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Short communication

Resistance of HepG2 cells against the adverse effects of ethanol related to neutral lipid and phospholipid metabolism

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Abstract

The influence of both short- and long-term ethanol exposure on the lipid metabolism was determined in the human hepatoma cell line HepG2. Ethanol did not cause any cytotoxicity or lipid peroxidation even after 7 days of 100 mM ethanol treatment of HepG2 cells. Incubation of cells in the presence of [1-^{14}C]ethanol demonstrated that these cells actively metabolize ethanol to acetyl CoA, incorporating the radioactive label into neutral lipids and phospholipids. [1,2,3-^{3}H]glycerol was efficiently used in phospholipid and neutral lipid biosynthesis, showing higher radioactivity in phosphatidylcholine, phosphatidylethanolamine and triacylglycerols. Exposure of HepG2 cells to 100 mM ethanol for 24 hr did not significantly modify the incorporation of glycerol into newly synthesized phospholipids and neutral lipids, nor was lipid degradation affected by the presence of ethanol. When the alcohol treatment was prolonged for 7 days, incorporation of [1,2,3-^{3}H]glycerol into triacylglycerols and diacylglycerols showed a slight increase concomitantly with decreased radioactivity in the major phospholipids, phosphatidylcholine and phosphatidylethanolamine. In addition, these changes were associated with a greater release of radiolabeled triacylglycerols into the culture medium. These results indicate that ethanol does not cause in HepG2 cells the marked lipogenic stimulation widely shown in hepatocytes, and demonstrate that HepG2 cells strongly resist the adverse effects of ethanol. Since these cells lack the isoenzymatic form of cytochrome P_{450} mainly involved in the ethanol metabolism (namely cytochrome P_{450}2E1) and also are devoid of alcohol dehydrogenase activity, we propose that the toxic actions of ethanol on liver must be linked to the activity of one or both of these systems.

Keywords: Ethanol; Lipid metabolism; HepG2 cells

1. Introduction

It has been widely shown that ethanol consumption adversely affects hepatic and extrahepatic tissues, leading to the development of fatty liver as well as lipid alterations in other body organs [1,2].

Substantial controversy has arisen in the past as a result of attempts to explain the different biochemical mechanisms operative in various disorders associated with the actions of ethanol on different tissues. The effects of chronic ethanol consumption on several organs may be related in part to its metabolism. The main pathway for ethanol oxidation involves hepatic alcohol dehydrogenase (ADH), a cytosolic enzyme that catalyzes the transformation of ethanol into acetaldehyde, which is in turn converted to acetate through acetaldehyde dehydrogenase activity. Both enzyme reactions generate an excess of reducing equivalents in the liver, primarily in the form of NADH [3]. Thus, after prolonged ethanol consumption the change in the redox state promotes the synthesis of fatty acids and increases the concentration of α-glycerophosphate [4], which favors hepatic lipid accumulation by trapping fatty acids. Ethanol can also be metabolized by peroxisomal catalase activity, although this system of ethanol oxidation is believed to play in liver a minor part *in vivo* [5].

Moreover, long-term ethanol consumption is associated with the proliferation of hepatic microsomal membranes, suggesting that liver microsomes could be the site of a distinct and adaptive system of ethanol oxidation, called the microsomal ethanol-oxidizing system (MEOS). This

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Abbreviations: ADH, alcohol dehydrogenase; DAG, diacylglycerols; EMEM, Eagle's minimum essential medium; FCS, fetal calf serum; HepG2, human hepatoblastoma cells; MEOS, microsomal ethanol-oxidizing system; PBS, phosphate-buffered saline; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; TAG, triacylglycerols.

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system involves an alcohol-inducible isoform of cytochrome P_{450}, named P_{450}2E1 [6].

We have recently demonstrated that both chronic [7,8] and acute [9,10] ethanol treatments increase lipogenic activity in hepatocytes isolated from rat liver, but it is difficult to distinguish between the effects caused by ethanol itself and those caused by the oxidative metabolism of ethanol. Therefore, we have investigated the actions of ethanol in the ADH-deficient human hepatoblastoma cell line HepG2, which is extensively used in studies on human hepatic lipid metabolism [11,12]. It has been reported that HepG2 cells exhibit around 0.5% of ADH activity of freshly isolated rat hepatocytes [13]. Thus, the different ethanol metabolism exhibited by HepG2 prompted us to examine how ethanol exposure could alter the lipid synthesis in these cells. To do this, we have firstly analyzed ethanol metabolism using labeled ethanol, and secondly we have determined the influence of both short- and long-term ethanol exposure on the metabolism of neutral lipids and phospholipids, using labeled glycerol as an exogenous precursor.

