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## Role for Membrane Fluidity in Ethanol-Induced Oxidative Stress of Primary Rat Hepatocytes

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### ABSTRACT

The relationship between bulk membrane fluidizing effect of ethanol and its toxicity due to oxidative stress is still unknown. To elucidate this issue, membrane fluidity of primary rat hepatocytes was studied by measuring order parameter after inhibition of ethanol-induced oxidative stress. We showed that pretreating cells with either 4-methyl-pyrazole (to inhibit ethanol metabolism), thiourea [a reactive oxygen species (ROS) scavenger], or vitamin E (a free radical chain-breaking antioxidant) prevented the ethanol-induced increase in membrane fluidity, thus suggesting that ethanol metabolism and ROS formation were involved in this elevation. The effects of membrane stabilizing agents (ursodeoxycholic acid or ganglioside GM1), shown to prevent fluidification, next pointed to a role for this increase in membrane fluidity in the development of ethanol-

induced oxidative stress. Indeed, ROS production, lipid peroxidation, and cell death were all inhibited by these agents. In contrast, the fluidizing compounds Tween 20 or 2-(2-methoxyethoxy) ethyl 8-(*cis*-2-*n*-octylcyclopropyl) octanoate, which increased the membrane fluidizing effect of ethanol, enhanced the related oxidative stress. Using electron paramagnetic resonance to determine low molecular weight iron, we finally demonstrated that membrane fluidity influence proceeded through an increase in low molecular weight iron to enhance oxidative stress. In conclusion, the present findings clearly highlight the pivotal role of membrane fluidity in ethanol-induced oxidative stress and the potential therapeutic effect of membrane stabilizing compounds.

Oxidative damage to lipids, proteins, and DNA after ethanol intoxication of the liver are very well described (Nordmann et al., 1992; Bailey and Cunningham, 2002). Since oxidative stress generated in hepatocytes is involved in processes as varied as ethanol-induced apoptosis (Kurose et al., 1997; Minama et al., 2002), immune reactions toward the liver (Albano, 2002), and activation of stellate cells and hence fibrogenesis (Nieto et al., 2002), this phenomenon seems to contribute to the pathogenesis of alcoholic liver diseases. Consequently, this emphasizes the necessity to fully characterize the molecular mechanisms of ethanol-induced oxidative stress in hepatocytes, especially during the early stages of the disease. It can result from ethanol metabolism in hepatocytes that triggers reactive oxygen species (ROS) pro-

duction during both acute and chronic alcoholism (Sergent et al., 1995; Bailey and Cunningham, 1998). At the very early stage of the disease, changes in membrane fluidity could also play a role, because an increase in plasma membrane fluidity has been described after acute ethanol intoxication of WRL-68 hepatic cell lines (Gutierrez-Ruiz et al., 1995) or of primary rat hepatocytes (Benedetti et al., 1994). What is particularly interesting is that liver is the only organ to maintain this increase in membrane fluidity during chronic intoxication (Yamada and Lieber, 1984; Polokoff et al., 1985), even though chronic ethanol exposure of animals or liver cells would make membranes more resistant to the disordering effects of ethanol in vitro (Polokoff et al., 1985; Gutierrez-Ruiz et al., 1995). However, to our knowledge, no study has looked for a close association between ROS production and the increase in liver membrane fluidity due to ethanol intoxication. Actually, the main findings have focused on mitochondrial inner membrane and have highlighted a role for a decreased fluidity in the impairment of the mitochondrial

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**ABBREVIATIONS:** ROS, reactive oxygen species; GM1, monosialoganglioside 1; H<sub>2</sub>FDA, dihydrofluorescein diacetate; UDCA, sodium ursodeoxycholate; 12-DSA, 12-doxyl stearic acid; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; A<sub>2</sub>C, 2-(2-methoxyethoxy) ethyl 8-(*cis*-2-*n*-octylcyclopropyl) octanoate; EPR, electron paramagnetic resonance.

transport of reduced glutathione, one of the most important antioxidants in cells (Lluís et al., 2003).

In the present study, we have performed a series of experiments in which bulk membrane fluidity was manipulated by incorporating membrane stabilizing agents or membrane fluidizing compounds in membranes of rat hepatocytes. This has allowed assessment of the effect of membrane fluidity changes on ethanol-induced oxidative stress. In addition, membrane fluidity was also measured after inhibition of ethanol-induced oxidative stress to determine whether an early oxidative stress can influence the physical state of hepatocyte membranes.

## Materials and Methods

**Chemicals.** Minimum Eagle's medium and medium 199 with Hanks' salts were purchased from Biomedica (Boussens, France). Fetal calf serum was from Biowest (Nuaille, France). Liberase was from Roche Diagnostics (Meylan, France). Ethanol and thiourea were obtained from Prolabo (Paris, France). Monosialoganglioside 1 sodium salt (GM1) was from Alexis (Coger, Paris, France). Dihydrofluorescein diacetate (H<sub>2</sub>FDA) was provided by Molecular Probes (Interchim, Montluçon, France). Ursodeoxycholate sodium salt (UDCA) was from Calbiochem (Meudon, France). 12-Doxyl stearic acid (12-DSA), Hoechst 33342,  $\alpha$ -tocopherol, desferrioxamine, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), 4-methylpyrazole, xanthine, xanthine oxidase, polyoxyethylene sorbitan monolaurate (Tween 20), and 2-(2-methoxyethoxy) ethyl 8-(*cis*-2-*n*-octylcyclopropyl) octanoate (A<sub>2</sub>C) were all purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

**Cell Isolation and Culture.** Briefly, adult rat hepatocytes were isolated from 2-month-old Sprague-Dawley animals by perfusion of the liver as described previously, except that a 17  $\mu$ g/ml liberase solution was used for dissociation of liver parenchymal cells instead of a collagenase solution (Guguen-Guillouzo et al., 1983). Routinely, more than 90% of the freshly isolated hepatocytes excluded trypan blue, and 500 million of hepatocytes could be obtained from a single rat liver perfusion. Cells obtained using this protocol were shown to synthesize albumin, a specific function of hepatocytes, and to form bile canalicular-like elements (Guguen-Guillouzo et al., 1983). Cells were seeded at densities of  $30 \times 10^3$  cells/well in 96-well Falcon microplates (ROS evaluation),  $3 \times 10^6$  cells in 25-cm<sup>2</sup> Falcon flasks (lipid peroxidation evaluation),  $10 \times 10^6$  cells in 75-cm<sup>2</sup> Falcon flasks (membrane fluidity determination), and  $20 \times 10^6$  cells in 175-cm<sup>2</sup> Falcon flasks (low molecular weight iron measurement) and cultured in a medium composed of 75% minimum Eagle's medium and 25% medium 199 with Hanks' salts, supplemented with 10% fetal calf serum and containing 50  $\mu$ g of streptomycin, 5  $\mu$ g of penicillin, 5  $\mu$ g of bovine insulin, 1 mg of bovine serum albumin, and 2.2 mg of NaHCO<sub>3</sub> per milliliter. The cells were kept at 37°C in an atmosphere of 5% CO<sub>2</sub> and 95% air. The medium was changed 3 h after seeding and substituted with the same medium as described above but deprived of serum. Culture treatments were in all cases started the day after seeding.

