Hepatotoxicity of Tacrine: Occurrence of Membrane Fluidity Alterations without Involvement of Lipid Peroxidation

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ABSTRACT
Tacrine (THA), used in the treatment of Alzheimer’s disease, is known to induce hepatotoxicity, the mechanisms of which remain to be fully established. We have previously shown that THA reduced intracellular glutathione concentration in rat hepatocytes in primary culture, thus pointing to a possible role for oxidative stress in THA toxicity. To test this, the effects of antioxidant molecules, namely, the flavonoids silibinin, silibinin dihydrogensuccinate, and silymarin, were evaluated on the toxicity of THA in cultured rat hepatocytes. This toxicity was investigated after a 24-h treatment over a concentration range from 0 to 1 mM, in the presence or absence of antioxidant (1 and 10 μM). We found that simultaneous treatment of hepatocytes with any of the antioxidants and THA remained ineffective on the lactate dehydrogenase release induced by THA. Then, the production of lipid-derived radicals (to estimate lipid peroxidation) was measured in THA (0.05–0.50 mM)-treated cells using a spin-trapping technique coupled to electron paramagnetic resonance (EPR) spectroscopy. No increase of the EPR signal was observed over the period of 30 min to 24 h. In contrast, treatment of cells with the spin label 12-doxyl stearic acid followed by EPR spectroscopy showed that THA (0.05 and 0.25 mM) rapidly increased hepatocyte membrane fluidity. Extracellular application of GM1 ganglioside (60 μM) both reversed this increase in fluidity and partially reduced lactate dehydrogenase release on THA exposure. In conclusion, this work indicates that early alterations of membrane fluidity, not resulting from lipid peroxidation, are likely to play an important role in the development of THA toxicity.

Alzheimer’s disease is a progressive, degenerative dementia characterized by decreased cognitive functions with associated decline in cholinergic transmission. Drug therapy to increase cholinergic transmission has been one strategy to combat this disease. It is in this context that tacrine (1,2,3,4-tetrahydro-9-aminoacridine; THA), a centrally active acetylcholinesterase inhibitor, has been developed (Farlow et al., 1992). Unfortunately, the clinical use of this molecule has been demonstrated to induce hepatotoxicity (Forsyth et al., 1989; Watkins et al., 1994), the mechanisms of which remain to be fully established.

Recent works by Berson et al. (1996) and Robertson et al. (1998) have shown that a mitochondrial dysfunction is elicited by THA. However, as raised by the latter group, mitochondrial dysfunction might not be the only factor involved, because the clinical manifestations of THA do not resemble those typically associated with mitochondrial cytopathies (Feuer and de la Iglesia, 1996). Moreover, although we have clearly shown an effect of THA on the intermediary metabolism of hepatocytes, this could rather represent a late event in the cascade leading to cell death. Indeed, alterations in cell membrane integrity [as revealed by lactate dehydrogenase (LDH) leakage] were detected before any decrease in intracellular ATP (Lagadic-Gossmann et al., 1998), thus suggesting the involvement of other factors.

The fact that THA was shown to alter intracellular glutathione concentration in cultured hepatocytes (Lagadic-Gossmann et al., 1998) might suggest the involvement of generation of reactive oxygen species (ROS) and lipid peroxidation, because intracellular glutathione represents an important protective factor against oxidative damage. With respect to this hypothesis, it previously has been shown that THA, at high concentrations (≥1 mM), induced lipid peroxidation in isolated rat hepatocytes, which was prevented by preincubating the cells with vitamin E, a naturally occurring antioxidant (Dogterom et al., 1988). Under those conditions, the toxicity of THA was then markedly decreased. The occur-

ABBREVIATIONS: THA, tacrine; DSA, doxyl stearic acid; EPR, electron paramagnetic resonance; LDH, lactate dehydrogenase; pH, intracellular pH; POBN, α-(4-pyridyl 1-oxide)-N-tet-butyl-nitron; SDH, silibinin dihydrogensuccinate; SNARF-1, seminaphtorhodafluor-1; ROS, reactive oxygen species; Fe-NTA, ferric nitriloacetate.

