0026-895X/01/6004-742–752$3.00
MOLECULAR PHARMACOLOGY
Vol. 60, No. 4
903/930961
Mol Pharmacol 60:742–752, 2001
Printed in U.S.A.
Copyright © 2001 The American Society for Pharmacology and Experimental Therapeutics

Synergistic Toxicity of Iron and Arachidonic Acid in HepG2 Cells Overexpressing CYP2E1

ANDRES A. CARO and ARTHUR I. CEDERBAUM

Department of Biochemistry and Molecular Biology, Mount Sinai School of Medicine, New York, New York

Received February 21, 2001; accepted June 26, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Priming of the liver for ethanol-induced injury, by nutrients such as polyunsaturated fat and iron, plays a key role in alcoholic liver disease. The objective of this work was to evaluate the effect of the combination of Fe-nitrilotriacetic acid (Fe-NTA) and arachidonic acid (AA) on the viability of HepG2 cells (E47 cells) transfected to express human CYP2E1. Cells were plated, preloaded with arachidonic acid, washed, and exposed to Fe-NTA for variable periods. Fe-NTA (10 μM) or AA (5 μM) alone showed low toxicity to E47 cells (18 and 8%, respectively, at 24 h), whereas the combination produced synergistic injury (62% toxicity at 24 h). Exposure of cells not expressing any cytochrome P450 (P450), or HepG2-C3A4 cells (expressing CYP3A4) to 10 μM Fe-NTA plus 5 μM AA produced lower toxicity (14 and 32%, respectively), demonstrating a role for P450, and in particular CYP2E1, in the development of toxicity by exposure to Fe + AA. Lipid peroxidation was induced in the E47 cells exposed to Fe plus arachidonic acid and the synergistic toxicity was prevented by antioxidants, which also decreased lipid peroxidation. Damage to mitochondria plays a role in the CYP2E1-dependent toxicity of Fe + AA, because the mitochondrial transmembrane potential decreased early in the process, and cyclosporin A prevented the toxicity. Toxicity in E47 cells exposed to Fe + AA is mainly necrotic in nature. Hepatocytes from pyrazole-treated rats, with high levels of CYP2E1, were more sensitive to Fe + AA toxicity than were saline control hepatocytyes. The results presented suggest that low concentrations of Fe and AA can act as priming or sensitizing factors for CYP2E1-induced injury in HepG2 cells, and such interactions may play a role in alcohol-induced liver injury.

Oxidative stress is implicated in the pathogenesis of alcoholic liver injury (Tsukamoto, 2000). Although there are several sources of reactive oxygen species (ROS) in the liver, several findings incriminate the ethanol-inducible cytochrome P450 2E1 (CYP2E1) as one of the systems leading to cell damage (Morimoto et al., 1993) but also see Kono et al. (1999). CYP2E1 is effective in catalyzing the production of ROS and lipid peroxidation in vitro (Ekstrom and Ingelman-Sundberg, 1989); antibody against CYP2E1 blocks lipid peroxidation, which is accentuated by ethanol feeding (Castillo et al., 1992); a close correlation exists between induction of CYP2E1 and experimental alcoholic liver disease (Nanji et al., 1994a); inhibition of CYP2E1 ameliorates alcohol-induced liver injury (Morimoto et al., 1995); and HepG2 cells transfected with a CYP2E1 expression vector show a role for CYP2E1 in alcohol-induced ROS production and cell death (Wu and Cederbaum, 1996). However, several observations suggest that other factors probably interact with alcohol consumption in causing liver damage.

These studies were supported by National Institute on Alcohol Abuse and Alcoholism Grant AA06610.

The intragastric ethanol infusion technique in rodents has demonstrated the critical role of nutritional factors such as polyunsaturated fat and iron in determining sensitization and priming of the liver for ethanol-induced injury (Tsukamoto, 2000). In this model, intake of polyunsaturated fat in ethanol-fed rats but not in pair-fed controls produced many of the pathological features of alcoholic liver injury (Morimoto et al., 1993, 1995; Nanji et al., 1994a,b), although saturated fat did not cause this priming effect. An association between iron and alcoholic liver injury has been proposed (Powell, 1975), probably reflecting synergistic induction of oxidative stress. Dietary iron supplementation (in concentrations that did not induce iron overload) in rats fed ethanol plus a high polyunsaturated fat diet exacerbated hepatocyte damage through accentuation of oxidative stress (Tsukamoto et al., 1995). Several studies have shown that ethanol feeding mildly increases liver iron content, that ethanol feeding sensitizes the liver to iron-catalyzed oxidative injury, and that addition of iron to rats chronically consuming ethanol exacerbates liver injury (Tsukamoto et al., 1995; Stal et al., 1996; Valerio et al., 1996). An oral iron chelator lowered ethanol-

ABBREVIATIONS: ROS, reactive oxygen species; PUFA, polyunsaturated fatty acid; NTA, nitrilotriacetic acid; P450, cytochrome P450; PBS, phosphate-buffered saline; MEM, minimal essential medium; AA, arachidonic acid; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase; Rh123, rhodamine 123; PI, propidium iodide; ANOVA, analysis of variance; TBARS, thiobarbituric acid reactive substances; DMSO, dimethyl sulfoxide; PT, permeability transition; TNF, tumor necrosis factor.

induced lipid peroxidation and fat accumulation (Sadrzadeh et al., 1994). In epidemiological studies in humans, a high intake of polyunsaturated fat promoted alcoholic liver disease, whereas a high intake of saturated fat was relatively protective, and the dietary intake of iron was significantly associated with the risk of cirrhosis in alcohol consumers (Corrao et al., 1998). Clinical evidence suggests a possible synergistic hepatotoxic effect between alcohol ingestion and iron overload, because alcohol consumption may accelerate the development of fibrosis seen in genetic hemochromatosis (Loreal et al., 1992). Although liver iron concentration is elevated only mildly in some patients with alcoholic liver disease (Chapman et al., 1982), there is growing evidence that only mildly increased or even normal amounts of iron can cause or enhance toxicity to the liver in the presence of alcohol (Bonkovsky et al., 1996).

Together, this information suggests a synergistic hepatotoxic effect between alcohol ingestion and nutritional factors such as iron and polyunsaturated fat that may reduce the threshold concentration of hepatic iron and PUFA for developing liver damage in patients with alcoholic liver disease.

The objective of this work was to establish whether there is a synergistic toxic effect of iron and arachidonic acid (a representative polyunsaturated fatty acid) in hepatocytes that overexpress CYP2E1. To test this hypothesis, HepG2 cells transduced to express human CYP2E1 were exposed to Fe-NTA, or arachidonic acid, or a combination of iron and arachidonic acid, and the effect on cell viability was compared with the effect in control HepG2 cells or HepG2 cells expressing CYP3A4 (the principal form of P450 in human liver). This cellular model was previously shown to be successful in demonstrating increased toxicity of Fe-NTA itself (Sakurai and Cederbaum, 1998) or arachidonic acid itself (Chen et al., 1997) in HepG2 cells that overexpress CYP2E1. The toxicity of iron and arachidonic acid was also evaluated in rat hepatocytes with high levels of CYP2E1 (isolated from pyrazole-treated rats), and compared with controls (isolated from saline-treated rats). In the current study, the working concentrations of Fe-NTA and arachidonic acid were chosen such that the toxicity of these compounds by themselves was kept to a minimum. The mode of cell death by the combination of iron and arachidonic acid was also evaluated, as well as the development of oxidative stress and mitochondrial damage in this model.

Materials and Methods

Chemicals. Caspase inhibitor I was purchased from Calbiochem (La Jolla, CA). PBS was from Roche Molecular Biochemicals (Indianapolis, IN). G418 was from Invitrogen (Carlsbad, CA). Ethanol 95% was from Pharmaco Products (Brookfield, CT). Protein concentration was measured using the Bio-Rad DC protein assay (Hercules, CA). The rest of the chemicals used were from Sigma (St. Louis, MO). The iron-NTA complex (1:3 Fe/NTA) was prepared as described previously (Sakurai and Cederbaum, 1998).

Culture and Treatment of Cells. Three human hepatoma HepG2 cell sublines, described in Chen and Cederbaum (1998) and Mari and Cederbaum (2000), were used as models in this study: E47 cells, which constitutively express human CYP2E1; C3A4 cells, which constitutively express human CYP3A4 (obtained from Dr. Dennis Feierman, Mount Sinai School of Medicine, New York, NY), and C34 cells, which are HepG2 cells transfected with the empty pCI vector. P450 (evaluated from the carbon monoxide complex formation) could not be detected in C34 cells, whereas P450 was detectable in the E47 and C3A4 cells; the content of P450 was similar in the E47 and C3A4 cells (15 and 18 pmol/mg of protein, respectively). NADPH cytochrome *c* reductase activity in C34, E47, and C3A4 cells was not significantly different (20.7, 28.3, and 27.0 nmol/min/mg of protein, respectively). All cell lines were grown in MEM containing 10% fetal bovine serum and 0.5 mg/ml G418 supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere in 5% CO_2 at 37°C. Cells were subcultured at a 1:5 ratio once a week. For the experiments, cells were plated at a density of 30,000 cells/ml and incubated for 12 h, in MEM supplemented with 5% FBS and 100 units/ml penicillin and 100 μg/ml streptomycin (MEM_{exps}). After this period, the medium was replaced with MEM_{exps} supplemented with arachidonic acid (or other fatty acids) (from 0 to 10 μM). After 12 h of incubation at 37°C, the medium was removed and the cells were washed once with PBS to remove unincorporated arachidonic acid. The cells were incubated for an additional 12-h period with MEM_{exps}. Then, Fe-NTA was added (from 0 to 25 μM), with or without other additions (e.g., cyclosporin A, caspase inhibitor I, antioxidants), and the cells were incubated for variable periods (up to 36 h) before the biochemical analyses. This protocol of adding AA, allowing it to be incorporated into the cells, followed by removing medium containing free, unincorporated AA before the addition of Fe-NTA, rather than adding AA and Fe-NTA at the same time, was designed to minimize extracellular oxidation of the fatty acid and extracellular generation of free radicals.