**Culture Treatments.** Ethanol at a final concentration of 50 mM was added to rat hepatocytes and cultures maintained at 37°C for 0.5 to 24 h. It should be noted that over our test ethanol exposure period, no significant evaporation could be found in culture media without cells (Sergent et al., 1995). In one set of experiments, some cultures were preincubated either for 1 h with membrane stabilizing agents (100  $\mu$ M UDCA or 60  $\mu$ M GM1) or for 0.5 h with membrane fluidizers (600  $\mu$ g/ml Tween 20 or 5  $\mu$ M A<sub>2</sub>C), and then the medium was discarded before adding ethanol. In the experiments seeking the mechanisms underlying the fluidizing effect of ethanol, hepatocyte cultures were pretreated either for 12 h with 250  $\mu$ M  $\alpha$ -tocopherol, a lipid peroxidation inhibitor, for 1 h with 100 mM thiourea, a ROS scavenger, or for 0.5 h with 1 mM 4-methylpyrazole, an ethanol

metabolism inhibitor. These latter agents were kept in medium during the incubation with ethanol.

**Measurement of Membrane Fluidity.** The fluidity of hepatocyte bulk membranes (i.e., plasma membrane and possibly endosome and lysosome membranes) was determined by a spin-labeling method using electron paramagnetic resonance (EPR), as described previously in other cell cultures (Ogura et al., 1988). At the end of each incubation period, the lipid bilayer of hepatocyte membranes were spin labeled by incubation, for 15 min at 37°C, of hepatocyte suspensions with 50  $\mu$ g/ml 12-DSA, a fatty acid exhibiting a stable nitroxide radical ring at the C12-position. Cells were then washed three times with phosphate-buffered saline to remove the free spin label. The resultant pellet was then transferred to a disposable glass capillary. The EPR spectra of labeled samples were acquired at ambient temperature on a Bruker 106 EPR spectrometer operating at 3495-G center field, 20-mW microwave power, 9.82-GHz microwave frequency, 1.771-G modulation amplitude, and 100-kHz modulation frequency. The fluidity of the labeled membrane was quantified by calculating the order parameter *S* according to equations described previously (Ogura et al., 1988). An increase in the value of *S* is interpreted as a decrease in membrane fluidity, whereas a decrease of this parameter reflects an increase in membrane fluidity.

**Determination of Reactive Oxygen Species Production.** Intracellular levels of ROS were measured using the nonfluorescent probe, H<sub>2</sub>FDA, as described previously (Hempel et al., 1999). Oxidation by ROS causes a conversion of this probe into fluorescent fluorescein. Briefly, hepatocytes were loaded with 50  $\mu$ M H<sub>2</sub>FDA in HEPES-buffered solution for 30 min at 37°C, washed with HEPES, and then treated with 50 mM ethanol in usual culture medium. After a last wash, fluorescence was directly recorded by a SpectraMax Gemini spectrofluorimeter (Molecular Devices, Saint-Grégoire, France) using 485-nm excitation and 530-nm emission wavelengths.

**Evaluation of Lipid Peroxidation.** Lipid peroxidation was analyzed by measurement of extracellular free malondialdehyde as described previously (Morel et al., 1990). Briefly, malondialdehyde was measured by size exclusion chromatography after ultrafiltration of the culture media through 500-Da membrane (Millipore, Saint-Quentin-les-Yvelines, France).

**Measurement of Low Molecular Weight Iron.** Measurement of intracellular low molecular weight iron was based upon the capacity of desferrioxamine to chelate only low molecular weight iron and to give a paramagnetic chelate, which had the advantage to be directly detectable by EPR in whole hepatocytes (Sergent et al., 1997a).

**Cell Death Determination by Microscopy.** Because neither caspase activity nor lactic dehydrogenase release could be detected under our experimental conditions (our unpublished results), cell death was studied by visualization of nuclear morphology (condensed and fragmented chromatin) and by estimation of lysosome disruption. Nuclear fragmentation was monitored by Hoechst 33342 staining (excitation, 330–380 nm; emission, 460 nm). After 5 h of incubation with ethanol, cells were stained in PBS with 2 mg/ml Hoechst dye for 15 min at 37°C in the dark. Cells were examined using an inverted Olympus microscope, and the percentage of cells with fragmented nuclei was calculated as (cells with fragmented nuclei/total cells)  $\times$  100. Total population was always  $>300$  cells.

Lysosome disruption was estimated from acridine orange-stained hepatocytes, knowing that an increase in green acridine orange fluorescence reflected such a disruption (Zhao et al., 2000). Briefly, cultures were incubated with 5  $\mu$ M acridine orange for 20 min at 37°C before being detached by incubation with trypsin/EDTA. After resuspension of cells in culture medium, cell suspensions were immediately analyzed by flow cytometry on an FACS Calibur flow cytometer (BD Biosciences, San Jose, CA). Fluorescence emission from acridine orange was detected at FL1 (green acridine orange fluorescence) after excitation at 488 nm. Each measurement was conducted on 20,000 events and analyzed on Cell Quest software (BD Biosciences).