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rence of such a phenomenon was also hypothesized in a recently developed animal model of THA-induced hepatotoxicity (Stachlewitz et al., 1997) and in HepG2 cells (Osseni et al., 1999).

In this study, we have tested the possibility that oxidative stress might be involved in the THA-induced toxicity previously observed using rat hepatocytes in primary culture. One way to evaluate the involvement of such a phenomenon is to use antioxidant molecules and to look for any prevention of toxic effects. Silibinin and derivatives have long been known as hepatoprotective molecules, mainly due to their antioxidant properties (Muriel et al., 1992; Rauen et al., 1997), and are used in clinical trials (Ferenci et al., 1989; Velussi et al., 1997). Therefore, they have been tested here against THA-induced toxicity. In a second set of experiments, we used a more direct means to more thoroughly evaluate the involvement of oxidative stress, more specifically, lipid peroxidation. This was carried out by using a spin-trapping technique in conjunction with electron paramagnetic resonance (EPR), allowing the direct detection of the radicals involved in the lipid peroxidation process (Morel et al., 1995). If such a process were to occur on THA, then alterations of membrane fluidity might have been expected. In this regard, it has been reported that alterations of this parameter are responsible for the toxicity of molecules such as alcohol (Rubin and Rotenberg, 1982). Moreover, THA has been shown to bind to acidic phospholipid-containing membranes and to locate in the interfacial region of the lipid bilayer (Lehtonen et al., 1996). Therefore, we decided to use EPR, coupled to a spin-labeling method, to estimate the effects of THA on hepatocyte membrane fluidity. Finally, because alteration of cell membrane properties may affect ion transporters and, consequently, homeostasis of, e.g., H⁺ ions (Dudeja et al., 1987; Simkiss, 1998), the effects of this molecule on steady-state cytoplasmic pH were also tested in primary hepatocyte cultures using the intracellular fluorophore carboxy-sensaminephorhodafluor-1 (SNARF-1). In this regard, we have recently evidenced such alterations in a rat liver biliary epithelial cell line following treatment with THA (Lagadic-Gossmann et al., 1999).

This work shows that THA elicits early alterations in both membrane fluidity and intracellular pH (pHᵢ), without concomitant induction of lipid peroxidation. Prevention of membrane fluidity alterations affords partial protection to hepatocytes against THA toxicity.

Materials and Methods

Chemicals. Williams’ E medium was purchased from Eurobio (Les Ulis, France). Fetal calf serum was from Dominique Dutcher SA (Brumath, France). Liberase was from Boehringer Mannheim (Germany). THA hydrochloride hydrate (THA), α-(4-pyridyl 1-oxide)-N-tet-butyl-nitrotrine (POBN), 5-doxyl stearic acid (5-DSA), 12-DSA and monosialoganglioside GM1 were purchased from Sigma (St. Louis, USA). Silibinin, silybinin dihydrogen succinate (SDH) and silimarin were all given by Madaus AG (Cologne, Germany). LD kit (for LDH determination) was from Bayer-Diagnostics (Putaux, France).