Hepatocytes were isolated from pyrazole- or saline-treated rats as described previously (Wu and Cederbaum, 2000). The culture protocol was the same as described above, except that cells were initially incubated for 2 h after seeding, exposed to AA for 8 h, and immediately after washing with PBS, exposed to Fe-NTA for 8 h. The incubation time was decreased because CYP2E1 content in these cells declines rapidly.

Cytotoxicity Measurements. Cells were plated onto 24-well plates and after the corresponding treatment, the medium was removed, and cell viability was evaluated by the MTT test. To the cells in each well, 300 μl of a 1-mg/ml solution of MTT in MEM_{exps} lacking fetal bovine serum was added, and the plate was incubated for 2 h at 37°C. At the end of this period the medium was removed, and 1 ml of 1-propanol was added to each well. The plate was vigorously shaken to solubilize the blue formazan, and the absorbance of the converted dye was measured at a wavelength of 570 nm with background substraction at 630 nm. Viability was expressed as ($100 \times (\Delta A_{570-630}$ sample/$\Delta A_{570-630}$ control)). Control refers to incubations in the absence of arachidonic acid and Fe-NTA and was considered as the 100% viability value.

Another index of cytotoxicity used was the leakage of lactate dehydrogenase. Cells were plated onto six-well plates and at the end of the treatment 1 ml of the medium was collected to measure LDH activity released into the medium (LDH out) using the Sigma LDH-20 diagnostic kit. Cells were washed with PBS, harvested by scraping in 1.0 ml of PBS, and sonicated (20 s, duty cycle 40%, output control 50%). The suspension was centrifuged and the supernatant was assayed for LDH activity (LDH in). Cytotoxicity was expressed as percentage of LDH release: [100 × (LDHout/LDH out + LDH in)]. Cell morphology was also assessed under the light microscope (20× magnification) as a third index of viability.

Lipid Peroxidation Assay. Cells were plated onto 10-mm Petri dishes, and at the end of the treatment the cells were washed twice with PBS, and removed by scraping in PBS plus 0.5 mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), followed by low-speed centrifugation. The cell pellets were resuspended in PBS plus 0.5 mM trolox (to prevent nonspecific lipid peroxidation during sample preparation). The protein concentration of the cell suspension was determined using a protein assay kit based on the Lowry assay (Bio-Rad DC kit). The cell suspension was mixed with twice its volume of 15% trichloroacetic acid, 0.375% thiobarbituric acid, 0.24 N HCl plus 0.5 mM trolox, and heated for 15 min at 100°C. After

centrifugation, the absorbance of the supernatant was measured at 535 nm, and the concentration of MDA calculated from a standard curve prepared using malonaldehyde bisdimethylacetal (Esterbauer and Cheeseman, 1990).

Flow Cytometry Analysis of Mitochondrial Membrane Potential. The mitochondrial transmembrane potential was analyzed from the accumulation of rhodamine 123, a membrane-permeable cationic fluorescent dye. Cells were plated onto six-well plates, and at the end of the treatment the medium was replaced with MEM_{exps} containing 5 μg/ml Rh123, and incubated at 37°C for 1 h. The cells were then harvested by trypsinization, washed with PBS, and resuspended in 1 ml of fetal bovine serum–free MEM_{exps}. The intensity of fluorescence from Rh123 was determined using a BD FACSCalibur flow cytometer (San Jose, CA) as described previously (Bai et al., 1999).

Flow Cytometry to Determine DNA Fragmentation. Cells were plated onto six-well plates and after the corresponding treatment, harvested by trypsinization. Detached cells were also included by centrifugation of the medium. Cells were washed with PBS, resuspended in 80% ethanol, and stored at 4°C for 24 h. After this period, cells were washed twice with PBS, and resuspended in PBS containing 100 μg/ml RNase A. After 30 min at 37°C, the cells were stained with PI (50 μg/ml), and analyzed by flow cytometry for DNA analysis as described previously (Bai et al., 1999).

ATP Assay. The ATP content was determined by the luciferin-luciferase method. Cells were plated onto six-well plates, and after the corresponding treatment, harvested by trypsinization. The cells were washed twice with PBS and resuspended in the same buffer. An aliquot of the cell suspension was assayed for ATP using the Sigma Chemical Luciferase ATP assay kit. The amount of ATP in experimental samples was calculated from a standard curve prepared with ATP, and expressed as nanomoles per milligram of protein. The protein concentration in the cellular suspension was determined as described previously.

Statistics. Data are expressed as mean ± standard error of the mean from three to five independent experiments. One-way analysis of variance (ANOVA) with subsequent post hoc comparisons by Scheffé's test was performed. A $p < 0.05$ was considered statistically significant.

Results

Synergistic Toxicity of Iron and Arachidonic Acid in HepG2 Cells Overexpressing CYP2E1. E47 and C34 cells were preincubated during 12 h with 0 or 10 μM arachidonic acid, washed, and then exposed for variable periods (0 to 36 h) to MEM medium supplemented with either 0 or 25 μM Fe-NTA, as described under *Materials and Methods*. Although the incubation with Fe-NTA or arachidonic acid alone at these concentrations showed low toxicity to E47 cells (19 and 14% at 36 h, respectively), the combination showed a pronounced synergistic toxicity (82% loss of viability at 36 h) (Fig. 1). This synergistic effect in E47 cells was observed starting from early incubation times (3 h) and was not observed in C34 cells (Fig. 1), where overall toxicity in any condition never exceeded 20%. NTA (75 μM) was not toxic to E47 cells preexposed or not to 5 μM arachidonic acid (data not shown), demonstrating the role of iron in the toxicity of the Fe-NTA complex. Concentration curves for arachidonic acid over the range of 0 to 10 μM (with 0 or 10 μM Fe-NTA; Fig. 2A) and Fe-NTA over the range of 0 to 25 μM (with 0 or 5 μM arachidonic acid; Fig. 2B) were performed. The curves confirm the lack of synergism of the combination of iron and arachidonic acid in producing toxicity in C34 cells at these concentrations, and show that in E47 cells the synergistic toxic effect of the combination of iron and arachidonic acid can be observed even at low concentrations of arachidonic acid (2 μM, with 10 μM Fe-NTA), and Fe-NTA (2 μM, with 5 μM arachidonic acid).

Because C34 cells do not contain significant levels of P450, we also evaluated the synergistic effectiveness of AA plus Fe-NTA in a HepG2 cell line that expresses a different P450 than CYP2E1. C3A4 cells express human CYP3A4 at the same level of total P450 as the E47 cells express CYP2E1. Moreover, activity of NADPH-cytochrome P450 reductase was the same between C3A4 and E47 cells (see *Materials and Methods*). Figure 2 shows the toxicity of AA alone or Fe-NTA alone or the combination of AA plus Fe-NTA in the C3A4 cells. Little toxicity by AA alone or Fe-NTA alone at the concentrations used was observed with the C3A4 cells (analogous to results with C34 and E47 cells), whereas the combination of AA plus Fe-NTA produced toxicity. This toxicity in C3A4 cells was greater than that found with C34 cells but

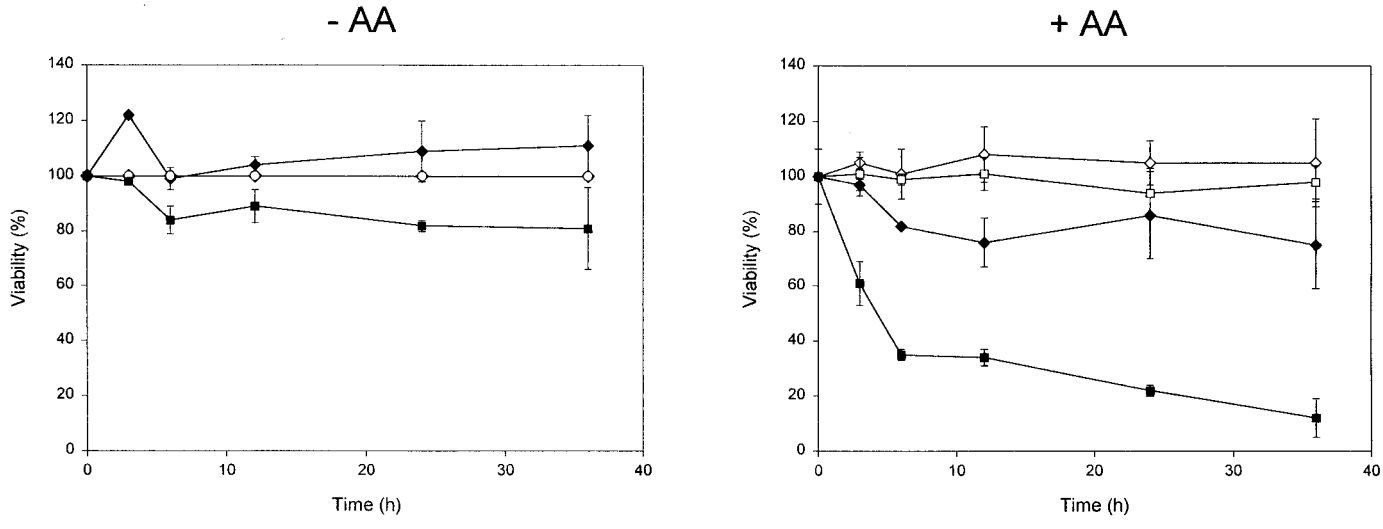

Fig. 1. Synergistic toxicity of iron and arachidonic acid in E47 cells. C34 cells (diamonds) and E47 cells (squares) were incubated for 12 h in MEM medium (−AA), or in MEM medium supplemented with 10 μM arachidonic acid (+AA). After washing, cells were cultured for variable periods in MEM medium (open symbols) or in MEM medium supplemented with 25 μM Fe-NTA (filled symbols). Viability was assessed by MTT reduction activity as described under *Materials and Methods*. The loss of viability in the E47 cells incubated with AA plus iron was significantly different than the other incubations at all time points evaluated ($p < 0.05$).

was less than that observed with the E47 cells. For example, toxicity in cells exposed to 5 μM AA plus 10 μM Fe-NTA over a 24-h period was 14, 32, and 62% in C34, C3A4, and E47 cells, respectively (Fig. 2A). These results suggest that expression of a cytochrome P450, and in particular CYP2E1 (at least relative to CYP3A4), is a key contributor to the synergistic toxicity of AA plus Fe-NTA.