2. Materials and methods

2.1. Reagents and chemicals

[1-^{14}C]ethanol (55 mCi/mmol) and [1,2,3-^{3}H]glycerol (40 Ci/mmol) were purchased from American Radiolabeled Chemicals Inc. EMEM and TLC plates were acquired from Sigma-Aldrich. FCS was obtained from Roche Diagnostics. All other reagents were of analytical grade.

2.2. Cell culture

The human hepatoma cell line HepG2 was obtained from European collection of animal cell cultures. Cells were routinely grown in EMEM supplemented with 10% (v/v) heat-inactivated FCS, 2 mM L-glutamine, 1% non-essential amino acids, 100 units/mL penicillin and 100 μg/mL streptomycin, at pH 7.4. Cells were seeded in tissue culture plates (Nunc^{TM}) and incubated at 37° in a humidified atmosphere of 95% air, 5% CO_2.

In the ethanol-treatment studies, cells were incubated in the presence or absence of 100 mM ethanol for 24 hr (acute doses) as well as for 7 days (chronic treatment). Medium of the ethanol and control dishes was renewed every 24 hr.

The toxicity of ethanol against HepG2 cells was determined by the trypan blue exclusion assay, by measuring the release of cytosolic lactate dehydrogenase (LDH) into the extracellular medium, and *via* the 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Lipid peroxidation was measured by using the thiobarbituric acid test.

Cultures were tested at regular time intervals for the presence of mycoplasma contamination, by using the bisbenzimidazole fluorochrome stain for DNA.

2.3. Metabolic labeling experiments: incorporation of [1-^{14}C]ethanol or [1,2,3-^{3}H]glycerol

When cells reached about 70% confluence, they were deprived of serum for 12 hr before the metabolic labeling assays. The metabolic fate of the ethanol was determined by incubating HepG2 cells for 2.5 hr in serum-free EMEM medium containing [1-^{14}C]ethanol (10 mM, 0.5 mCi/mmol), in the presence or absence of 10 mM metyrapone. Lipid biosynthetic activity was estimated from the rate of incorporation of [1,2,3-^{3}H]glycerol (25 μM, 0.2 Ci/mmol) into cellular lipids.

For the analysis of lipid degradation, pulse-chase experiments were performed. Cells were labeled with [1,2,3-^{3}H]glycerol (25 μM, 0.2 Ci/mmol) for 24 hr in serum-free medium. The radioactive medium was then replaced with fresh serum-free medium alone (control) or containing 100 mM ethanol and radioactivity was quantified in the course of 24 hr in the cellular and secreted lipid fractions, for ethanol-treated and control cells.

2.4. Lipid extraction and analysis

Medium was collected and centrifuged at 50 *g* for 10 min to remove cell debris. Cells were harvested by scraping and the lipids were extracted from the cells and the medium, following the procedure of Bligh and Dyer [14]. The main phospholipids were separated by TLC using a mixture of chloroform/methanol/acetic acid/water (60:50:1:4, by vol.) as solvent, while phosphatidate and phosphatidylethanol were separated using the solvent chloroform/methanol/acetic acid/water (80:13:8:0.3, by vol.). The different neutral lipids were separated using a solvent of *n*-hexane/ethyl ether/acetic acid (80:20:2, by vol.). Cholesterol and DAG were separated using the mixture benzene/isopropanol/water (100:10:0.25, by vol.).

The spots were scraped and transferred to scintillation vials to measure the radioactivity with a Beckman 6000-TA liquid scintillation counter. The amount of cell protein was determined by the method of Bradford [15].

2.5. Statistical analysis

Statistical comparisons were made by student's *t*-test. For time-course experiments, statistical analyses were performed using a multivariant ANOVA test with the software SPSS 9.0.

