased cell peroxides. Under normal circumstances, the superoxide anion radical formed by the respiratory chain is dismutated by manganese superoxide dismutase (MnSOD) into hydrogen peroxide, which is detoxified by glutathione peroxidase, whereas GSH is oxidized into GSSG (Kowaltowski and Vercesi, 2001). Normally, GSSG is then reduced back to GSH by glutathione reductase and NADPH (Kowaltowski and Vercesi, 2001). However, when cells lack NADPH, the impaired glutathione reductase activity increases cell GSSG (Eklöw et al., 1984). High GSSG levels cause its extrusion from cells (Lauterburg et al., 1984) and GSH depletion (Brigelius et al., 1982; Eklöw et al., 1984). GSH depletion impairs glutathione peroxidase activity and increases ROS release by mitochondria (Han et al., 2003). Collectively, these data indicate that the uncoupling nimesulide causes NAD(P)H oxidation, GSH oxidation, GSH depletion, and increased cellular ROS in HUH-7 cells incubated without albumin (Fig. 8A).

The nimesulide-induced redox changes were associated with MPT, as indicated by a cyclosporin A-inhibited loss in ΔΨm (Fig. 4), loss of mitochondrial cytochrome c (Fig. 5), and demonstration of mitochondrial swelling by electron microscopy (Fig. 6). Several intertwined factors have been shown to increase MPT in other circumstances and may help trigger nimesulide-induced MPT (Fig. 8A), including the decreases in ΔΨm and NAD(P)H (Kowaltowski and Vercesi, 2001; Kowaltowski et al., 2001) and the 7.4-fold increase in the GSSG/GSH ratio. Indeed, the MPT pore is tuned by the oxidation-reduction state of vicinal thiols (Petronilli et al., 1994) due to the formation of disulfide bridges in the adenine nucleotide translocator or other proteins (Halestrap et al., 1997; Costantini et al., 1998), and MPT is enhanced by GSH depletion (Haouzi et al., 2001).

MPT occurring in some mitochondria may trigger vicious cycles triggering MPT in other mitochondria (Fig. 8A). Cytochrome c egress from mitochondria blocks electron flow, causes over-reduction of upstream respiratory chain complexes, and increases mitochondrial ROS formation (Cai and Jones, 1998; Tay et al., 2005), which may trigger MPT in still unaffected mitochondria. Severe ATP depletion inhibits apoptosis and triggers necrosis. In keeping with a major role of MPT and ATP depletion in the toxicity of nimesulide, necrosis was prevented by either cyclosporin A or the combination of the glycolysis substrate, fructose-1,6-diphosphate (F-1,6-dP), and oligomycin, an inhibitor of ATP synthase. B, in the presence of albumin, only the small fraction of nimesulide (1%), which is not bound to albumin, enters HUH-7 cells. The ΔΨm is unchanged, and toxicity is not detected.

**Fig. 8.** Suggested effects of nimesulide in HUH-7 cells. A, in the absence of albumin, nimesulide (1 mM) enters HUH-7 cells, where this weak acid translocates protons into the mitochondrial matrix and decreases the ΔΨm. The decreased ΔΨm unleashes the flow of electrons in the respiratory chain and increases mitochondrial respiration and the reoxidation of NADH. The resulting NAD(P)H depletion increases GSSH, decreases GSH, and contributes to the increase in cellular ROS by decreasing their inactivation. Together with the decreased ΔΨm, these redox changes and the increase in ROS may start to trigger MPT in some mitochondria. MPT releases cytochrome c from mitochondria, which activates caspases, and also partially blocks the flow of electrons in the respiratory chain. The over-reduction of upstream respiratory chain complexes increases mitochondrial ROS formation, which may trigger MPT in still unaffected mitochondria. MPT also prevents ATP generation by the affected mitochondria. As the percentage of damaged mitochondria increases, severe ATP depletion occurs and inhibits Ca²⁺-ATPases, thus increasing cell calcium, which may trigger MPT in still undamaged mitochondria. Severe ATP depletion inhibits apoptosis and triggers necrosis. In keeping with a major role of MPT and ATP depletion in the toxicity of nimesulide, necrosis was prevented by either cyclosporin A or the combination of the glycolysis substrate, fructose-1,6-diphosphate (F-1,6-dP), and oligomycin, an inhibitor of ATP synthase.
the $\Delta \psi_m$ (Fig. 4), suggesting that a major effect of oligomycin is to indirectly prevent MPT, possibly by preventing ATP depletion (Fig. 4), which might prevent the increase in cell calcium and calcium-induced MPT (Fig. 8A).