Other polyunsaturated fatty acids (docosahexaenoic acid, eicosapentaenoic acid) also showed synergistic toxicity in E47 cells when combined with Fe-NTA (Table 1). The degree of toxicity in E47 cells of the combination PUFA + Fe-NTA seems to depend on the degree of unsaturation of the fatty acid chain, being high with fatty acids containing at least four double bonds but less significant with linoleic acid (two double bonds). The synergistic effect was not observed in C34 cells exposed to Fe-NTA and any PUFA, and these cells showed very low, nonsignificant toxicities under all of the conditions tested (Table 1).

LDH leakage as an index of viability evaluates the permeability of the plasma cell membrane. Loss of plasma membrane integrity is a characteristic feature of necrotic or late apoptotic cell death (Zamzami et al., 1997). The toxicity of iron and arachidonic acid in E47 cells was further confirmed by observing an increase in LDH leakage in E47 cells exposed to this combination (Fig. 3). LDH release increased early after the exposure to Fe-NTA, indicating an early loss of plasma membrane integrity. On the contrary, LDH release in C34 cells incubated with 10 μM Fe-NTA and 5 μM AA did not change significantly from control values at 24 h (data not shown). E47 cells exposed for 24 h to Fe-NTA and arachidonic acid showed substantial morphological changes (Fig. 4H) with respect to control cells (Fig. 4E): cells were swollen and dispersed, with accumulation of intracellular vesicles, and many cells were detached and floated to the top of the culture dish. No changes were detected in C34 and E47 cells exposed to Fe-NTA alone (Fig. 4, C and G) or arachidonic acid alone (Fig. 4, B and F), and in C34 cells exposed to the combination of iron and arachidonic acid (Fig. 4D), with respect to control cells (Fig. 4A).

Lipid Peroxidation and Cytotoxicity of Iron Plus Arachidonic Acid. Transition metals such as iron are powerful catalysts of lipid peroxidation processes, and polyunsaturated fatty acids such as arachidonic acid provide basic substrates for this reaction (Halliwell and Gutteridge, 1984). Therefore, the hypothesis that the toxicity of iron and arachidonic acid in E47 cells is mediated by lipid peroxidation-dependent reactions was evaluated. The content of TBARS in the cellular suspension was measured as an index of lipid peroxidation. Fe-NTA (10 μM) or 5 μM arachidonic acid did not show significant toxicity in E47 and C34 cells and did not significantly change the levels of TBARS with respect to controls (Table 2A). The combination of iron and arachidonic acid produced a small, nonsignificant increase in loss of viability and production of TBARS in C34 cells, but produced significant toxicity and a pronounced increase in TBARS (10-fold increase) in E47 cells, with respect to control cells (Table 2A). Supplementation of the medium with α-tocopherol phosphate enhanced the viability of E47 cells incubated with Fe-NTA plus arachidonic acid in association with a decline in the levels of TBARS to near control levels (Table 2B). These results suggest that lipid peroxidation is a major contributor to the toxicity in E47 cells exposed to Fe and arachidonic acid. Other antioxidants that block lipid peroxidation such as trolox and α-tocopherol completely restored the viability in E47 cells that were exposed to Fe and arachidonic acid, confirming the role of lipid peroxidation in the toxicity (Table 3). A partial protective effect was seen in the presence of externally added superoxide dismutase and catalase, suggesting a role for $O_2^{\cdot-}$ and H_2O_2 in the toxicity (Table 3). Ethanol and DMSO, which are effective hydroxyl radical scavengers, were inefficient in restoring viability of E47 cells exposed to iron and arachidonic acid (Table 3).

Effect of Arachidonic Acid and Fe-NTA on Mitochondrial Activity in E47 Cells. Damage to mitochondrial func-

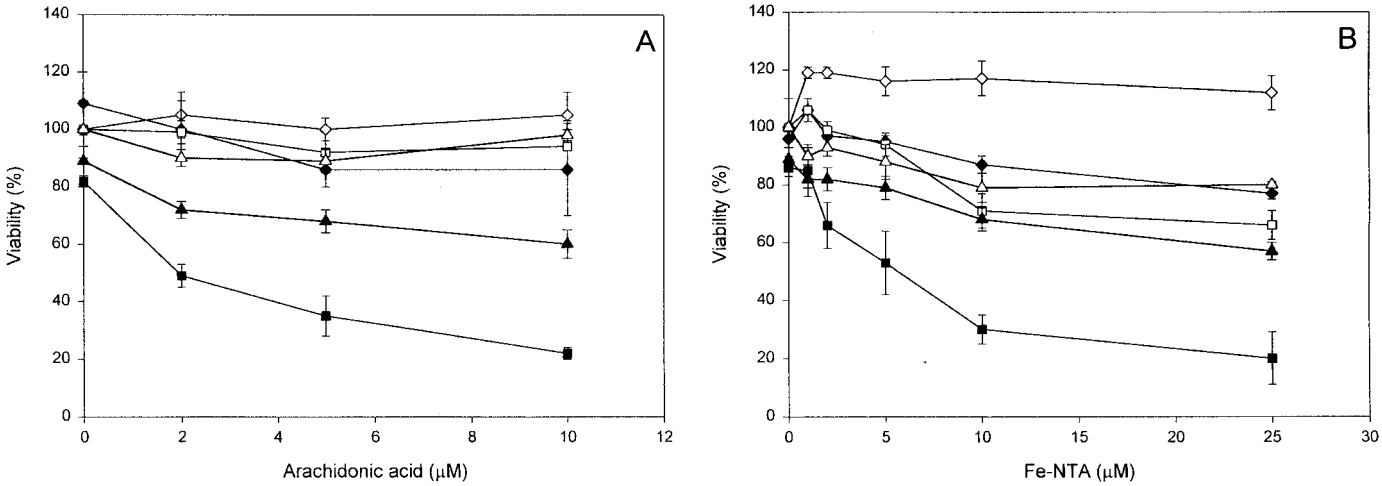

Fig. 2. Concentration curve for arachidonic acid and Fe-NTA-induced cytotoxicity. A, arachidonic acid dose dependence curve. C34 cells (diamonds), E47 cells (squares), and C3A4 cells (triangles) were cultured in MEM medium supplemented with arachidonic acid at concentrations ranging from 0 to 10 μM for 12 h. After washing, the cells were incubated for 24 h either with MEM medium (open symbols) or with MEM medium supplemented with 10 μM Fe-NTA (filled symbols). Viability was assessed by MTT reduction activity as described under *Materials and Methods*. B, Fe-NTA dose dependence curve. C34 cells (diamonds), E47 cells (squares), and C3A4 cells (triangles) were cultured either in MEM medium (open symbols) or in MEM medium supplemented with 5 μM arachidonic acid (filled symbols) for 12 h. After washing, the cells were incubated for 24 h in MEM supplemented with increasing concentrations of Fe-NTA from 0 to 25 μM. Viability was assessed by MTT reduction activity as described under *Materials and Methods*.

tion with a subsequent loss of ATP production is one of the key features of the necrotic mode of cell death (Tsujimoto, 1997; Lemasters et al., 1999). We therefore evaluated mitochondrial membrane potential and ATP content in the HepG2 cells. E47 cells exposed to Fe-NTA and arachidonic acid showed a time-dependent increase of the number of cells with low rhodamine fluorescence (M1 cells) (Fig. 5), suggesting a decrease in the mitochondrial transmembrane potential. This decrease could be observed within a short time interval after addition of Fe-NTA to the arachidonic acid-preloaded cells [e.g., 3 to 6 h] (Fig. 5). Because one important regulator of an increased membrane permeability transition (PT) is a decreased mitochondrial membrane potential, the possibility that a membrane permeability transition played a role in the Fe-NTA/arachidonic acid toxicity in E47 cells was evaluated by studying the effects of an inhibitor, cyclosporin A (Zoratti and Szabo, 1995). Cyclosporin A alone produced some toxicity to the E47 cells and partially prevented toxicity in E47 cells exposed to Fe-NTA and arachidonic acid, increasing the viability of the cells almost to levels found with cyclosporin A alone (Fig. 6). Higher concentrations of cyclosporin A could not be evaluated because of increased toxicity. The partial protection suggests the involvement of the permeability transition pore in the cytotoxicity. Onset of the mitochondrial permeability transition can cause mitochondrial uncoupling, inhibition of mitochondrial ATP formation and accelerated ATP hydrolysis by the mitochondrial ATPase. ATP-depletion results in necrotic cell death, whereas apoptosis develops when the PT occurs without exhaustion of ATP (Tsujimoto, 1997; Lemasters et al., 1999). In E47 cells exposed to Fe-NTA and arachidonic acid, ATP levels were decreased early in the toxic process with respect to controls (Fig. 7). Under these experimental conditions, a decrease in mitochondrial membrane potential mirrored the decline in ATP levels, suggesting that both events may be important for CYP2E1-dependent hepatocyte cell death, in the presence of iron plus a polyunsaturated fatty acid.