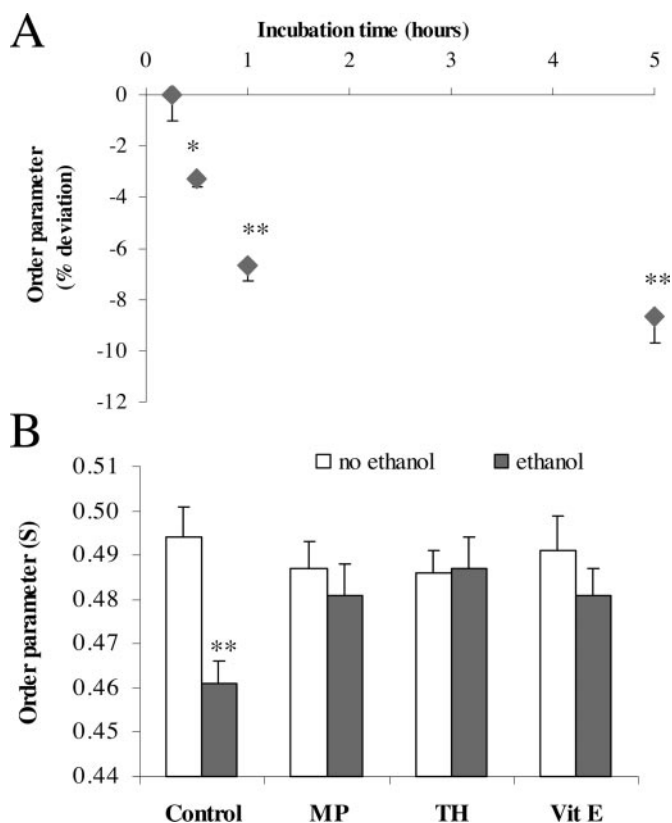
**Free Radical Scavenging Properties.** Scavenging properties of membrane stabilizing agents toward hydroxyl radical ( $^{\circ}\text{OH}$ ) and superoxide anion ( $\text{O}_2^-$ ) were examined by spin trapping using DMPO as a spin trap. For the  $^{\circ}\text{OH}$  radical generation, the so-called Fenton reaction was used. Briefly, reaction mixture was constituted as follows: 30  $\mu\text{l}$  of distilled water, 50  $\mu\text{l}$  of 40 mM  $\text{H}_2\text{O}_2$ , 10  $\mu\text{l}$  of 800 mM DMPO, and 90  $\mu\text{l}$  of 1 mM ferrous sulfate supplemented with either 20  $\mu\text{l}$  of distilled water or 20  $\mu\text{l}$  of 1 mM UDCA, or 20  $\mu\text{l}$  of 600  $\mu\text{M}$  GM1. For the spin trapping experiments of the  $\text{O}_2^-$ , the xanthine/xanthine oxidase reaction was used to generate  $\text{O}_2^-$  by preparing the following reaction mixture: 30  $\mu\text{l}$  of 26 U/ml xanthine oxidase, 30  $\mu\text{l}$  of 5 mM xanthine, 20  $\mu\text{l}$  of 400 mM DMPO, and 100  $\mu\text{l}$  of ethanol supplemented with either 20  $\mu\text{l}$  of distilled water or 20  $\mu\text{l}$  of 1 mM UDCA, or 20  $\mu\text{l}$  of 600  $\mu\text{M}$  GM1. Immediately after a vigorous stirring, the reaction mixtures were injected inside a glass capillary. For  $^{\circ}\text{OH}$  or  $\text{O}_2^-$  detection, EPR spectra were recorded after 1 or 2 min, respectively, on a Bruker 106 EPR spectrometer at room temperature with the EPR settings as follows: 3495-G field center, 9.81-GHz microwave frequency, 20-mW microwave power, 100-kHz frequency modulation, 0.804-G modulation amplitude, 81.92-ms conversion time, and 1.28-ms time constant. The intensities of EPR spectra were represented by the double integration of the lines of lowest field. Free radical scavenging activities of UDCA and GM1 were expressed as percentage of decrease in the EPR intensity of the control performed with distilled water.

**Statistical Analysis.** Values were expressed as means  $\pm$  S.D. Hepatocytes obtained from three different isolations were generally used for all the protocols carried out. Multiple comparisons among the groups were performed using analysis of variance followed by the parametric Student-Newman-Keuls test or the nonparametric Mann-Whitney test, depending on the results of the normality test. Differences were considered significant at  $p < 0.05$ .

## Results

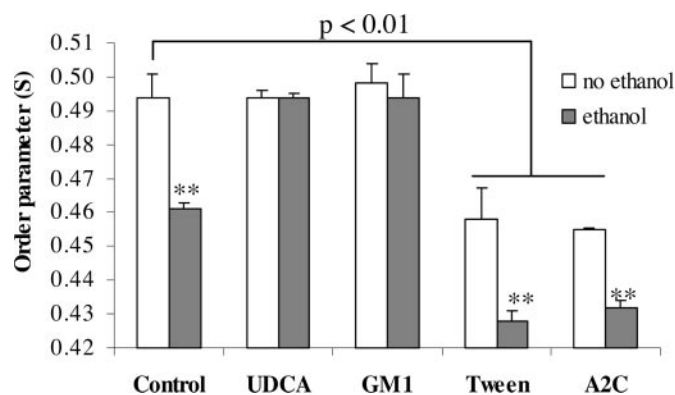
**Early Increase in Membrane Fluidity upon Ethanol Exposure Involved Oxidative Stress.** To investigate the kinetics of ethanol-induced membrane fluidizing effect, rat hepatocytes were treated with 50 mM ethanol for different incubation times (Fig. 1A). The values were expressed as percentage of deviation from the order parameter calculated from untreated cells. An early increase (+3.3%) in membrane fluidity of rat hepatocytes upon ethanol exposure was detectable after 30 min of incubation. Longer incubation times resulted in a more extensive rise, with a deviation percentage of + 8.7% at 5 h. For the 24-h-incubation time, no change could be observed compared with control hepatocytes (data not shown). Because ethanol metabolism is well known to induce an oxidative stress that may affect membrane fluidity, the effect of ethanol on membrane fluidity was also evaluated after pretreatment of hepatocytes either by 4-methyl-pyrazole, an inhibitor of ethanol metabolism; by thiourea, an ROS scavenger; or by vitamin E, a free radical chain-breaking antioxidant. It should be noted that 4-methyl-pyrazole, at this concentration, inhibited ethanol elimination by 95% (our unpublished results). As shown in Fig. 1B, all these compounds prevented the increase in membrane fluidity due to ethanol. Indeed, in 4-methyl-pyrazole-, thiourea-, or vitamin E-pretreated hepatocytes, no significant differences in the order parameter were found between ethanol-treated and untreated cultures.