Cell Isolation and Culture. All procedures were in accordance with the regulations laid down by the Ministère de l’Agriculture et de la Forêt, France, for the care and use of laboratory animals. Hepatocytes from adult male Sprague-Dawley rats weighing 180 to 200 g were isolated by perfusion of the liver as previously described except that a liberase solution (23.3 μg/ml) was used for dissociation of liver parenchymal cells instead of a collagenase solution (Guguen-Guillouzo et al., 1983). Following dissociation, cell viability was estimated by the trypan blue exclusion test and was above 80%. Cells were seeded at densities of 15 × 10⁴ cells/well in 24-well microplates (LDH determinations), 10 × 10⁶ cells onto 100 mm-diameter culture plastic dishes (EPR studies) and 1 × 10⁶ cells on 25 mm-diameter glass coverslips (pHᵢ measurement), and cultured in Williams’ E medium supplemented with 10% fetal calf serum and (per milliliter) 5 IU penicillin, 5 μg of streptomycin, 1 mg of BSA, and 1 μg of bovine insulin. The cells were kept at 37°C in an atmosphere of 5% CO₂ and 95% air. The medium was discarded 4 h after cell seeding to remove unattached cells. The fresh serum-containing medium was then supplemented with 5 μM hydrocortisone hemisuccinate. Treatments of the cultures were in all cases started the day after seeding.

Drug Application Protocol. THA was first prepared as a stock solution (20 mM) in culture medium and kept at 4°C for up to 15 days. Media with THA at the test concentrations were prepared just before experiments. For LDH determinations and EPR studies, cells were treated with THA for 24 h. When using the antioxidant molecules, stock solutions in dimethyl sulfoxide were prepared for silibinin and silymarin, whereas SDH was dissolved directly in culture medium. Controls received the same volume of vehicle (final concentration <0.1%). These antioxidant molecules were added simultaneously to THA and were present during the whole period of treatment with THA. Stock solution (4 mM) of monosialoganglioside GM1 was prepared in PBS. GM1 at the test concentrations was applied 2 h before THA treatment, and was maintained for an additional 24 h.

Cytotoxicity Assays. THA-mediated cell death was determined by measuring the leakage of cellular LDH into the medium. LDH activity was measured both in media and cell extracts using a kit for LDH determination (LD kit; Bayer-Diagnostics). Before the addition of the reagents, cell monolayers were lysed in 0.2% Triton X-100 in water for 15 min at room temperature. Optical density was then measured at 340 nm. To determine the dose of THA inducing 50% of cell mortality (IC₅₀), the basal extracellular LDH/total LDH ratio (LDH/HELDHot, i.e., in absence of THA) was assigned to zero mortality, whereas ratios above 0.9 were assigned to 100% mortality (confirmed by light microscopic observations).

Detection of Lipid-Derived Radicals by EPR Spectroscopy. The technique used in this study has been described previously in detail (Morel et al., 1995). Briefly, following treatment with THA, cells were immediately lysed in PBS by an ultrasonic homogenizer. The spin-trap POBN was then added to cell homogenates at a final concentration of 160 mM. The mixture was finally transferred into a Pasteur pipette adapted for EPR analysis. EPR spectra were recorded on a Bruker ESP 106 spectrometer at ambient temperature, with the following instrument conditions: 9.82 GHz frequency, 20 mW microwave power, 1.8 G modulation amplitude, 100 kHz modulation frequency, three scans accumulated. At the end of analysis, the remaining cell homogenate was used for protein content estimation. The results were obtained as arbitrary units given by computer double integration of the low field doublet of the spectra and were related to per milligram protein content for each sample.

Determination of Membrane Fluidity by an EPR Spin-Labeling Method. The membrane fluidity of hepatocytes was determined by a spin-labeling method using EPR, as described previously (Ogura et al., 1988; Sakanashi et al., 1988). Briefly, after treatment, the cell suspension collected in PBS was placed in a test tube and incubated with the spin label (5 μg in 100-μl suspension) for 15 min at 37°C. In this study, two different spin labels were used, depending on their location in the membrane: the 5-DSA and the 12-DSA, which incorporate at the outer and inner layers, respectively. An ethanol solution of each spin label was prepared at a concentration of 500 μg/ml and stored at −20°C. Following incorporation of the spin labels, cells were then washed three times with PBS to eliminate the free spin labels. The final pellet was kept on ice to prevent any spin
label reduction until transfer into a Pasteur pipette adapted for EPR analysis. The EPR spectrum was obtained at room temperature (20°C), on the same spectrometer as above; EPR measurement was done after the sample temperature had reached an equilibrium with room temperature. In that case, the spectrometer settings were the following: 9.82 GHz frequency, 20 mW microwave power, 1,771 G modulation amplitude and 100 kHz modulation frequency. The values of outer and inner hyperfine splitting EPR spectra, typical for each DSA spin label, were observed to calculate the order parameter (S) according to equations that have been described previously (Ogura et al., 1988; Sakanashi et al., 1988). An increase in the order parameter reflects a decrease in membrane lipid fluidity, whereas a decrease of this parameter reflects an increase in membrane fluidity. 