TABLE 1

Cytotoxicity produced by iron and fatty acids in C34 and E47 cells

C34 and E47 cells were supplemented with or without 10 μM of the following fatty acids: arachidonic acid, docosahexaenoic acid, eicosapentaenoic acid, and linoleic acid, and incubated for 12 h. After a washing step and 12 h of incubation in MEM_{exps}, the cells were supplemented with or without 25 μM Fe-NTA, and incubated for an additional 24-h period. Viability of the cells was then determined by the MTT assay.

Addition	Viability (%)	
	C34	E47
None	100 ± 7	100 ± 8
Fe-NTA 25 μM	88 ± 5	74 ± 4*
Arachidonic acid 10 μM	96 ± 7	89 ± 10
Docosahexaenoic acid 10 μM	90 ± 10	91 ± 12
Eicosapentaenoic acid 10 μM	85 ± 8	81 ± 12
Linoleic acid 10 μM	91 ± 5	97 ± 10
Fe-NTA 25 μM + arachidonic acid 10 μM	90 ± 12	41 ± 5*
Fe-NTA 25 μM + docosahexaenoic acid 10 μM	78 ± 15	27 ± 4*
Fe-NTA 25 μM + eicosapentaenoic acid 10 μM	94 ± 10	51 ± 6*
Fe-NTA 25 μM + linoleic acid 10 μM	96 ± 12	70 ± 3*

* Significantly different ($p < 0.05$, ANOVA) compared with the viability of the corresponding control without any addition.

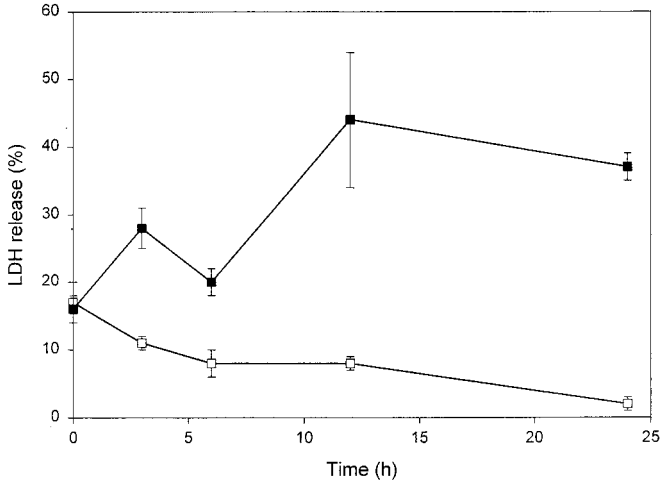

Fig. 3. Effect of arachidonic acid and Fe-NTA on LDH leakage in E47 cells. E47 cells were either not exposed (□, control cells) or exposed first to 5 μM arachidonic acid for 12 h, washed, and then incubated with 10 μM Fe-NTA for variable periods (■). LDH leakage was determined as described under *Materials and Methods*.

DNA Fragmentation Induced by Fe-NTA and Arachidonic Acid in E47 Cells. DNA fragmentation as assessed by the staining with PI increased significantly in E47 cells exposed to iron and arachidonic acid after 12 h of incubation, with respect to control cells (Fig. 8). However, DNA fragmentation did not seem to be an early event in the overall toxicity (e.g., no DNA fragmentation was seen at 6 h). No increase in DNA fragmentation was observed in C34 cells exposed to iron and arachidonic acid up to 24 h (data not shown). If DNA

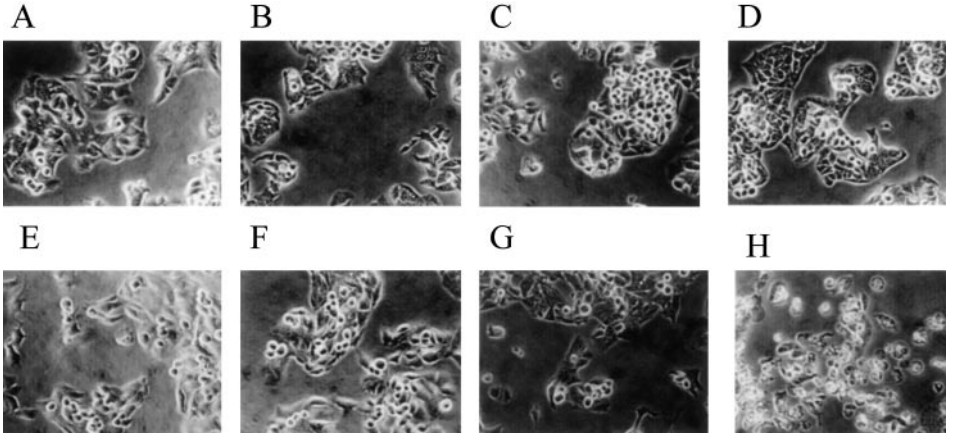

Fig. 4. Morphological changes in C34 (A–D) and E47 (E–H) cells. Cells were either not exposed to Fe-NTA or arachidonic acid (A and E), exposed to only 5 μM arachidonic acid (B and F), or only 10 μM Fe-NTA (C and G), or 5 μM arachidonic acid and 10 μM Fe-NTA (D and H), following the culture protocol described under *Materials and Methods*. Cell morphology changes were observed under the light microscope (20× magnification) with no staining.

degradation in E47 cells is a result of caspase activation as part of an apoptotic process then pan caspase inhibitor I should block DNA degradation and cell toxicity. Caspase inhibitor I did not inhibit Fe/arachidonic acid toxicity (data not shown), or DNA degradation (data not shown) in E47 cells exposed to Fe/AA, suggesting that DNA degradation in this model is not a result of caspase activation during an apoptotic process. A control experiment was run, where cytotoxicity by an apoptotic stimulus in E47 cells (TNF-α + cycloheximide) was effectively blocked by caspase inhibitor I, validating the effectiveness of this inhibitor in preventing caspase-dependent, apoptotic cell death. A DNA ladder could not be observed in the E47 cells treated with iron plus arachidonic acid (data not shown), further suggesting that the DNA fragmentation may not be part of an apoptotic cell death, but rather may reflect DNA degradation as a result of cellular necrosis.

Synergistic Toxicity of Iron and Arachidonic Acid in Hepatocytes from Pyrazole-Treated Rats. The levels of CYP2E1 were 2- to 3-fold higher per milligram of protein in hepatocytes from pyrazole-treated rats compared with levels in saline control rats. Hepatocytes isolated from pyrazole-treated rats showed low toxicity when exposed to AA alone or Fe-NTA alone, but the combination of AA plus iron showed synergistic injury (e.g., the hepatocytes were 70% viable in the presence of 50 μM AA alone, 74% viable with 50 μM Fe-NTA alone, but only 17% viable with 50 μM AA plus 50 μM Fe-NTA) (Fig. 9). In saline-treated controls, AA alone or Fe-NTA alone also produced low toxicity, but the combination of AA plus Fe-NTA did produce toxicity; however, this toxicity was considerably less than that observed with hepatocytes from pyrazole-treated rats, at various concentrations of AA or of iron evaluated (Fig. 9).

Discussion

The primary goal of the present study was to evaluate the possible synergism between iron and arachidonic acid in CYP2E1-induced injury in a hepatic cell line. This study was prompted by the necessity for other factors to prime or sensitize the liver to alcohol-induced liver injury; current data supports a critical role for nutritional factors such as PUFA and transition metals such as iron as such sensitizing factors. Our results showed that the combination of iron and arachidonic acid (in concentrations that produced low toxicity by themselves), produced synergistic toxicity in cells overexpressing CYP2E1, because the combination showed much more toxicity than expected from either agent acting alone. The toxicity was not specifically related to AA itself, because other PUFAs also showed this effect. The lower toxicity observed in E47 cells exposed to Fe-NTA plus linoleic acid (with respect to arachidonic acid, docosahexaenoic acid, and eicosapentaenoic acid) can be explained by an increased substrate availability for lipid peroxidation, because oxidative deterioration is more likely to occur in fatty acids with a high degree of unsaturations (Nanji et al., 1994a). In this respect, alcohol-induced liver pathology in the intragastric model of ethanol feeding was more pronounced when rats were fed a diet enriched in fish oil, with long-chain PUFAs such as eicosapentaenoic acid, than rats fed a diet enriched in corn oil, containing high levels of linoleic acid (Nanji et al., 1994a). This synergistic toxicity was most prominent in HepG2 cells expressing CYP2E1, because C34 cells (no significant expression of P450) or C3A4 cells (expression of CYP3A4) showed significantly less toxicity when exposed to these agents. The enhanced role of CYP2E1 in the synergistic toxicity of iron plus AA in liver cells is further suggested by the fact that the toxicity of AA plus iron is more pronounced with hepatocytes from pyrazole-treated rats (high CYP2E1) with respect to control rats with basal levels of CYP2E1. The pronounced toxicity in the E47 cells could be achieved when these cells were challenged with low concentrations of Fe-NTA and arachidonic acid (<5 μM) and just after several hours (3 to 6) of incubation. The linkage observed in this simple cell culture model between CYP2E1, cytotoxicity, iron, and PUFA, mimics some of the key features involved in the exacerbation of liver injury observed by iron/high-PUFA diet in the intragastric infusion model of ethanol toxicity. Although one role for iron sensitization has been suggested to be priming hepatic macrophages for nuclear factor-$\kappa\beta$ activation and expression of cytokines (Tsukamoto et al., 1999), the results obtained in this work suggest that another source for the increased sensitivity to iron-catalyzed oxidant stress in animals fed ethanol/high fat diet is CYP2E1 expression.