**The Membrane Fluidizing Effect of Ethanol Was Responsible for an Enhancement of Oxidative Stress.** To determine whether the increase in membrane fluidity could participate in oxidative stress induced by ethanol, membrane



**Fig. 1.** Ethanol increased membrane fluidity in rat hepatocytes. A, changes in the membrane fluidity of hepatocytes treated with 50 mM ethanol for 0.25, 0.5, 1, 5, or 24 h. The membrane fluidity was monitored by EPR analysis of 12-DSA embedded in membranes. This analysis resulted in the obtention of the order parameter S that is inversely related to membrane fluidity. Data are expressed as a percentage of deviation from those obtained in untreated control cultures. B, effects of 4-methyl-pyrazole, an inhibitor of ethanol metabolism; thiourea, an ROS scavenger; and vitamin E, a free radical chain-breaking antioxidant, on the increase of membrane fluidity in hepatocytes treated with 50 mM ethanol for 1 h. Some rat hepatocyte cultures were preincubated with 1 mM 4-methyl-pyrazole (4MP) for 0.5 h, 100 mM thiourea (TH) for 1 h, or with 250  $\mu\text{M}$  vitamin E (Vit E) for 12 h. Results were obtained as in A, except that data were directly expressed as order parameter (S). Values are the mean  $\pm$  S.D. of three independent experiments. Ethanol-treated versus untreated cultures: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

stabilizing agents such as GM1 ganglioside or UDCA were added before addition of ethanol. These agents were first confirmed as being capable of blocking the membrane fluidizing effect of ethanol since, after pretreatment of cells with GM1 or UDCA, ethanol remained without effect on the order parameter (Fig. 2). In rat hepatocytes treated with ethanol, a rise in ROS production was detectable as soon as 15 min (Fig. 3A). After 1 and 5 h of incubation, the level of ROS was further increased, by nearly 2-fold, compared with untreated cells (Fig. 3, B and C, respectively); no significant effect of ethanol was detected at 24 h (data not shown). Interestingly, membrane stabilizing agents displayed no effect on ROS formation induced by a 15-min exposure to ethanol (Fig. 3A), whereas after 1 or 5 h of treatment, a significant protection against ROS production was observed (Fig. 3, B and C). Besides, we found that 4-methyl-pyrazole inhibited ROS formation as soon as 15 min (Fig. 3A). Note that ROS production increases in all groups over time. This is due to the primary culture senescence. Indeed, it is well known that many antioxidant enzymes, such as Cu,Zn superoxide dismutase, glu-

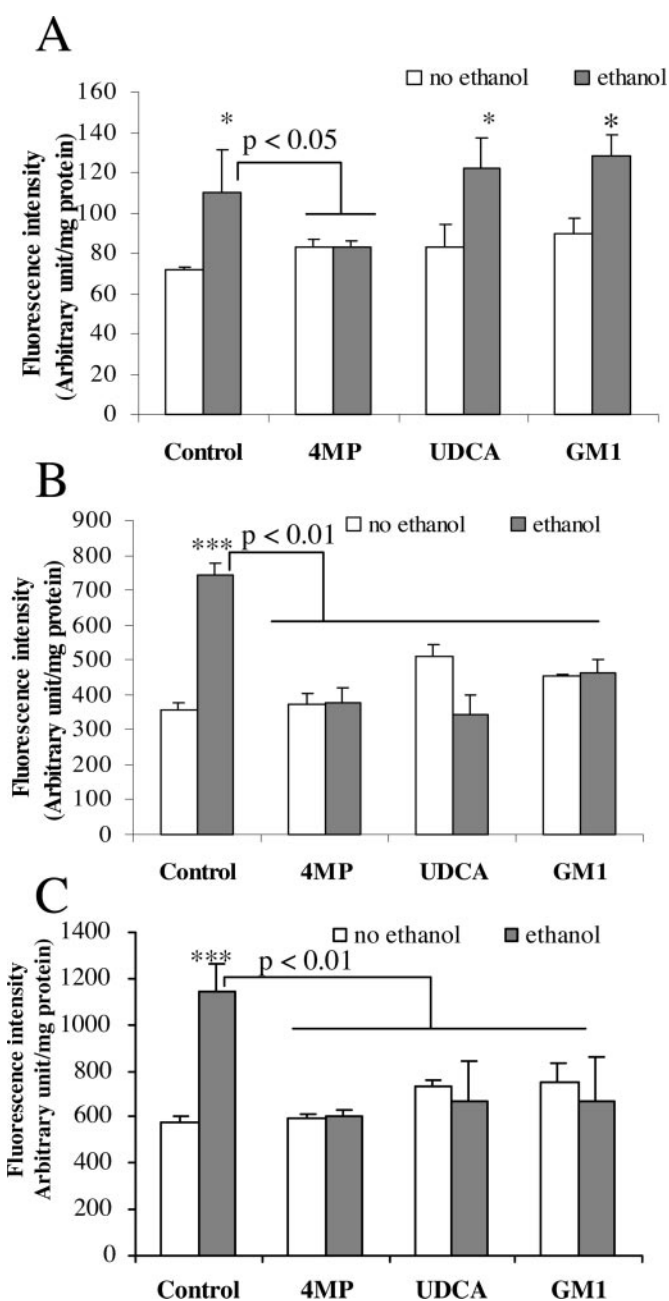


**Fig. 2.** Effects of membrane stabilizing agents (UDCA or GM1) or membrane fluidizers (Tween 20 or A<sub>2</sub>C) on the membrane fluidizing effect of ethanol. The membrane fluidity was monitored by EPR analysis of 12-DSA embedded in membranes. This analysis allowed the estimation of the order parameter S that is inversely related to membrane fluidity. Rat hepatocytes were incubated or not with 50 mM ethanol for 1 h. Some cultures were pretreated, for 1 h, with membrane stabilizing agents (100  $\mu$ M UDCA or 60  $\mu$ M GM1) or for 0.5 h with membrane fluidizers (600  $\mu$ g/ml Tween 20 or 5  $\mu$ g/ml A<sub>2</sub>C). Values are the mean  $\pm$  S.D. of three independent experiments. Ethanol-treated versus untreated cultures: \*\*,  $p < 0.01$ .

tathione peroxidase, or catalase, are less expressed over incubation time after isolation of rat hepatocytes (Antras-Ferry et al., 1997).