Measurements of $pH_i$. The $pH$ of hepatocytes cultured on glass coverslips was monitored using the pH-sensitive fluorescent probe, carboxy-SNARF-1 (Molecular Probes, Eugene, OR) (Buckler and Vaughan-Jones, 1990). Cells were loaded with SNARF-1 by incubating them in a 5 μM solution of the acetoxy-methyl ester for 20 min at 37°C.

SNARF-1-loaded cells were placed in a continuously perfused recording chamber (at a temperature of 36 ± 1°C) mounted on the stage of an epifluorescence microscope (Nikon Diaphot; Nikon France, Champigny sur Marne, France). Hepatocytes were then excited with light at 514 nm, and fluorescence from the trapped probe was measured at 590 and 640 nm. The necessary monochromator and photometers to produce and detect the fluorescence from a small area of the coverslip (representing approximately three to four cells in the field of view) were part of a Photon Technology International (PTI; Monmouth Junction, NJ) DeltaRAM system, and the software systems to control the monochromator and both acquire and process the data were also supplied by PTI. The emission ratio 640:590 obtained from intracellular SNARF-1 was calculated and converted to a linear pH scale using in situ calibration data obtained by the nigericin technique described elsewhere (Thomas et al., 1979; Buckler and Vaughan-Jones, 1990). Nigericin (Sigma) calibration solutions used in this study have been described elsewhere (Lagadic-Gossmann et al., 1992).

In the course of these experiments, the composition of the perfusion solution used was as follows: 111.8 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, 1.0 mM CaCl₂, 2 mM glutamine, 10 mM glucose, 23 mM NaHCO₃. This bicarbonate-buffered solution, containing or not containing THA, was equilibrated with 5% CO₂, 95% O₂ and had a pH of 7.4 at 37°C.

Statistics. All data are quoted as mean ± S.E. along with the number of observations, n, corresponding, if not otherwise stated, to the number of separate cultures used. Cells obtained from three different isolations were generally used for all the protocols carried out. Unless otherwise stated, statistical significance was estimated by ANOVA followed by Student-Newman-Keuls test to locate differences between groups. Differences were considered significant at the level of $P < .05$.

Results

Effects of Silibinin and Derivatives on THA-Induced Toxicity in Cultured Hepatocytes. With the aim of seeking the possibility of an oxidative stress induced by THA, the effects of known hepatoprotective, antioxidant molecules, i.e., a flavonoid-containing extract (silymarin), its main constituent (silibinin), and a water-soluble derivative thereof (SDH), were evaluated in rat hepatocytes in primary culture. To avoid any cytotoxic interference of these compounds with THA-induced toxicity, we first determined the influence of the three test compounds on the viability of cultured hepatocytes using the LDH leakage assay, confirmed by light-microscopic observations. Under our culture conditions and following a 24-h exposure, cytotoxic effects were obvious at concentrations above 10 μM, whatever the molecule tested (data not shown). Therefore, the two concentrations of sili- binin and derivatives chosen for the following experiments were 1 and 10 μM.