The toxicity in E47 cells exposed to iron and arachidonic acid was mainly necrotic in nature, based on morphology, early disruption of plasma membrane integrity, depletion of

TABLE 2

Lipid peroxidation and cytotoxicity induced by iron and arachidonic acid in C34 and E47 cells

After exposure to either 5 μM arachidonic acid or 10 μM Fe-NTA, or a combination of both, following the protocol described under *Materials and Methods*, cells were harvested and assayed for lipid peroxidation, or tested for viability as MTT reduction activity (A). The same experiment as in A was performed, with the addition of 25 μM α-tocopherol phosphate (TP) in the last 24-h incubation period, with 10 μM Fe-NTA (B).

	Addition			Viability		TBARS	
	Fe	AA	TP	C34	E47	C34	E47
				%		*nmol/mg of protein*	
A	−	−	−	100 ± 10	100 ± 10	0.030 ± 0.005	0.044 ± 0.003
	+	−	−	99 ± 6	74 ± 11	0.028 ± 0.006	0.049 ± 0.003
	−	+	−	97 ± 3	94 ± 2	0.029 ± 0.005	0.049 ± 0.008
	+	+	−	75 ± 4	29 ± 9[a,b]	0.039 ± 0.010	0.43 ± 0.14[c]
B	−	−	+	117 ± 5	130 ± 13	0.022 ± 0.006	0.032 ± 0.001
	+	−	+	118 ± 7	109 ± 10	0.017 ± 0.004	0.033 ± 0.007
	−	+	+	115 ± 9	113 ± 6	0.020 ± 0.003	0.036 ± 0.006
	+	+	+	94 ± 2	70 ± 15	0.027 ± 0.001	0.070 ± 0.025

[a] Significantly different ($p < 0.05$, ANOVA) compared with the viability of the corresponding control without any addition.
[b] Significantly different ($p < 0.05$, ANOVA) compared with the viability of cells incubated in the same condition, with αTP.
[c] Significantly different ($p < 0.05$, ANOVA) compared with the TBARS content of the rest of the treatments; no statistical difference was obtained between any other treatment.

ATP levels, and nonsignificant DNA degradation at early periods, where toxicity was already apparent (e.g., 3 to 6 h). The characteristic features of necrosis include cell swelling, vacuolization of cytoplasm, early damage of plasma membrane with leakage of cell content, and random DNA cleavage as a late event in the process (Collins et al., 1992; Dong et al., 1997). The morphological features observed by light microscopy in E47 cells exposed to Fe-NTA and arachidonic acid, and the early increase of LDH leakage are consistent with the development of a necrotic event. The presence of cells with low DNA stainability (sub-G1 peaks, A0 cells) has been considered a marker of cell death by apoptosis, although the sub-G1 peak can also represent mechanically damaged cells (Wolfe et al., 1996). DNA fragmentation after exposure to Fe-NTA, in E47 cells preloaded with arachidonic acid, might reflect autolytic DNA breakdown as a late event in necrosis. DNA degradation was only significant after 12 h of incubation, even though cellular toxicity was evident starting from 3 h. Internucleosomal DNA cleavage in the apoptotic process is often the result of endonuclease activation involving the participation of caspases. Caspase inhibitor I (z-vad-fmk) is a direct inhibitor of several caspases and the apoptotic-inducing factor AIF (Susin et al., 1998). It inhibited TNF-α/cycloheximide induced apoptosis in E47 cells (see *Results*), staurosporine-induced cell death in corneal epithelial cells (Joo et al., 1999), and Fas-mediated apoptosis in Jurkat T cells (Chow et al., 1995). However, caspase inhibitor I was not able to inhibit the DNA fragmentation observed in E47 cells exposed to iron and arachidonic acid, nor did it prevent the cytotoxicity. This seems to rule out the participation of (the more typical, e.g., caspase 3) caspases in the Fe + AA toxic process, and DNA degradation by caspase-activated endonucleases, and are thus consistent with the conclusion that necrosis is the principal mode of cellular death in this model.

Necrosis was the dominant mode of cell death produced by the synergistic interactions between AA and Fe-NTA with E47 cells. Previous experiments showed that apoptosis did

TABLE 3

Effect of antioxidants on the cytotoxicity produced by iron and arachidonic acid in E47 cells

E47 cells were cultured for 12 h in MEM supplemented with or without 5 μM arachidonic acid, and after a washing period, the cells were incubated for 24 h in MEM medium supplemented with or without 10 μM Fe-NTA. After this period, viability of the cells was determined by the MTT assay. The α-tocopherol (αT) was dissolved in ethanol (final concentration of ethanol, 9.7 mM).

Addition	Viability	
	−FeNTA −AA	+FeNTA +AA
	%	
None	100 ± 10	42 ± 3
αT 25 μM (ethanol)	105 ± 4	102 ± 13*
Control ethanol 9.7 mM	107 ± 5	52 ± 12
Trolox 25 μM	108 ± 2	103 ± 14*
SOD 1000 U/ml	95 ± 1	63 ± 7
CAT 2000 U/ml	109 ± 5	71 ± 5*
Ethanol 50 mM	107 ± 3	54 ± 10
DMSO 25 mM	104 ± 5	56 ± 12

CAT, catalase; SOD, superoxide dismutase.

* Significantly different ($p < 0.05$, ANOVA) compared with the viability of Fe-NTA + AA-treated cells without any addition.

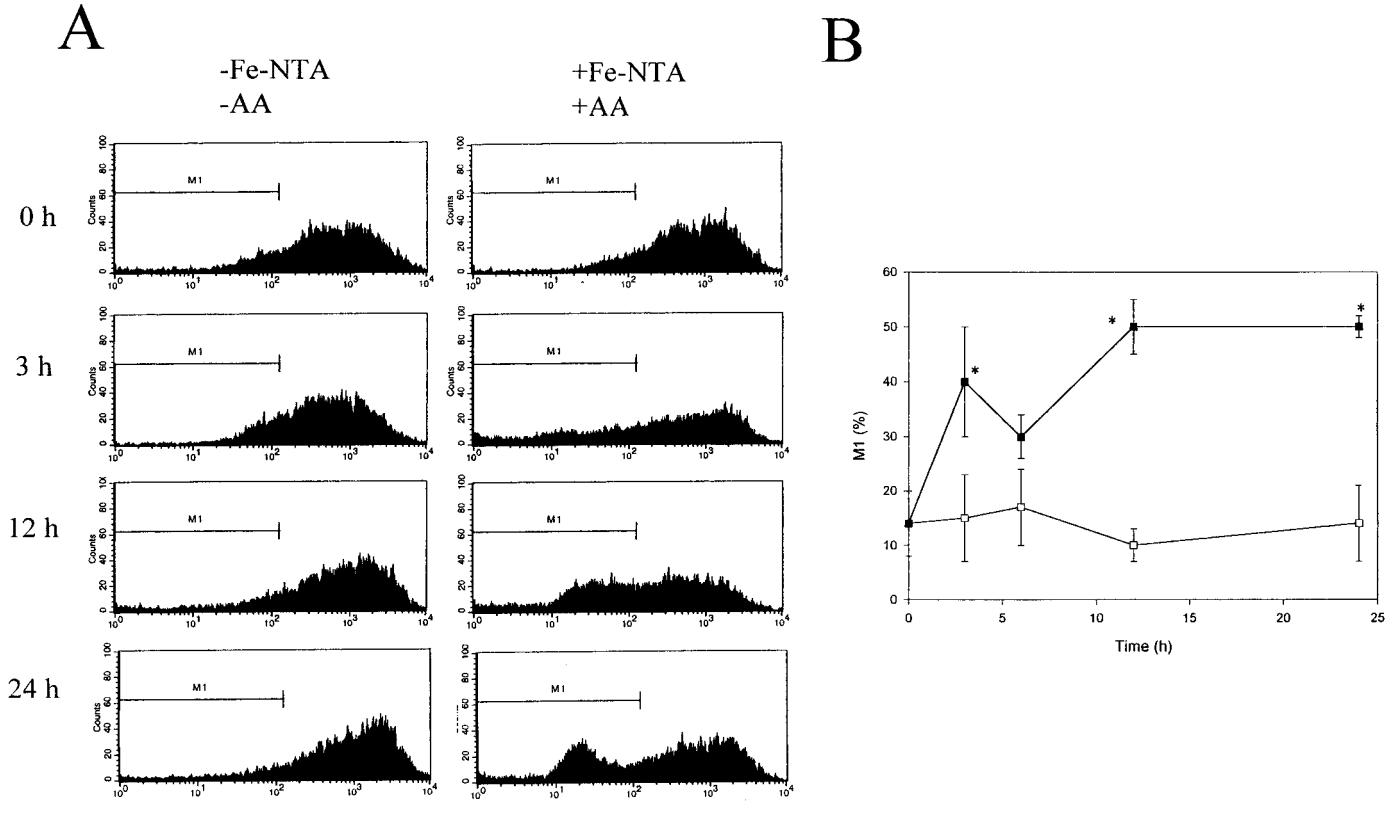

Fig. 5. Flow cytometry analysis of the mitochondrial membrane potential. E47 cells were incubated in MEM medium (□) or in MEM medium with 5 μM arachidonic acid/10 μM Fe-NTA (■) for variable times. After this period, the cells were incubated for 1 h in MEM containing 5 μg/ml Rh123. The cells were harvested by trypsinization and resuspended in 1 ml of MEM. A, typical histograms showing the decrease in cellular Rh123 fluorescence in E47 cells incubated with Fe + AA. B, percentage of cells in the M1 fraction (low Rh123 fluorescence) is shown as a function of incubation time. *, significantly different ($p < 0.05$, ANOVA) compared with the M1 percentage of cells incubated without Fe-NTA + AA for the same time period.