3. Results

3.1. Cytotoxic effects of ethanol

Initial experiments evaluated any potential cytotoxic effect of ethanol by a variety of techniques such as leakage of LDH from the cytosol of damaged cells, uptake of

Table 1
Incorporation of radiolabeled ethanol into the different phospholipids and neutral lipids in HepG2 cells

Cell lipids	Incorporation (nmol/mg protein)	Percentage of newly synthesized total lipids (%)
Phospholipids		
PtdCho	125.9 ± 4.5	31.8
PtdEtn	26.5 ± 0.7	6.7
PtdSer	5.5 ± 0.4	1.4
PtdIns	4.5 ± 0.3	1.1
Sphingomyelin	3.8 ± 0.2	1.0
Phosphatidate	1.3 ± 0.4	0.3
Phosphatidylethanol	0.7 ± 0.2	0.2
Neutral lipids		
TAG	160.7 ± 5.0	40.6
Free cholesterol	31.2 ± 0.7	7.9
DAG	19.6 ± 0.9	4.9
Fatty acids	10.8 ± 1.1	2.7
Esterified cholesterol	4.7 ± 0.1	1.2
Fatty acid ethyl esters	1.1 ± 0.2	0.3

HepG2 cells were seeded onto 60-mm dishes. Subconfluent cells were incubated for 2.5 hr in serum-free EMEM medium containing [1-^{14}C]ethanol (10 mM, 0.5 mCi/mmol). Lipids were extracted from the cells and separated by TLC. Values are expressed as mean ± SEM for four determinations.

trypan blue dye, or formazan production from MTT. Chronic or acute ethanol treatment caused no significant toxicity in HepG2 cells. In addition, lipid peroxidation determined as the content of thiobarbituric acid reacting substances was not detected after 24 hr or 7 days incubation of HepG2 cells with 100 mM ethanol, compared to the control cells (results not shown).

3.2. Metabolism of ethanol

To characterize the metabolic fate of ethanol, we incubated HepG2 cells with [1-^{14}C]ethanol and determined its incorporation *via* acetyl CoA into the different cell lipids. As reflected in Table 1, the phospholipid fraction reached its highest radioactive levels in PtdCho and PtdEtn, while the other phospholipids showed low levels of radioactivity. In these experimental conditions we have also found a minimal radioactive label in phosphatidylethanol, indicative of a low activity of transphosphatidylation catalyzed by phospholipase D.

Results of the analysis of [1-^{14}C]ethanol incorporation into the different neutral lipids are also shown in Table 1, revealing a substantial incorporation of radioactivity into TAG, while DAG were labeled to a much lesser degree. Notably, a high radiolabel resulted from [1-^{14}C]ethanol in the cholesterol fraction, even higher than that found in the DAG fraction, while a low level of incorporation into fatty acid ethyl esters was detected.

To study more thoroughly the pathway by which HepG2 cells catabolize ethanol and to quantify the contribution of MEOS to ethanol metabolism, we analyzed the incorporation of [1-^{14}C]ethanol into lipids in the presence of metyrapone, a potent inhibitor of cytochrome P_{450} [16]. Incubation of cells with 10 mM metyrapone decreased the incorporation of radiolabeled ethanol into lipids by 95% with respect to untreated HepG2 cells (265.1 ± 10.5 for control cells vs. 11.2 ± 1.0 for metyrapone-treated cells, expressed as nanomole of labeled lipids per milligram of protein; $n = 4$).

3.3. Time-course incorporation of [1,2,3-^{3}H]glycerol

The results of the time-course incorporation of [1,2,3-^{3}H]glycerol into different phospholipids (Fig. 1) indicate a substantial uptake of this substrate into the major phospholipids, PtdCho and PtdEtn, although PtdIns and PtdSer also show significant labeling. It is noticeable that the profile of distribution of radioactivity is similar for all the phospholipids analyzed, i.e. an increase in its labeling over the period assayed, reaching a maximum at around 8 hr of incubation with [1,2,3-^{3}H]glycerol.

With regard to neutral lipids, glycerol is markedly incorporated in the TAG fraction, while DAG show a low radioactive label. Incorporation of radioactivity into TAG rose steadily up to 8 hr of incubation holding this value afterwards (approximately 2000 pmol/mg protein), while the DAG increased steeply up to 2 hr of incubation and afterwards the rate of radioactive uptake stabilized (approximately 150 pmol/mg protein).