Cytotoxicity of THA toward hepatocytes was investigated after a 24-h exposure time over a concentration range from 0 to 1 mM, in the presence or absence of either antioxidant molecule. Figure 1A illustrates the effects of silymarin (1 and 10 μM) on the LDH leakage induced by THA. As in our previous study (Lagadic-Gossmann et al., 1998), an increase in the release of LDH was detected from 0.25 mM THA. However, it was clear from Fig. 1A that simultaneous treatment of the cells with silymarin and THA remained without effect on the dose-dependent increase in LDH release induced by THA. Similar results were obtained with silibinin and SDH (not shown). This was also confirmed when determining the IC₅₀ of THA under the different conditions of treatment tested (Table 1); indeed, no significant increase of IC₅₀ was apparent between control cells and those treated with antioxidants, whatever the concentration used. It could be argued that these antioxidants were actually ineffective against THA-induced toxicity due to the low concentrations used. This led us to verify that these two concentrations were capable of preventing or at least limiting the oxidative stress.

![Image](https://via.placeholder.com/150)
TABLE 1
Effects of silibinin and derivatives used at two concentrations on IC50 of THA in rat hepatocytes in primary culture

<table>
<thead>
<tr>
<th>IC50 of THA (mM)</th>
<th>-Antioxidant-</th>
<th>1 µM</th>
<th>10 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silibinin</td>
<td>0.275 ± 0.013</td>
<td>0.279 ± 0.016</td>
<td>0.270 ± 0.033</td>
</tr>
<tr>
<td>SDH</td>
<td>0.275 ± 0.015</td>
<td>0.283 ± 0.010</td>
<td>0.287 ± 0.011</td>
</tr>
<tr>
<td>Silimarmin</td>
<td>0.272 ± 0.013</td>
<td>0.274 ± 0.013</td>
<td>0.288 ± 0.010</td>
</tr>
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induced by a known oxidant. This was carried out using a 24-h exposure to ferric nitroacetate (Fe-NTA, 100 µM), which has been shown to induce an increase of both ROS and LDH leakage in rat hepatocytes in primary culture (Morel et al., 1995). Figure 2B shows the data obtained using silymarin. It is clear from this figure that silymarin partially protected hepatocytes against Fe-NTA-induced toxicity under our culture conditions, with a reduction by ~20% and 45% of LDH release at 1 and 10 µM, respectively, validating the results described above. Similar results were obtained with SDH whereas the protection afforded by silibinin was lower (by ~20% at 10 µM; not shown). Taken together, these results therefore suggested that the toxicity of THA in hepatocytes was not related to the induction of an oxidative stress.

Effects of THA on Production of Lipid-Derived Radicals. To fully rule out the possibility of a THA-induced oxidative stress leading to lipid peroxidation, a spin-trapping technique (with POBN as spin-trap) in conjunction with EPR spectroscopy (Morel et al., 1995) was used to directly measure the production of lipid-derived radicals in cells treated with different concentrations of THA and for different times of exposure. Before performing such measurements, validity for using this technique was confirmed by inducing lipid peroxidation in cultured hepatocytes on exposure to Fe-NTA (100 µM; Fig. 2A). As expected from previous studies (Morel et al., 1995), this treatment increased the EPR signal (corresponding to POBN/lipid-derived radicals) by ~3.5- and 9.5-fold, when compared with untreated cells, after 8 and 24 h, respectively. With respect to THA, the EPR signal recorded under different treatment conditions is illustrated in Fig. 2B and is given as a percentage of that obtained in control untreated cultures. As clearly shown by this graph, THA did not significantly increase the EPR signal, that is, the production of radicals, at all concentrations tested whatever the time of treatment. At the highest concentrations tested (0.25 and 0.50 mM), a slight decrease (not significant) of the EPR signal, rather than an increase, was even observed. Therefore, these data argue against the involvement of lipid peroxidation in THA-induced cytotoxicity, at least during the early stages.