occur upon the addition of AA alone (Chen et al., 1997) or Fe-NTA alone (Sakurai and Cederbaum, 1998) to CYP2E1-expressing HepG2 cells (although higher concentrations of AA or iron were necessary to observe toxicity compared with the experiments in this report). It has been suggested that apoptosis and necrosis may share some common upstream events (Li et al., 1999), such as oxidative stress or impairment of mitochondrial function. Damage to mitochondrial function with a subsequent loss of ATP production is one of the key features of the necrotic mode of cell death, because ATP depletion results in necrotic cell death, whereas apoptosis develops when the PT occurs without a significant loss of ATP (Tsujimoto, 1997; Lemasters et al., 1999; Samali et al., 1999). Oxidants such as H_2O_2 may cause apoptosis at low concentrations and necrosis at high concentrations (Hampton and Orrenius, 1997; Samali et al., 1999). Similarly, nitric oxide at high levels can inhibit apoptosis or switch cell toxicity into necrosis (Melino et al., 2000). It is likely that the powerful synergistic interactions between AA and Fe-NTA in the E47 cells, and the resulting high levels of lipid peroxidation and oxidative stress, coupled to mitochondrial damage and the early and profound depletion of ATP levels in E47 cells, produce a necrotic mode of cell death when these two prooxidants are combined.

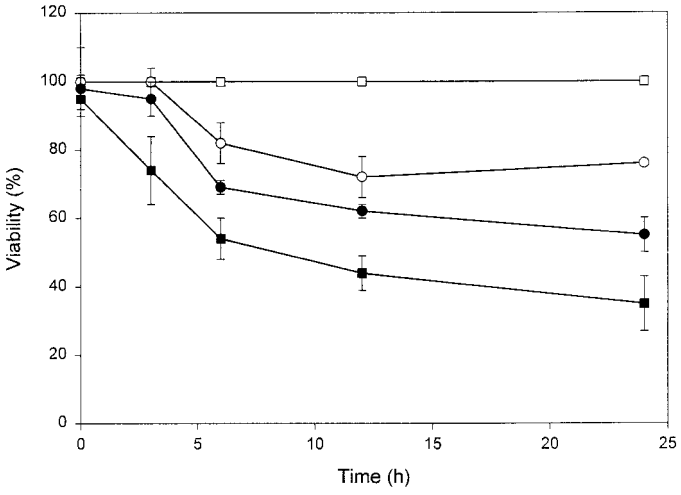

Fig. 6. Cyclosporin A decreases cytotoxicity in E47 cells exposed to Fe-NTA and arachidonic acid. E47 cells were incubated with or without 5 μM arachidonic acid for 12 h, washed, and then incubated for variable periods with either 2 μg/ml cyclosporin A or cyclosporin A plus 10 μM Fe-NTA. □, control; ○, control plus cyclosporin A; ■, Fe-NTA plus arachidonic acid; ●, Fe-NTA plus arachidonic acid plus cyclosporin A. The loss of viability in the E47 cells incubated with Fe-NTA plus arachidonic acid plus cyclosporin A was significantly less than that produced by Fe-NTA plus arachidonic acid, at all time points evaluated ($p < 0.05$).

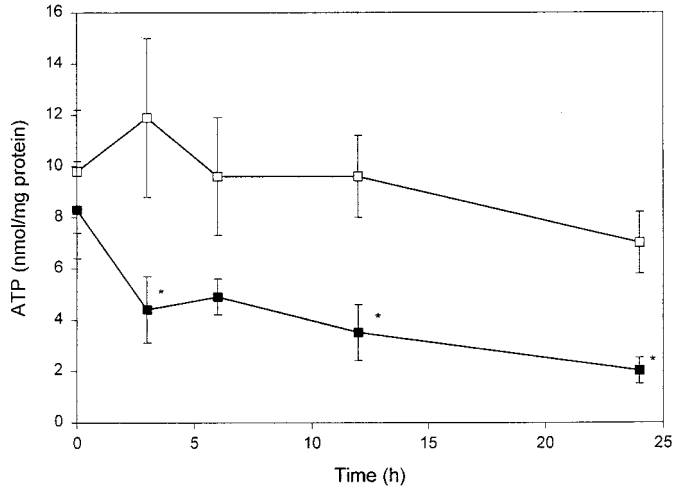

Fig. 7. Effect of arachidonic acid and Fe-NTA on ATP levels in E47 cells. Cells were either not exposed (□, control cells) or exposed first to 5 μM arachidonic acid for 12 h, washed, and then incubated with 10 μM Fe-NTA for variable periods (■). Levels of ATP were determined as described under *Materials and Methods*. *, significantly different ($p < 0.05$, ANOVA) compared with the ATP concentration of cells incubated without Fe-NTA + AA for the same time period.

There is increasing evidence that ethanol toxicity is linked to the increased production of ROS as evidenced by enhanced lipid peroxidation (Nordmann et al., 1992). Lipid peroxidation is not only a reflection of tissue damage but may also play a pathogenic role, for example, by promoting collagen production (Kamimura et al., 1992). Lipid peroxidation was increased in E47 cells after exposure to Fe-NTA and arachidonic acid, and it was linked as a cause of cytotoxicity by the prevention of cell death and TBARS accumulation by the use of antioxidants. The results obtained suggest that increased lipid peroxidation and cytotoxicity induced by ethanol intoxication in liver cells can be the result of a complex interaction between CYP2E1, iron, and PUFA, with increased production of ROS and oxidative stress. The mechanism seems to involve the generation of superoxide and H_2O_2 by CYP2E1, which in the presence of iron would produce oxidants such as hydroxyl radical and/or ferryl-perferryl species. These reactive species would interact with peroxidizable substrates such as PUFAs, giving rise to lipid peroxidation products, including reactive lipid aldehydes, which are likely to play a central role in the cellular toxicity. The protection of added catalase may reflect removal of CYP2E1-derived H_2O_2 from the E47 cells, down a concentration gradient directed outwards, especially in the presence of an external sink such as catalase (Bai et al., 1999). The poor ability of ethanol or DMSO to prevent the AA/iron toxicity, however, does not rule out a role for hydroxyl radical-like oxidants in the overall toxic process, but may reflect a low accessibility of the compounds to the intracellular membrane sites where oxygen free radicals are active in catalyzing peroxidation reactions, the generation of toxic secondary radicals (α-hydroxyethyl radical for ethanol, methyl radical for DMSO), or the increase in content of CYP2E1 by ethanol and DMSO, which were shown to stabilize CYP2E1 against degradation in the HepG2 cells (Yang and Cederbaum, 1997). The latter could offset potential antioxidant effects of ethanol and DMSO.

Mitochondria are a main source for generating ROS and target for damage by ROS also. In numerous in vitro models of necrosis, after toxin exposure, the mitochondrial transmembrane potential $\Delta\psi_m$ dissipates before the plasma membrane disrupts and before cells manifest signs of damage (Kroemer et al., 1998). A major change in the redox balance (such as hyperproduction of ROS), or energy balance (such as depletion of ATP or disruption of the $\Delta\psi_m$) can provoke a permeability transition. It has been proposed that PT pore opening might have a major role in the pathogenesis of necrotic cell death, because the PT pore inhibitor cyclosporin A inhibits cell death in hepatocytes in different models of necrosis (Kroemer et al., 1998). The opening of the PT pore results in a dissipation of $\Delta\psi_m$ and major changes in cellular energy and redox potentials, thus establishing self-amplify-