Incubation of HepG2 cells with 100 mM ethanol during 24 hr did not significantly modify the kinetics of [1,2,3-^{3}H]glycerol incorporation into the different phospholipids (Fig. 1) and neutral lipids (results not shown) analyzed.

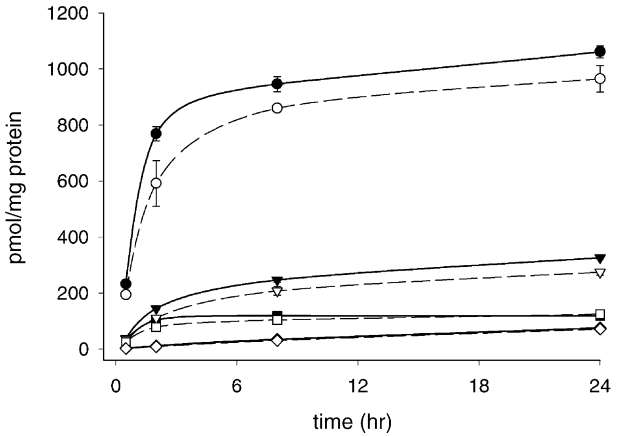

Fig. 1. Time-course of [1,2,3-^{3}H]glycerol incorporation into cellular phospholipids. HepG2 cells were treated with 100 mM ethanol for 24 hr and then incubated with radiolabeled glycerol (25 μM, 0.2 Ci/mmol). At selected times, the incorporation of [1,2,3-^{3}H]glycerol into different newly synthesized phospholipids was analyzed in the acutely ethanol-treated cells (- - -) and control cells (—). Data are expressed as picomole of glycerol incorporated per milligram of cell protein. The different phospholipids analyzed are represented by the following symbols: PtdCho (●), PtdEtn (▼), PtdIns (■), PtdSer (◆) for controls, and PtdCho (○), PtdEtn (▽), PtdIns (□), PtdSer (◇) for ethanol. Values are mean ± SEM for five determinations.

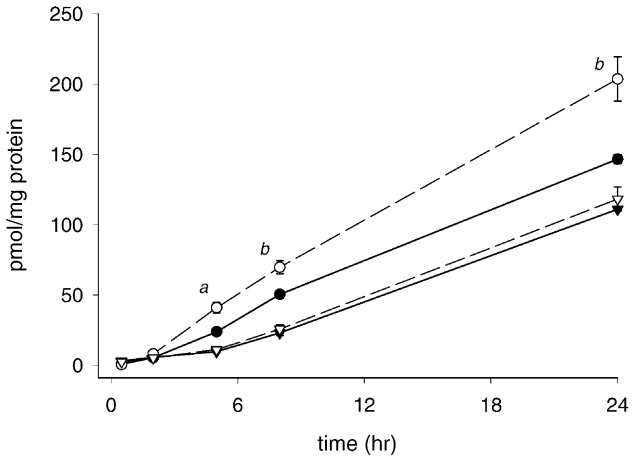

Fig. 2. Effect of ethanol on the release of lipids into the medium in HepG2 cells. Cells were treated with 100 mM ethanol for 24 hr and then incubated with [1,2,3-^{3}H]glycerol (25 μM, 0.2 Ci/mmol). At different times, we analyzed the radioactivity of TAG (circles) and PtdCho (triangles) secreted into the medium by acutely ethanol-treated cells (- - -) and control cells (—). The lipids analyzed are represented by the following symbols: TAG (●) and PtdCho (▼) for controls, and TAG (○), PtdCho (▽) for ethanol. Data are expressed as picomole of glycerol incorporated per milligram of cell protein. Values are mean ± SEM for five determinations. Statistical significance of differences from the controls is indicated by: (a) $P < 0.05$, (b) $P < 0.01$.

3.4. Effect of ethanol on the lipid secretion

We also analyzed the influence of ethanol on secretion of newly synthesized lipids into the medium. TAG and PtdCho were the only lipids that appeared labeled from [1,2,3-^{3}H]glycerol to a significant extent in the medium (Fig. 2). The profiles of TAG and PtdCho export did not follow those for the time-course incorporation of [1,2,3-^{3}H]glycerol into cellular lipids, and thus, release of labeled lipids was found to rise steadily over the 24-hr period. It is noteworthy that only a minor percentage of total TAG and PtdCho synthesized by the cells were exported to the medium. Exposure of the cells to the acute alcohol treatment significantly increased radioactivity of TAG into the medium from 5 hr, without altering the release of *de novo* synthesized PtdCho (Fig. 2).