Alterations of Cell Membrane Fluidity by THA. It has been shown that THA, due to its chemical structure, is capable of binding to acidic phospholipids of reconstituted monolayers; it then locates at the interfacial region (Lehtonen et al., 1996). In this context, we hypothesized that THA might affect membrane fluidity in cultured hepatocytes. To test this hypothesis, EPR spectroscopy was again used but this time coupled to a spin-labeling technique (Ogura et al., 1988). Two different stearic acid spin labels were chosen depending on their site of incorporation into membranes: the 5-DSA and the 12-DSA incorporated into the outer (hydrophilic) and inner (hydrophobic) layers of the membrane, respectively.
cultured hepatocytes, as determined using the spin-label 5-DSA, are illustrated as a function of time in culture on THA treatment; the values are expressed as percent deviation from the order parameter calculated in nontreated cells to establish the difference of membrane fluidity between THA-treated and nontreated hepatocytes. This graph showed that THA, even at a dose as high as 0.50 mM and following a 6-h treatment, did not modify the fluidity of the membrane outer layer. In contrast, the use of 12-DSA revealed a rapid and marked decrease of the order parameter, representative of an increase in the fluidity of the membrane inner layer, as soon as 30 min (Fig. 4). It was important to note that such an increase in membrane fluidity was observed at the concentration of 0.25 mM as well as at a lower concentration of THA (0.05 mM); this effect was still detected following a 24-h treatment.

**Reduction of THA-Induced Cytotoxicity by GM1 Ganglioside.** To find out whether the increase of membrane fluidity on THA treatment might underlie the cytotoxicity of this molecule, experiments were carried out using the GM1 ganglioside. Indeed, this complex lipid, a natural constituent of cell membranes, has been shown to afford protection against a variety of neural insults when extracellularly applied, this protection being related to a stabilizing effect of GM1 on membranes (Hungund and Mahadik, 1993). Figure 5 shows the effects of GM1 (added 2 h before and then simultaneously to THA) on the dose-dependent increase in LDH leakage induced by THA following a 24-h treatment. Although not totally prevented, the THA-elicited LDH release was significantly reduced in the presence of 60 μM GM1 by about 30% (average results from three independent experiments). Results with 80 μM GM1 were obtained from two independent experiments and showed that protection afforded by the ganglioside was not enhanced at a higher concentration. With the aim of testing whether this effect of GM1 was due to an effect on membrane fluidity, EPR signals were next recorded in cultured hepatocytes treated or not with THA (0.25 mM) and/or GM1 (60 μM). Figure 6 shows that, although GM1 did not change the basal cell membrane fluidity, it fully reversed the effect of THA on this parameter. Similar results were obtained in two independent experiments.

**Effects of THA on pH i.** The effects of short-term (about 15-min) treatments with THA were evaluated on the steady-state pH i of cultured hepatocytes. Figure 7A shows a representative pH i recording obtained using carboxy-SNARF-1 in hepatocytes superfused with a HCO3-/CO2-buffered solution. Under these conditions, the steady-state pH i of these cells was 7.29 ± 0.03 (n = 24 coverslips seeded from three different cell isolations). On application of THA (0.25 mM), a stable intracellular alkalinization was elicited. This effect was found to be less marked at a lower concentration (Fig. 7B).