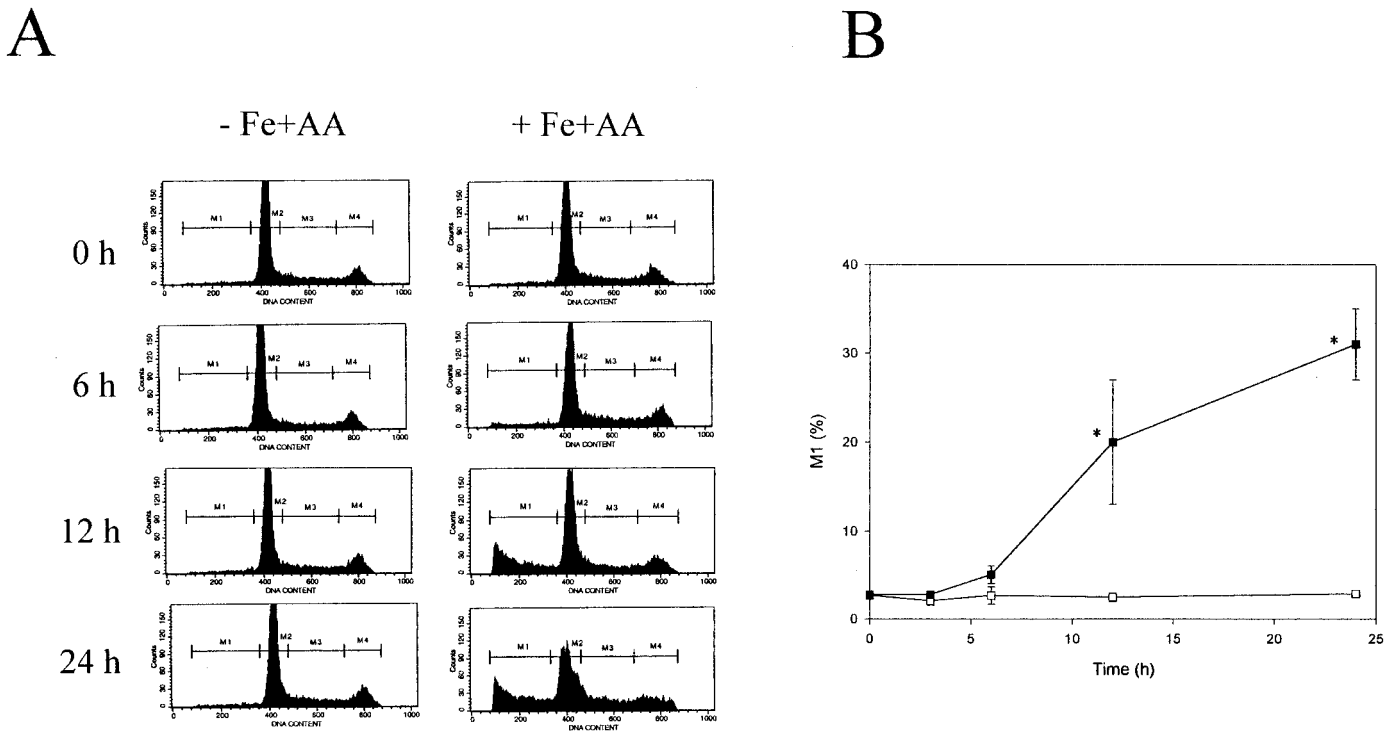

Fig. 8. DNA analysis by flow cytometry. E47 cells were cultured in MEM medium (□) or in MEM medium with 5 μM arachidonic acid/10 μM Fe-NTA (■) for variable times. The cells were harvested by trypsinization, fixed with ethanol, and stained with 50 μg/ml PI, and processed as described under *Materials and Methods* for flow cytometry analysis. A, typical histograms showing the increase in DNA fragmentation in E47 cells incubated with Fe + AA. B, percentage of cells in the sub-G_0/G_1 fraction (M1 zone, hypodiploid area) is shown as a function of incubation time. *, significantly different ($p < 0.05$, ANOVA) compared with the value of DNA fragmentation of cells incubated without Fe-NTA + AA for the same time period.

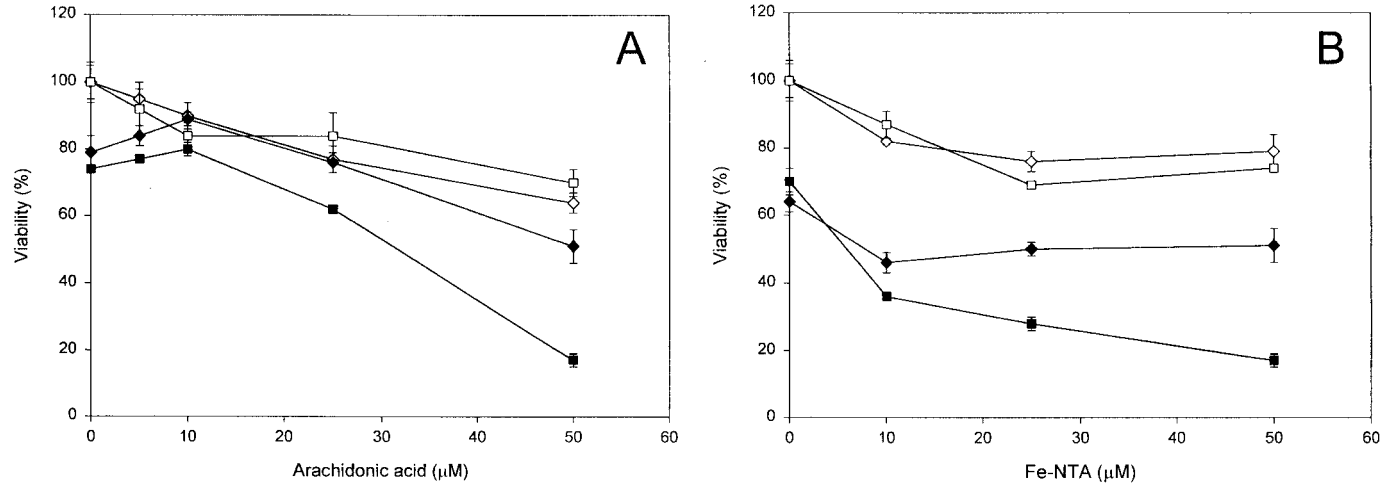

Fig. 9. Dose-response curve of arachidonic acid and Fe-NTA cytotoxicity in hepatocytes isolated from pyrazole-treated or saline-treated rats. A, arachidonic acid-dose dependence curve. Hepatocytes from pyrazole-treated (squares) or saline-treated (diamonds) rats were cultured in MEM medium supplemented with arachidonic acid at concentrations ranging from 0 to 50 μM for 8 h. The cells were immediately incubated for 8 h either with MEM medium (open symbols) or with MEM medium supplemented with 50 μM Fe-NTA (filled symbols). Viability was assessed by MTT reduction activity as described under *Materials and Methods*. B, Fe-NTA dose-dependence curve. Hepatocytes from pyrazole-treated (squares) or saline-treated (diamonds) rats were cultured either in MEM medium (open symbols) or in MEM medium supplemented with 50 μM arachidonic acid (filled symbols) for 8 h. The cells were immediately incubated for 8 h in MEM supplemented with increasing concentrations of Fe-NTA from 0 to 50 μM. Viability was assessed by MTT reduction activity as described under *Materials and Methods*.

ing loops that locks the cell in an irreversible stage (Kroemer et al., 1998). The early $\Delta\psi_m$ collapse detected in E47 cells exposed to Fe-NTA and arachidonic acid suggests that damage to mitochondria plays a role in the CYP2E1-dependent toxicity. Damage to mitochondrial function after chronic ethanol treatment has been recognized (Cunningham et al., 1990) and it is interesting to speculate that mitochondria may be a target for CYP2E1-derived ROS. In this respect, mitochondria isolated from rats chronically fed ethanol were more sensitive to induction of a PT by a variety of agents than were control mitochondria (Pastorino et al., 1999), and ethanol was shown to potentiate TNF-α cytotoxicity to a

greater extent in E47 cells than C34 cells by promoting induction of the PT (Pastorino and Hoek, 2000). Cell death in E47 cells exposed to Fe-NTA and arachidonic acid was partially prevented by cyclosporin A (Fig. 6), suggesting that CYP2E1-dependent production of ROS may interact with the mitochondria to cause a PT, uncoupling of mitochondrial energy transduction, ATP depletion, and necrosis. The $\Delta\psi_m$ collapse PT pore opening process may represent a critical event in CYP2E1-dependent, Fe/arachidonic acid-induced toxicity.

In summary, low concentrations of iron and AA that are not cytotoxic by themselves can act as priming or sensitizing factors for CYP2E1-dependent loss of viability in HepG2 cells or rat hepatocytes. This synergistic toxicity was associated with elevated lipid peroxidation and could be prevented by antioxidants, which prevent lipid peroxidation. Damage to mitochondria by CYP2E1-derived oxidants seems to be an early event in the overall pathway of cellular injury. Relatively low concentrations of iron or AA were effective in promoting toxicity in the CYP2E1-expressing cells, suggesting that interactions between CYP2E1, iron, and polyunsaturated fatty acids may lower the threshold concentrations for these reactive nutrients for inducing a state of oxidative stress, which may play a role in the development of alcohol-induced liver injury.

Acknowledgments

We thank Dr. Dennis Feierman (Department of Anesthesiology, Mount Sinai School of Medicine) for providing the C3A4 cells, and Dr. Defeng Wu (Department of Biochemistry and Molecular Biology, Mount Sinai School of Medicine, New York, New York) for preparing hepatocytes from pyrazole-treated and saline control rats.

References

Bai J, Rodriguez AM, Melendez JA, and Cederbaum AI (1999) Overexpression of catalase in cytosolic or mitochondrial compartment protects HepG2 cells against oxidative injury. *J Biol Chem* **274:**26217–26224.

Bonkovsky H, Banner BF, Lambrecht RW, and Rubin RB (1996) Iron in liver diseases other than hemochromatosis. *Semin Liver Dis* **16:**65–82.

Castillo T, Koop DR, Kamimura S, Triadafilopoulos G, and Tsukamoto H (1992) Role of cytochrome P-450 2E1 in ethanol-, carbon tetrachloride-, and iron-dependent microsomal lipid peroxidation. *Hepatology* **16:**992–996.

Chapman RW, Morgan MY, Laulicht M, Hoffbrand AV, and Sherlock S (1982) Hepatic iron stores and markers of iron overload in alcoholics and patients with idiopatic hemochromatosis. *Dig Dis Sci* **27:**909–916.

Chen Q and Cederbaum AI (1998) Cytotoxicity and apoptosis produced by cytochrome P4502E1 in HepG2 cells. *Mol Pharmacol* **53:**638–648.

Chen Q, Galleano M, and Cederbaum AI (1997) Cytotoxicity and apoptosis produced by arachidonic acid in HepG2 cells overexpressing human cytochrome P4502E1. *J Biol Chem* **272:**14532–14541.

Chow SC, Weiss M, Kass GE, Holmstrom TH, Eriksson JE, and Orrenius S (1995) Involvement of multiple proteases during Fas-mediated apoptosis in T lymphocytes. *FEBS Lett* **364:**134–138.