3.5. Degradation and secretion of pre-labeled endogenous lipids

Taking into account that ethanol could also affect the lipid catabolism, we examined the degradation of phospholipids and neutral lipids in the presence of none (control) or 100 mM ethanol in the medium. After pre-labeling cells with [1,2,3-^{3}H]glycerol for 24 hr, medium was replaced with non-radioactive medium. The amount of radioactivity in the different lipids was determined in both cells and culture medium at selected times.

Interestingly, ethanol alters neither the degradation rate of the different phospholipids or neutral lipids analyzed nor the release of pre-labeled endogenous TAG and PtdCho into the culture medium (results not shown).

Table 2
Effect of chronic ethanol treatment on the incorporation of radiolabeled glycerol into cellular and secreted lipids

Cell lipids	Incorporation (pmol/mg protein)	
	Control	100 mM ethanol
Phospholipids		
PtdCho	899.0 ± 5.5	774.7 ± 4.7[b]
PtdEtn	295.1 ± 4.9	224.0 ± 5.8[b]
PtdIns	26.3 ± 2.9	33.7 ± 1.8
PtdSer	21.4 ± 3.9	22.0 ± 0.6
Phosphatidate	12.7 ± 1.0	13.1 ± 1.8
Neutral lipids		
TAG	1304.2 ± 32.5	1609.6 ± 59.2[a]
DAG	127.6 ± 2.9	196.2 ± 4.7[b]
Secreted lipids		
PtdCho	18.1 ± 0.6	20.1 ± 0.6
TAG	31.8 ± 1.8	40.9 ± 1.2[a]

HepG2 cells were seeded onto 60-mm dishes and treated with 100 mM ethanol for 7 days. Afterwards, cells were incubated for 7 hr in serum-free EMEM containing [1,2,3-^{3}H]glycerol (25 μM, 0.2 Ci/mmol). Lipids were extracted from the cells and separated by TLC as described in Section 2. Values are expressed as mean ± SEM for at least three determinations.

[a] Statistical significance of differences from the controls is indicated by $P < 0.05$.

[b] Statistical significance of differences from the controls is indicated by $P < 0.01$.

3.6. Effect of chronic ethanol treatment on the incorporation of [1,2,3-^{3}H]glycerol into different phospholipids and neutral lipids

The effects of cell exposure to 100 mM of ethanol for 7 days on the incorporation of radiolabeled glycerol into phospholipids are shown in Table 2. As might be expected, in control cells the highest levels of radioactivity were found in PtdCho and PtdEtn. Ethanol treatment resulted in a significant reduction of radioactivity incorporated into these two phospholipids, compared to the control cells, while the radioactive levels of PtdSer, PtdIns or phosphatidate were unaltered by ethanol.

Results concerning the effects of ethanol on the incorporation of isotopically labeled glycerol into neutral lipids are also shown in Table 2. The incubation of HepG2 cells with 100 mM ethanol for 7 days resulted in a slight but significant increase in the uptake of [1,2,3-^{3}H]glycerol into both DAG and TAG, compared to the control cells. In addition, exposure to ethanol provokes a significant increase in the radioactivity associated to TAG released into the medium without affecting the radiolabel of PtdCho.

4. Discussion

Despite decades of study, the mechanisms of the adverse effects of alcohol in liver are not fully established. In the present work we evaluated firstly the effects of ethanol exposure on cellular viability and toxicity in HepG2 cells, showing that chronic or acute ethanol treatment did not

produce alterations in plasma membrane permeability, mitochondrial function, or cell viability. These findings clearly contrast with those of Neuman *et al*. [17] reporting that 50 mM ethanol for 24 hr exerts a toxic effect on HepG2 cells. Other authors demonstrate that cytotoxicity of ethanol is found in transfected HepG2 cells that express cytochrome P_{450}2E1 activity but not in the cells lacking this isoform [18,19], suggesting that some of the hepatotoxic effects of ethanol are associated with intensified lipid peroxidation [20]. In this sense, we found that both acute or chronic ethanol treatment fails to cause any detectable peroxidation in this cell line lacking cytochrome P_{450}2E1.