Although nimesulide triggered mostly necrosis, there were a few apoptotic cells, as shown by some sub-G1 cells (Fig. 5) and apoptotic bodies (Fig. 6). Cyclosporin A afforded partial protection against sub-G1 cell development (Fig. 5), suggesting MPT involvement. MPT causes matrix expansion and outer membrane rupture, which releases cytochrome c from mitochondria. In the presence of ATP, cytochrome c associates with apoptotic protease-activating factor-1 to activate caspase-9, which activates caspase-3 (Fig. 5) and other effectors that can trigger apoptosis. Predominance of necrosis over apoptosis could explain why cytosolic cytochrome c was paradoxically decreased, despite evidence for its escape from mitochondria (Fig. 5). Necrosis causes plasma membrane rupture (Fig. 6), which allows cytochrome c to leak out of hepatocytes, whereas $\beta$-actin, which is partly associated with the cytoskeleton, is better retained. A major leak of cytosolic cytochrome c from a large number of necrotic cells may mask an increase in cytosolic cytochrome c in a few apoptotic cells.

In humans, the recommended dose of nimesulide is 100 mg twice daily, giving peak nimesulide plasma concentrations of 2.9 to 6.5 mg/l (i.e., 10–20 times the therapeutic concentration of albumin, all added drug is free and is able to enter the cytoplasm of the total drug concentration, i.e., 0.10 to 0.20 $\mu$M (Bernaegggi, 1998). However, because 99% of nimesulide is albumin-bound, the free drug concentration able to enter cells and mitochondria is only 1% of the total drug concentration, i.e., 0.10 to 0.20 $\mu$M (Bernaegggi, 1998). When in vitro experiments are performed without albumin, all added drug is free and is able to enter mitochondria. Therefore, one cannot directly compare the in vitro drug concentrations having an effect on mitochondria or cells with the total drug concentrations in human plasma. This comparison can be made, however, if in vitro experiments are performed with the albumin concentration present in human plasma. An important observation of the present study is that nimesulide, even at a high concentration (50 $\mu$M), had no effect on mitochondrial respiration, $\Delta \psi_m$, or Ca$^{2+}$-induced MPT in mitochondria incubated with a physiological concentration of albumin (Tables 1–3). Likewise, an extremely high concentration (1 mM) of nimesulide had no effect on $\Delta \psi_m$ or cell survival when HUH-7 cells were incubated with albumin (Fig. 7).

These observations may suggest that nimesulide may not damage hepatocytes, unless genetic or acquired predisposing factors are also present, thus explaining the rarity of nimesulide-induced hepatitis. Nimesulide administration (10 mg/kg twice daily for 4 weeks) increased mitochondrial carboxyls and decreased mitochondrial aconitase activity in hetrozygous MnSOD+/− knockout mice, but not in wild-type mice (Ong et al., 2006). A human genetic polymorphism may modulate MnSOD activity (Nahon et al., 2005). However, in view of the rarity of nimesulide-induced hepatitis contrasting with the 16% frequency of subjects homozygous for the low-activity MnSOD allele (Nahon et al., 2005), this polymorphism may have at most a modest role in the susceptibility to develop nimesulide-induced hepatitis.

In conclusion, the present findings show that, in the absence of albumin, nimesulide transduces protons into the mitochondrial matrix, decreases the $\Delta \psi_m$, and increases mitochondrial respiration. The increased respiration triggers progressive NADPH depletions, GSH oxidation to GSSG, GSH depletion, increased cellular ROS levels, onset of MPT, ATP depletion, increased cell calcium, and liver cell necrosis in human hepatoma cells incubated without albumin (Fig. 8A). However, in the presence of albumin, only 1% of nimesulide can enter cells and organelles. The $\Delta \psi_m$ is unchanged, and cell death does not occur (Fig. 8B).

References


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