**Discussion**

This work shows that the toxicity of THA in rat hepatocytes in primary culture does not result from an early oxida-
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1) in the latter study, a fluorescent technique was used to detect the production of ROS while we directly estimated lipid peroxidation using a spin-trapping method in conjunction to EPR; 2) the cells used in the two studies were different (rat hepatocytes in primary culture compared with cells (HepG2) isolated from a human hepatocellular carcinoma). Finally, in an in vivo model of THA hepatotoxicity, production of ROS was assumed to result from an effect of THA on the sympathetic nervous system rather than a direct effect on parenchymal cells (Stachlewitz et al., 1997). Concerning the absence of effects of antioxidants, it could be argued that simultaneous addition of the flavonoids with THA did not allow sufficient time for efficient prevention of the oxidative stress. In this regard, it should be emphasized that a recent clinical study performed in de novo THA-treated patients with Alzheimer’s disease has shown that coadministration of silymarin with THA (the flavonoid was given 1 week before THA, at a dose known to be effective in vivo, see e.g., Ferenci et al., 1989) does not prevent the THA-induced elevation of serum liver transaminase, thus arguing against a determinant role for oxidative stress in vivo (Allain et al., 1999). Finally, another argument against the occurrence of early lipid peroxidation was the increase of membrane fluidity presently detected. Indeed, lipid peroxidation is generally associated with a decrease of this parameter (Sakanashi et al., 1988; Masaki et al., 1992). However, whether THA induces lipid peroxidation following long-term treatments is not excluded and remains to be tested.

As stated above, this work demonstrated that THA increased the fluidity of plasma membrane in cultured rat hepatocytes. More precisely, by using two different spin labels, 5-DSA and 12-DSA, which incorporate at different sites in the lipid bilayer (Ogura et al., 1988; Sakanashi et al., 1988), we showed that THA specifically altered the inner membrane whereas the outer (interfacial) layer remained unchanged. Such a differential effect on the two regions of the membrane bilayer previously has been obtained when exposing cells, for example, to an increase of extracellular calcium (Storch and Schachter, 1985), to bile acids (Nakashima et al., 1993), or to ethanol (Chin and Goldstein, 1981). The observation that the hydrophobic region was preferentially affected by THA was actually quite surprising because this molecule has been described to bind to acidic phospholipid-containing membranes and, as amphipathic, is expected to reside in the interfacial region of the bilayer (Lehtonen et al., 1996). As yet, there is no information regarding the mechanisms by which THA is capable of eliciting changes in membrane fluidity. One might suppose either an effect of THA on membrane lipid composition via an action on phospholipases or an effect of the THA-induced alkalization on the membrane architecture. Considering the former hypothesis, it has been reported that, in a neuronal cell line, stimulation of phospholipase A2 by ATP depletion changed membrane fluidity via a release of arachidonic acid (Ray et al., 1994). Concerning an effect of H+ ions, the membrane fluidity of rat liver mitochondria has been shown to decline as pH was shifted from 7.35 to 7.0 (Zimmer et al., 1990). Similarly, Astarie and coworkers (1992) have observed that platelet cytosolic acidification was accompanied by a decrease of membrane fluidity. In this context, we may suppose a rapid increase of hepatocyte membrane fluidity due to

tive stress leading to lipid peroxidation. In line with this, an increase rather than a decrease of cell membrane fluidity was detected very rapidly after THA exposure. This early increase, paralleled by pHI changes, is likely to play a determinant role in THA toxicity because prevention of membrane fluidity alterations partly reduced this toxicity.

The possibility for an oxidative stress to be involved in the THA hepatotoxicity was first raised by Dogterom and co-workers (1988) who showed that vitamin E, a natural antioxidant, delayed the toxicity of THA in isolated rat hepatocytes, through prevention of lipid peroxidation. However, the THA concentrations used in that study were high (>1 mM) compared with those expected in vivo (from 0.05 to 2.5 μM at the onset of the hepatic sinusoid; Berson et al., 1996); therefore, those results might have been merely artificial. Here, at lower concentrations (<0.5 mM), no production of lipid radical could be detected, at least up to 24 h following treatment of cultured hepatocytes. Moreover, experiments carried out with antioxidant molecules (silibinin and derivatives) did not show any prevention or reduction of THA toxicity under our experimental conditions, thus ruling out any involvement of an oxidative stress. This is actually in contrast to a recent study carried out on HepG2 cells (Osseni et al., 1999). These controversial results could stem from different factors:
the intracellular alkalization, which developed early on THA exposure.