To fully demonstrate the involvement of the increase in membrane fluidity in ethanol induced-oxidative stress, lipid peroxidation and also cell death were next followed, since oxidative stress generated by ethanol metabolism participates in ethanol-induced cell death (Kurose et al., 1997; Minama et al., 2002). Using malondialdehyde as a marker of lipid peroxidation, an increase by about 160% of lipid peroxidation was observed in hepatocyte cultures treated for 5 h with ethanol (Fig. 4A). This elevation of malondialdehyde content was inhibited by pretreating cells with membrane stabilizing agents GM1 or UDCA (Fig. 4A). In addition, both these agents prevented ethanol-induced cell death (Fig. 4, B and C). For lower concentrations of ethanol (10, 20, and 30 mM), UDCA exhibited a similar protection on both ROS formation and lipid peroxidation (data not shown).

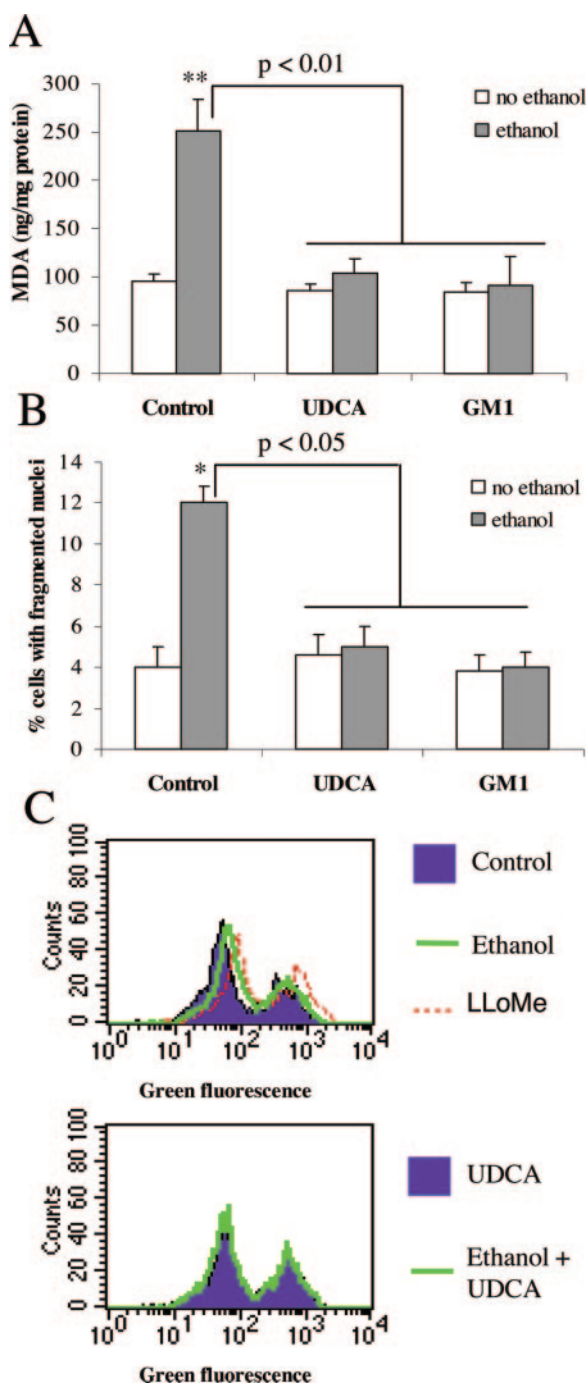
The involvement of the membrane fluidizing effect of ethanol in its capacity to generate oxidative stress was further confirmed by adding Tween 20, a mild neutral detergent, or A<sub>2</sub>C, a fatty acid-like compound, to hepatocytes before ethanol treatment. Indeed, Tween 20 and A<sub>2</sub>C were demonstrated to increase membrane fluidity under our experimental conditions (Fig. 2), both in the presence or absence of ethanol. This elevation of membrane fluidity might be responsible for potentiation of ethanol-induced oxidative stress (Fig. 5, A and B) and cell death (Fig. 5C) in cultures pretreated by Tween 20 or A<sub>2</sub>C. Note also that Tween 20 or A<sub>2</sub>C alone induced slight but significant effects on oxidative stress ( $p < 0.01$ ). In addition, the supplementation of hepatocytes with UDCA, before exposure to Tween 20 or A<sub>2</sub>C, protected cells from the potentiation by Tween 20 or A<sub>2</sub>C of ethanol-induced ROS formation (Fig. 5A). Similar effect was observed with GM1 (data not shown). It should be added that membrane stabilizing agents or fluidizing compounds did not change the rate of ethanol uptake by hepatocytes (data not shown).



**Fig. 3.** Effects of membrane stabilizing agents (UDCA or GM1) or of 4-methyl-pyrazole, an inhibitor of ethanol metabolism, on ROS production induced by ethanol. ROS production was followed by the fluorescence of H<sub>2</sub>FDA. Rat hepatocytes were incubated or not with 50 mM ethanol for 15 min (A), 1 h (B), or 5 h (C). Some cultures were pretreated for 1 h with 100  $\mu$ M UDCA or 60  $\mu$ M GM1. Values are the mean  $\pm$  S.D. of three independent experiments. Ethanol-treated versus untreated cultures: \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ .

### The Protection Afforded by Membrane Stabilizing Agents Was Not Due to a Direct Antioxidant Property.

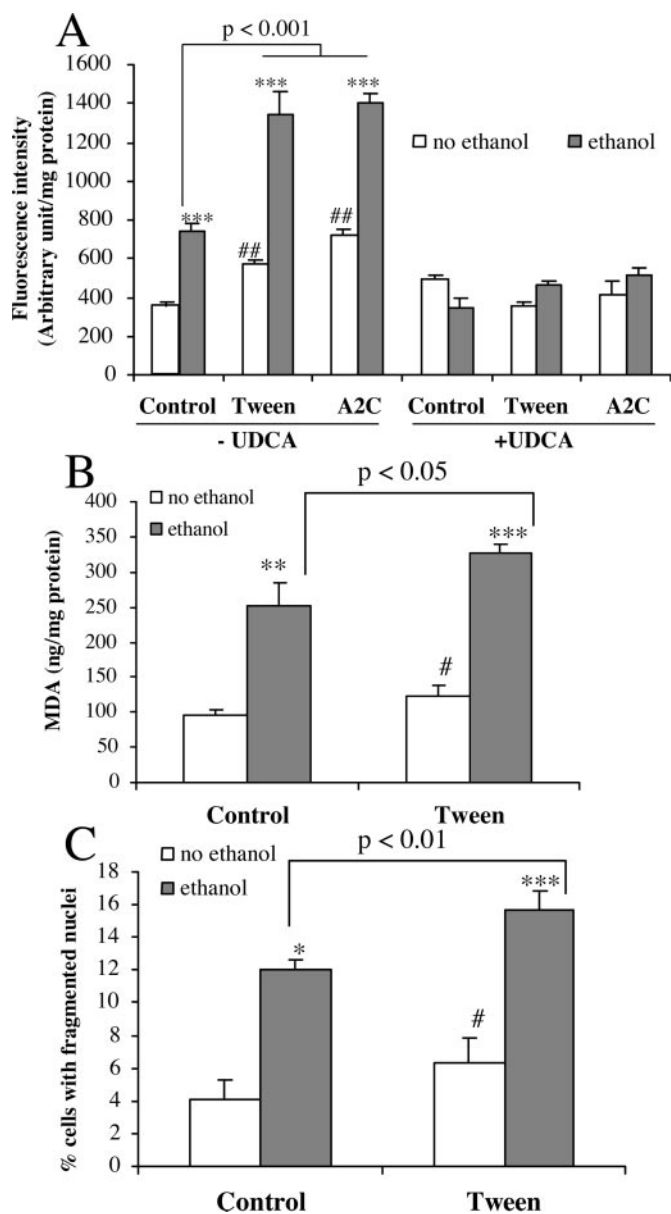
With the aim of seeking a possible direct antioxidant property of membrane stabilizing agents, which might have explained the above-mentioned results, UDCA and GM1, at the same concentrations as those used above, were tested by EPR for their capacity to scavenge superoxide anion or hydroxyl radical. UDCA and GM1 did not display a potent scavenging capacity (data not shown). Indeed, UDCA was the only agent to scavenge superoxide anion,



**Fig. 4.** Membrane stabilizing agents (UDCA or GM1) prevented ethanol-induced lipid peroxidation (A) and cell death (B and C). Lipid peroxidation was estimated by measurement of free malondialdehyde (MDA). Cell death was identified by staining nuclear chromatin with Hoechst 33342 (B) and or by staining lysosomes with acridine orange (C). L-leucyl-L-leucine methylester (LLeMe) (500  $\mu$ M, 20 min at 37°C) was used as a positive control of lysosome disruption. Rat hepatocytes were incubated or not with 50 mM ethanol for 5 h. Some cultures were pretreated for 1 h with 100  $\mu$ M UDCA or 60  $\mu$ M GM1. Values are the mean  $\pm$  S.D. of three independent experiments, except for nuclear fragmentation experiments ( $n = 3$  or 5). Ethanol-treated versus untreated cultures: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

and this superoxide anion scavenging capacity was very weak (21%).

To rule out the possibility of a direct antioxidant effect of UDCA or GM1, these drugs were also tested for their capac-



**Fig. 5.** Effects of membrane fluidizers (Tween 20 or A<sub>2</sub>C) on ROS formation (A), lipid peroxidation (B), and cell death (C) in rat hepatocytes incubated with ethanol. The membrane fluidity was monitored by EPR analysis of 12-DSA embedded in membranes. The estimated order parameter S is inversely related to membrane fluidity. ROS production was followed by the fluorescence of H<sub>2</sub>FDA. Lipid peroxidation was estimated by measurement of MDA. Cell death was identified by staining nuclear chromatin with Hoechst 33342. Concerning studies on ROS formation, rat hepatocytes were incubated or not with 50 mM ethanol for 1 h. For studies on lipid peroxidation and cell death, ethanol was added for 5 h. Some cultures were pretreated for 0.5 h with 600  $\mu$ g/ml Tween 20 or 5  $\mu$ g/ml A<sub>2</sub>C. Values are the mean  $\pm$  S.D. of three independent experiments, except for cell death experiments ( $n = 3$  or 5). Ethanol-treated versus untreated cultures: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . A<sub>2</sub>C- or Tween 20-treated versus untreated cultures: #,  $p < 0.05$ ; ##,  $p < 0.01$ .

ity to protect cells from lipid peroxidation induced by iron. This was carried out using a 5-h exposure to 20  $\mu$ M ferric nitriloacetate. Lipid peroxidation was followed by measuring malondialdehyde, a secondary end product of degradation of oxidized polyunsaturated fatty acids. UDCA and GM1 were unable to abolish the increase in malondialdehyde in hepatocytes treated by ferric nitriloacetate (control, 95  $\pm$  6 ng/mg protein; UDCA, 86  $\pm$  7 ng/mg protein; GM1, 79  $\pm$  4 ng/mg

protein; iron,  $1012 \pm 51$  ng/mg protein; UDCA + iron,  $944 \pm 130$  ng/mg protein; and GM1 + iron,  $915 \pm 87$  ng/mg protein;  $n = 3$  independent experiments). Even with ferric nitriloacetate at a lower concentration ( $5 \mu\text{M}$ ), which led to a lesser extent of lipid peroxidation, UDCA and GM1 did not succeed to reduce malondialdehyde levels to those of the controls (data not shown). Together, these data strongly emphasized that membrane stabilizing agents, at the concentration used in our experiments, did not exhibit any direct antioxidant property.