Radioactivity from [1-^{14}C]ethanol is actively incorporated into the different neutral lipids and phospholipids, demonstrating that in HepG2 cells ethanol can be used as a lipogenic substrate. In addition, we found that ethanol catabolism in HepG2 cells depends on the MEOS system since in the presence of metyrapone, an inhibitor of cytochrome P_{450}, the levels of incorporation of [1-^{14}C]ethanol into lipids fall to as much as 95% of that found in the absence of metyrapone.

Analysis of the incorporation of [1-^{14}C]ethanol into the different lipids shows that the synthesis of both PtdCho and TAG is especially active in this cellular type as described by other authors [21–23]. Acetyl CoA generated from ethanol is also used for sterol synthesis, since a quantitatively important radioactive label appears in the cholesterol fraction. It is noteworthy that the esterified cholesterol fraction, which must have radioactivity both in the sterol ring and in the fatty acyl moiety, shows only a slight radioactivity, suggesting a low activity of cholesterol esterification in HepG2 cells under our experimental conditions.

Interestingly, the rate of ethanol metabolism is not affected after 7 days of alcohol treatment, indicating that the system responsible for ethanol metabolism is not induced by chronic-ethanol treatment. Thus, in these cells ethanol seems to be metabolized by non-inducible forms of cytochrome P_{450}, and hence different from cytochrome P_{450}2E1.

Although it has been extensively reported that both chronic [7,8,24] and acute [4,9,10] ethanol treatments markedly augment lipogenic activity in liver, surprisingly acute exposure of HepG2 cells to ethanol does not produce significant change in the incorporation of radiolabeled glycerol into TAG. Moreover, long-term ethanol exposure of HepG2 cells resulted in only a 20% rise in the TAG and DAG synthesis. These results clearly contrast with our previous findings in which hepatocytes isolated from rat liver incorporate [1,2,3-^{3}H]glycerol into TAG at rates 16-fold higher than in control cells after chronic ethanol exposure [7].

The marked stimulation of lipogenesis in liver by ethanol has been ascribed mainly to the increase in the hepatocellular redox state [3,25]. In the present paper we demonstrate that ethanol is oxidized in HepG2 cells mainly through the P_{450}-dependent system. Alcohol metabolism by this system generates acetate but without changing the redox state of the cell and therefore the lipogenic activity is only slightly increased in HepG2 cells by ethanol, suggesting that the lipogenic stimulation produced by ethanol in the liver depends on alcohol metabolism *via* ADH.

Prolonged ethanol exposure substantially diminishes [1,2,3-^{3}H]glycerol incorporation into the main phospholipids, PtdCho and PtdEtn. This effect is not due to a reduction in the production of DAG, since the incorporation of [1,2,3-^{3}H]glycerol into DAG was even increased in ethanol-treated cells in comparison to controls. It suggests that ethanol exerts particular actions on the biosynthetic pathways of PtdCho and PtdEtn in HepG2 cells. In this sense, we and other authors have reported that ethanol significantly decreases PtdCho biosynthesis in different cell types [8,26–28]. Moreover, we demonstrate in the present study that ethanol acts on lipid metabolism exclusively at the level of the biosynthetic activity in HepG2 cells without affecting lipid degradation.

With regard to the influence of ethanol on the lipid secretion into the medium, our findings indicate that both acute or chronic ethanol exposure increases the radioactivity of TAG exported to the medium, without altering the release of PtdCho, an effect perhaps related to higher intracellular TAG concentration as well as secretion of apoB [16,29]. Notably, the results in our study show that ethanol exclusively alters the release of newly synthesized TAG without affecting the export of pre-labeled endogenous TAG.

In summary, our results allow us to conclude that the HepG2 cell line strongly resists the adverse effects of ethanol, such as membrane permeability alterations, peroxide formation, or activation of neutral lipid biosynthesis, which have been widely described in primary hepatocyte cultures or isolated hepatocytes. Since these cells lack cytochrome P_{450}2E1 and ADH activity, we propose that the hepatotoxic actions of ethanol must be linked to the activity of one or both of these systems. The only common feature with the hepatocytes is the inhibition by the ethanol of PtdEtn and PtdCho synthesis in HepG2 cells, suggesting that alcohol acts specifically interfering with the biosynthesis of these phospholipids.

Acknowledgments

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