Collins RJ, Harmon BV, Gobe GC, and Kerr JFR (1992) Internucleosomal DNA cleavage should not be the sole criterion for identifying apoptosis. *Int J Radiat Biol* **61:**451–453.

Corrao G, Torchio P, Zambon A, D'amicis A, Lepore AR. and di Orio F (1998) Alcohol consumption and micronutrient intake as risk factors for liver cirrhosis: a case-control study. The Provincial Group for the study of chronic liver disease. *Ann Epidemiol* **8:**154–159.

Cunningham CC, Coleman WB, and Spach PI (1990) The effects of chronic ethanol consumption on hepatic mitochondrial energy metabolism. *Alcohol Alcohol* **25:** 127–136.

Dong Z, Saikumar P, Weinberg JM, and Venkatachalam MA (1997) Internucleosomal DNA cleavage triggered by plasma membrane damage during necrotic cell death. *Am J Pathol* **151:**1205–1213.

Ekstrom G and Ingelman-Sundberg M (1989) Rat liver microsomal NADPH-supported oxidase activity and lipid peroxidation dependent on ethanol-inducible cytochrome P-450 (P450IIE1). *Biochem Pharmacol* **38:**1313–1319.

Esterbauer H and Cheeseman KH (1990) Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. *Methods Enzymol* **186:** 407–421.

Halliwell B and Gutteridge JMC (1984) Oxygen toxicity, oxygen radical, transition metals and disease. *Biochem J* **219:**1–14.

Hampton MB and Orrenius S (1997) Dual regulation of caspase activity by hydrogen peroxide: implications for apoptosis. *FEBS Lett* **414:**552–556.

Joo C, Cho K, Kim H, Choi JS, and Oh YJ (1999) Protective role for bcl-2 in experimentally induced cell death of bovine corneal endothelial cells. *Ophthalmic Res* **31:**287–296.

Kamimura S, Gaal K, Britton RS, Bacon BR, Triadafilopoulos G, and Tsukamoto H (1992) Increased 4-hydroxynonenal levels in experimental alcoholic liver disease: association of lipid peroxidation with liver fibrogenesis. *Hepatology* **16:**448–453.

Kono H, Bradford BU, Yin M, Sulik KK, Koop DR, Peters JM, Gonzalez FJ, McDonald T, Dikalova A, Kadiiska MB, et al. (1999) CYP2E1 is not involved in early alcohol-induced liver injury. *Am J Physiol* **277:**G1259–G1267.

Kroemer G, Dallaporta B, and Resche-Rigon M (1998) The mitochondrial death/life regulator in apoptosis and necrosis. *Annu Rev Physiol* **60:**619–642.

Lemasters JJ, Qian T, Bradham CA, Brenner DA, Cascio WE, Trost LC, Nishimura Y, Nieminen AL, and Herman B (1999) Mitochondrial dysfunction in the pathogenesis of necrotic and apoptotic cell death. *J Bioenerg Biomembr* **31:** 305–319.

Li YZ, Li CJ, Pinto AV, and Pardee AB (1999) Release of mitochondrial cytochrome c in both apoptosis and necrosis induced by beta-lapachone in human carcinoma cells. *Mol Med* **5:**232–239.

Loreal O, Deugnier Y, Moirand R, Lauvin L, Guyader D, Jouanolle H, Turlin B, Lescoat G, and Brissot P (1992) Liver fibrosis in genetic hemochromatosis. Respective roles of iron and non-iron-related factors in 127 homozygous patients. *J Hepatol* **16:**122–127.

Mari M, and Cederbaum AI (2000) CYP2E1 overexpression in HepG2 cells induces glutathione synthesis by transcriptional activation of gamma-glutamylcysteine synthetase. *J Biol Chem* **275:**15563–15571.

Melino G, Catani MV, Corazzari M, Guerrieri P, and Bernassola F (2000) Nitric oxide can inhibit apoptosis or switch it into necrosis. *Cell Mol Life Sci* **57:**612–622.

Morimoto M, Hagbjork AL, Nanji AA, Ingelman-Sundberg M, Lindros KO, Fu PC, Albano E, and French SW (1993) Role of cytochrome P4502E1 in alcoholic liver disease pathogenesis. *Alcohol* **10:**459–464.

Morimoto M, Hagbjork AL, Wan YJ, Fu PC, Clot P, Albano E, Ingelman-Sundberg M, and French SW (1995) Modulation of experimental alcohol-induced liver disease by cytochrome P450 2E1 inhibitors. *Hepatology* **21:**1610–1617.

Nanji AA, Zhao S, Lamb RG, Dannenberg AJ, Sadrzadeh SM, and Waxman DJ (1994b) Changes in cytochromes P-450, 2E1,2B1, and 4A, and phospholipases A and C in the intragastric feeding rat model for alcoholic liver disease: relationship to dietary fats and pathologic liver injury. *Alcohol Clin Exp Res* **18:**902–908.

Nanji AA, Zhao S, Sadrzadeh SMH, Dannenberg AJ, Tajan SR, and Waxman DJ (1994a) Markedly enhanced cytochrome P4502E1 induction and lipid peroxidation is associated with severe liver injury in fish oil-ethanol-fed rats. *Alcohol Clin Exp Res* **18:**1280–1285.

Nordmann R, Ribiere C, and Rouach H (1992) Implications of free radical mechanisms in ethanol-induced cellular injury. *Free Radic Biol Med* **12:**219–240.

Pastorino JG, and Hoek JB (2000) Ethanol potentiates TNFα cytotoxicity in hepatoma cells and primary rat hepatocytes by promoting induction of the mitochondrial permeability transition. *Hepatology* **31:**1141–1152.

Pastorino JG, Marcineviviute A, Cahill A, and Hoek JB (1999) Potentiation by chronic ethanol treatment of the mitochondrial permeability transition. *Biochem Biophys Res Commun* **265:**405–409.

Powell LW (1975) The role of alcoholism in hepatic iron storage disease. *Ann NY Acad Sci* **252:**124–134.

Sadrzadeh SMH, Nanji AA, and Price PL (1994) The oral iron chelator, 1,2-dimethyl-3-hydroxypyrid-4-one reduces hepatic free iron, lipid peroxidation and fat accumulation in chronically ethanol-fed rats. *J Pharmacol Exp Ther* **269:**632–636.

Sakurai K and Cederbaum AI (1998) Oxidative stress and cytotoxicity induced by ferric-nitrilotriacetate in HepG2 cells that express cytochrome P450 2E1. *Mol Pharmacol* **54:**1024–1035.

Samali A, Nordgren H, Zhivotovsky B, Peterson E, and Orrenius S (1999) A comparative study of apoptosis and necrosis in HepG2 cells: oxidant-induced caspase inactivation leads to necrosis. *Biochem Biophys Res Commun* **255:**6–11.

Stal P, Johansson I, Ingelman-Sundberg M, Hagen K, and Hultcrantz R (1996) Hepatotoxicity induced by iron overload and alcohol. *J Hepatol* **25:**538–546.

Susin SA, Zamzami N, and Kroemer G (1998) Mitochondria as regulators of apoptosis: doubt no more. *Biochim Biophys Acta* **1366:**151–165.

Tsujimoto Y (1997) Apoptosis and necrosis: intracellular ATP levels as a determinant for cell death modes. *Cell Death Differ* **4:**429–434.

Tsukamoto H (2000) CYP2E1 and ALD *Hepatology* **32:**154–155.

Tsukamoto H, Horne W, Kamimura S, Niemela O, Parkkila S, Yla-Herttuala S, and Brittenham GM (1995) Experimental liver cirrhosis induced by alcohol and iron. *J Clin Invest* **96:**620–630.

Tsukamoto H, Lin M, Ohata M, Giulivi C, French SW, and Brittenham G (1999) Iron primes hepatic macrophages for NF-$\kappa\beta$ activation in alcoholic liver injury. *Am J Physiol* **277:**G1240–G1250.

Valerio LG, Parks T, and Petersen DR (1996) Alcohol mediates increases in hepatic and serum nonheme iron stores in a rat model for alcohol-induced liver injury. *Alcohol Clin Exp Res* **20:**1352–1361.

Wolfe JT, Pringle JH, and Cohen GM (1996) Assays for the measurement of DNA fragmentation during apoptosis, in *Techniques in Apoptosis* (Cotter TG and Martin SJ eds) pp 51–106, Portland Press Ltd, London.

Wu D and Cederbaum AI (1996) Ethanol cytotoxicity to a transfected HepG2 cell line expressing human cytochrome P4502E1. *J Biol Chem* **271:**23914–23919.

Wu D and Cederbaum AI (2000) Ethanol and arachidonic acid produce toxicity in

hepatocytes from pyrazole-treated rats with high levels of CYP2E1. *Mol Cell Biochem* **204:**157–167.

Yang MX and Cederbaum AI (1997) Characterization of cytochrome P450 2E1 turnover in transfected HepG2 cells expressing human CYP2E1. *Arch Biochem Biophys* **341:**25–33.

Zamzami N, Hirsch T, Dallaporta B, Petit PX, and Kroemer G (1997) Mitochondrial implication in accidental and programmed cell death: apoptosis and necrosis. *J Bioenerg Biomembr* **29:**185–193.

Zoratti M and Szabo I (1995) Mitochondrial permeability transition. *Biochim Biophys Acta* **1241:**139–176.

Address correspondence to: Dr. Arthur I. Cederbaum, Department of Biochemistry and Molecular Biology, Box 1020, One Gustave L. Levy Place, Mount Sinai School of Medicine, New York, NY 10029. E-mail: arthur.cederbaum@mssm.edu