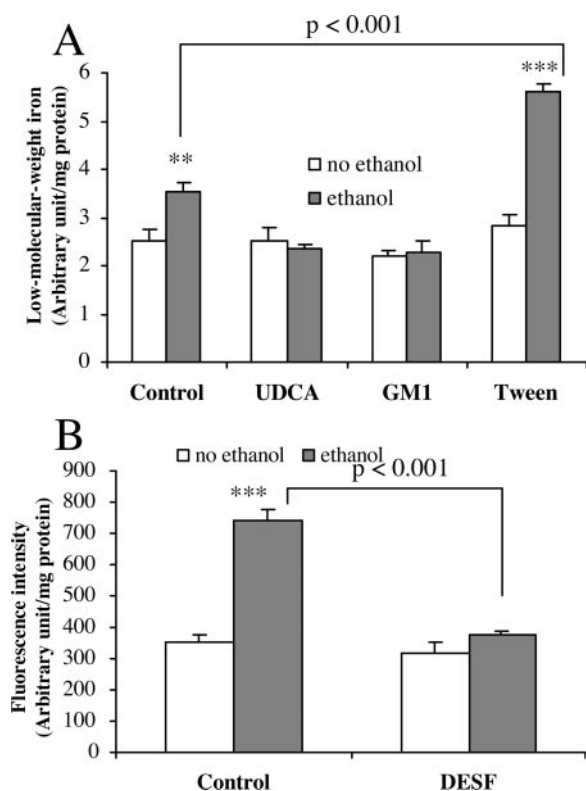
**The Involvement of Membrane Fluidity in Ethanol-Induced Oxidative Stress Proceeded through an Increase in Low Molecular Weight Iron Content.** Low molecular weight iron consists of iron species that are not contained in high molecular weight molecules, such as ferritin or mitochondrial ferroproteins, but are able to trigger oxidative stress by catalyzing the formation of a highly reactive free radical, the hydroxyl radical, via Fenton or Haber-Weiss reaction. An EPR technique was used to directly measure the level of low molecular weight iron in intact cells. As expected from previous studies (Sergent et al., 1995, 1997a), ethanol increased the content in low molecular weight iron by nearly 40% (Fig. 6A). UDCA and GM1 pretreatment totally

protected cells from this elevation, whereas Tween 20 pretreatment enhanced it (Fig. 6A). Because ROS formation could be inhibited by desferrioxamine, a low molecular weight iron chelator (Fig. 6B) and because ethanol-induced lipid peroxidation has been previously described to be inhibited by deferiprone, another low molecular weight iron chelator (Sergent et al., 1997b), it can be concluded from the present results that the membrane fluidizing effect of ethanol contributed to ethanol-induced oxidative stress by promoting the elevation of low molecular weight iron.

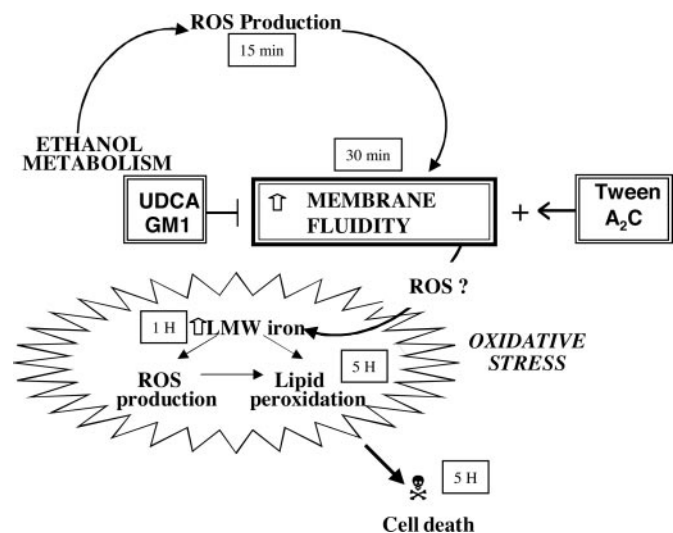
## Discussion

The present study demonstrates, for the first time, that an increase in bulk membrane fluidity can amplify the oxidative stress induced by ethanol in the liver. In addition, we have found a new interpretation based upon an early ROS production to explain how ethanol can very rapidly increase membrane fluidity. Thus, in rat hepatocytes, in only 30 min of incubation, ethanol elicited an early membrane fluidizing effect. Whereas the fluidizing effect of ethanol had been ascribed until now to the disruption of the orderly packing between adjacent acyl chains due to either direct entering of ethanol into the lipid portion of the membrane (Rottenberg, 1991), an increased phosphatidylcholine/sphingomyelin ratio (Polokoff et al., 1985), or a decreased cholesterol/phospholipid ratio (Yamada and Lieber, 1984) of liver plasma membranes, we showed here that oxidative stress due to ethanol metabolism also contributed to the elevation of membrane fluidity since this rise was prevented by both antioxidants and 4-methyl-pyrazole, an inhibitor of ethanol metabolism. Moreover, the earliest detectable ROS production, which was observed as soon as 15 min, preceded the increase in membrane fluidity detected only at 30 min and was inhibited by 4-methyl-pyrazole but not by GM1 or UDCA, suggesting that the early ROS formation was only related to ethanol metabolism. Thus, the proposed sequence of events linking ethanol metabolism to its membrane fluidizing effect is shown in Fig. 7. Although not directly addressed in this study, the possible molecular mechanism whereby oxidative stress influences membrane fluidity of rat hepatocytes can be discussed. Lipid peroxidation by-products have been widely recognized to increase membrane fluidity either by interacting with membrane proteins (Buko et al., 1996; Subramaniam et al., 1997) or more directly by their own rearrangement (Jain et al., 1994; Gabbita et al., 1998). ROS are also able, by oxidizing tubulin, to disrupt the microtubule cytoskeleton and thereby to increase membrane fluidity (Yoon et al., 1998; Remy-Kristensen et al., 2000). This remains to be tested under our conditions.

As stated above, the major finding of this study is the demonstration that an early increase in membrane fluidity can be crucially involved in the enhancement of oxidative stress. Indeed, UDCA and GM1 totally inhibited ethanol-induced ROS production, lipid peroxidation, and apoptosis in rat hepatocytes, whereas Tween 20 and  $\text{A}_2\text{C}$  enhanced them. The beneficial effects of UDCA and GM1 were found by EPR to be concomitant with a stabilizing effect on hepatocyte membranes, whereas Tween 20 enhanced the fluidizing effect of ethanol. It should be noted that UDCA and GM1 displayed a protection toward ethanol-induced ROS production only when ROS were evaluated after 1 or 5 h of incuba-



**Fig. 6.** Changes in low molecular weight iron content can account for the involvement of membrane fluidity in ethanol-induced oxidative stress. A, effects of membrane stabilizing agents (UDCA and GM1) and Tween 20 on low molecular weight iron content in rat hepatocytes treated with ethanol for 1 h. Low molecular weight iron was determined by EPR analysis after chelation by 1 mM desferrioxamine. Rat hepatocytes were preincubated either for 1 h with  $100 \mu\text{M}$  UDCA or  $60 \mu\text{M}$  GM1 or for 0.5 h with  $600 \mu\text{g/ml}$  Tween 20. Values are the mean  $\pm$  S.D. of three independent experiments. Ethanol-treated versus untreated cultures: \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . B, effect of desferrioxamine, a low molecular weight iron chelator, on ROS formation induced by ethanol. Rat hepatocytes were incubated with ethanol for 1 h or not. Some cultures were pretreated for 1 h with  $20 \mu\text{M}$  desferrioxamine (DESF). Ethanol-treated versus untreated cultures: \*\*\*,  $p < 0.001$ .