The observation that alterations in membrane fluidity were induced rapidly on THA exposure led us to seek a causal link between this early change and the cell-killing effects of THA. This supposition was reinforced by the fact that toxic molecules, such as butylated hydroxytoluene (Shertzer et al., 1991) or ethanol (Chen et al., 1996) also induced an increase of membrane fluidity. To test this hypothesis, we decided to use GM1 gangloside, a natural constituent of hepatocyte membranes, and to look for its effects on the LDH release elicited by THA. The rationale for performing such experiments actually came from studies dealing with ethanol neu­rotoxicity showing that exogenous GM1 decreased vulnera­bility to cellular injury caused by ethanol (Hungund and Mahadik, 1993) while stabilizing the cell membrane (Chen et al., 1996). Extracellular addition of GM1, simultaneously to THA, was shown to significantly reduce the LDH release from hepatocytes, indicating a partial protection against THA toxicity. By using EPR, we found that this protection was concomitant of a stabilizing effect of GM1 on hepatocyte membrane. Therefore, these results point to a determinant role for the early change of membrane fluidity in the development of THA-induced hepatotoxicity. As a consequence of this change, one might indeed suppose that the activity of several membrane-bound proteins important for cell survival [receptors, ion (e.g., H+ or Ca2+) carriers, . . . ] would be altered by THA, as previously shown for other toxic insults (López-Aparicio et al., 1994). In this context, pH alterations detected in this study, rather than affecting membrane fluidity as suggested above, might in contrast result from modifications of this fluidity (Dudeja et al., 1987). With respect to GM1, it would seem likely that membrane stabilization by this lipid may act to reduce the incidence of THA-induced cell death by maintaining normal membrane functions. This possibility is supported by studies reporting the ability of GM1 to preserve plasma membrane Na+/K+-ATPase activity, to maintain a normal intracellular Ca2+ homeostasis, or to reduce activation of phospholipase A2 (Hungund and Mahadik, 1993). Also, GM1 has been demonstrated to alter different protein kinase activities [phosphatidylinositol 3-kinase (Ryu et al., 1999), mitogen-activated protein kinase (Van Brocklyn et al., 1997), cyclic AMP-dependent protein kinase (Yates et al., 1989), . . . ], resulting in changes in membrane protein phosphorylation involved, e.g., in calcium transport.

The fact that THA-induced toxicity may partially result from membrane fluidity alterations raises an intriguing con­cern about the liver-specific toxicity of THA. Indeed, in this context, one could have expected THA to induce similar membrane alterations in many other tissues, leading to toxicity. In an attempt to solve this apparent contradiction, two important points have to be considered: 1) the membrane phospholipid composition differs among tissues, which might lead to differential effects on fluidity; and 2) first pass metabolism of THA in the liver results in the exposition of nonhepatic tissues to much lower concentrations, thus accounting for specific liver injury.

Finally, a major question concerns the in vivo relevance of the effects evidenced here. Indeed, plasma concentrations of THA associated with hepatotoxicity in humans are substantially lower than the concentrations presently used (e.g., Berson et al., 1996). These differences may actually originate from several factors: 1) liver THA concentrations exceed plasma concentrations (by ~6- to 10-fold); 2) the high THA concentrations required for in vitro cytotoxicity may be related to short exposure times relative to prolonged drug exposure in patients (the onset of expression of elevated transaminases in humans takes 6 to 8 weeks); and 3) older animals are more susceptible to THA-induced cellular alterations than younger ones (Robertson et al., 1998). In this regard, it is important to emphasize that the target population for THA therapy is older than the general population.

In conclusion, our data show that THA induces early alterations of membrane fluidity, likely playing an important role in the development of related cytotoxicity. Furthermore, these membrane changes do not result from THA-induced lipid peroxidation, which did not occur under our experimental conditions. Finally, this study is, to our knowledge, the first one to show that application of extracellular GM1 ganglioside affords some protection to hepatocytes against toxic insults (here THA) altering cell membrane fluidity.

References
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