**Fig. 7.** Proposed model for the role of early ROS-induced fluidizing effect of ethanol in the amplification of oxidative stress. In hepatocytes, ethanol metabolism can very rapidly lead to ROS production, which in turn increased membrane fluidity. This increase can promote the elevation of low molecular weight (LMW) iron content and therefore the enhancement of ROS production and lipid peroxidation to finally trigger cell death. Membrane fluidizers (Tween or  $A_2C$ ) by potentiating the increase in membrane fluidity enhanced oxidative stress, whereas membrane stabilizing agents (UDCA or GM1), by protecting from the fluidizing effect of ethanol, inhibited it.

tion with ethanol. At 15 min, no protection was afforded by membrane stabilizing agents, unlike the inhibitor of ethanol metabolism, 4-methyl-pyrazole. This has led us to postulate a sequence of events whereby the early ROS formation was mainly due to ethanol metabolism and the late phase might also be attributed to the increase in membrane fluidity (Fig. 7). To reinforce the determinant role for the increased membrane fluidity in the initiation of oxidative stress, we have further sought the mechanism whereby membrane fluidity can elicit oxidative stress. Using EPR determination with intact hepatocytes, we show that similarly to their effects on ethanol-induced ROS production and lipid peroxidation, UDCA and GM1 inhibited the increase in low molecular weight iron content induced by ethanol, whereas Tween 20 enhanced it. It should be noted that, in previous studies (Sergent et al., 1995), the increase in low molecular weight iron was shown to be detectable only after 30 min of incubation with ethanol. Together, these results strongly support the idea that oxidative injury due to the elevation of membrane fluidity was mediated by an increase in low molecular weight iron content. Indeed, low molecular weight iron consists of iron species that can trigger oxidative stress by catalyzing the formation of a highly reactive free radical, the hydroxyl radical. In our study, desferrioxamine, a low molecular weight iron chelator, was thus found to inhibit the ROS formation induced by ethanol in rat hepatocytes. These results agree with those of Rouach et al. (1994), who observed an early increase of hepatic iron uptake from transferrin related to ROS produced by ethanol, but without establishing a link with the membrane fluidizing effect of ethanol. In this regard, it should be emphasized that an increased transferrin receptor mobility can favor its internalization (Paccaud et al., 1993) and that the major pool of low molecular weight iron is located in lysosomes or late acidic endosomes (Yu et al., 2003). In our model, it cannot be excluded that the

elevation of low molecular weight iron content could be due to an increase ROS formation. Indeed, superoxide anion is well described to release iron from ferritin (Biemond et al., 1988) and oxidative stress can promote rupture of late acidic endosomes or lysosomes, thus liberating iron from these organelles (Yu et al., 2003). In this regard, our results on acridine orange-stained hepatocytes that suggested lysosome disruption would tend to support that point.

It should be kept in mind that the toxic effect of an increase in membrane fluidity, described in this study, concerns bulk membranes. Indeed, a decrease rather than an increase in fluidity has been demonstrated in inner membranes of mitochondria isolated from HepG2 cells treated with acetaldehyde, a product of ethanol metabolism (Lluis et al., 2003). Moreover, the addition of a membrane fluidizer such as  $A_2C$  to these mitochondria can restore levels of glutathione, an important antioxidant for cells (Lluis et al., 2003). In this context, the use of membrane fluidizers should be done with caution, since from our results, it seems that they can be injurious for hepatocytes. On the contrary, UDCA and its conjugates seem to be good candidates for a potential therapeutic use: due to their membrane stabilizing properties, they reestablish the normality in membrane fluidity for every type of membrane. Thus, in case of ethanol intoxication, they are able to prevent both the increase of bulk membrane fluidity, as we observed it, and the decrease in the mitochondrial membrane of hepatocytes from ethanol-fed rats, thereby restoring mitochondrial glutathione levels (Colell et al., 2001). Moreover, our results about the cytoprotection afforded by UDCA and GM1 agree with previous studies that showed the prevention by UDCA of ethanol-induced necrosis and apoptosis, respectively, in human Hep G2 cells (Neuman et al., 1995) and primary rat hepatocytes (Rodrigues et al., 1998) or by GM1 of ethanol-induced death in mice neural crest cells (Chen et al., 1996) and rat cerebellar granule neurons (Saito et al., 1999). UDCA was also found to prevent lipid peroxidation in the liver from chronic ethanol-fed rats (Montet et al., 2002). But none of these studies gave a clear understanding of the mechanism involved in the protection against oxidative injury. In our study, UDCA and GM1 actions, at the doses used in our experiments, cannot be attributed to direct antioxidant properties of these compounds since they were unable to scavenge superoxide anion and hydroxyl radical in vitro and to inhibit iron-induced lipid peroxidation of rat hepatocytes. This new mechanistic demonstration of a beneficial effect of UDCA is particularly interesting because UDCA is a therapeutically relevant bile acid, already used for preventing human chronic cholestatic liver diseases (Colombo et al., 1992; Poupon et al., 2003) that might be useful in the very early stages of the alcoholic liver diseases as well.

In conclusion, the present study shows that ethanol metabolism, by triggering an early ROS production, promotes a rapid increase in membrane fluidity. The fluidizing effect of ethanol is then responsible for a secondary elevation of ROS production that can then induce lipid peroxidation and apoptosis (Fig. 7). Consequently, early ethanol toxicity can be strongly enhanced by an increase in membrane fluidity due to ethanol metabolism itself or membrane fluidizers. The present findings clearly highlight the pivotal role of membrane fluidity in the early stages of alcoholic liver disease,



and, in this context, membrane stabilization might be a very effective strategy to protect hepatocytes from oxidative damage.

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