Cytotoxicity and Apoptosis Produced by Arachidonic Acid in Hep G2 Cells Overexpressing Human Cytochrome P4502E1*

Qi Chen‡§, Monica Galleano‡, and Arthur I. Cederbaum¶
From the Department of Biochemistry, Mount Sinai School of Medicine, New York, New York 10029 and §Physical Chemistry, School of Pharmacy and Biochemistry, University of Buenos Aires, 1113 Buenos Aires, Argentina

The goal of the current study was to evaluate the effects of arachidonic acid, as a representative polyunsaturated fatty acid, on the viability of a Hep G2 cell line, which has been transduced to express human cytochrome P4502E1 (CYP2E1). Arachidonic acid produced a concentration- and time-dependent toxicity to Hep G2-MV2E1-9 cells, which express CYP2E1, but little or no toxicity was found with control Hep G2-MV-5 cells, which were infected with retrovirus lacking human CYP2E1 cDNA. In contrast to arachidonic acid, oleic acid was not toxic to the Hep G2-MV2E1-9 cells. The cytotoxicity of arachidonic acid appeared to involve a lipid peroxidation type of mechanism since toxicity was enhanced after depletion of cellular glutathione; formation of malondialdehyde and 4-hydroxy-2-nonenal was markedly elevated in the cells expressing CYP2E1, and toxicity was prevented by antioxidants such as α-tocopherol phosphate, 6-hydroxy-2,5,7,8-tetramethylethylidene-2-carboxylic acid (trolox), propylgallate, ascorbate, and diphenylphenylenediamine, and the iron chelator desferrioxamine. Transfection of the Hep G2-MV2E1-9 cells with plasmid containing CYP2E1 in the sense orientation enhanced the arachidonic acid toxicity, whereas transfection with plasmid containing CYP2E1 in the antisense orientation decreased toxicity. The CYP2E1-dependent arachidonic acid toxicity appeared to involve apoptosis, as demonstrated by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling and DNA laddering experiments. Trolox, which prevented toxicity of arachidonic acid, also prevented the apoptosis. Transfection with a plasmid containing bcl-2 resulted in complete protection against the CYP2E1-dependent arachidonic acid toxicity. It is proposed that elevated production of reactive oxygen intermediates by cells expressing CYP2E1 can cause lipid peroxidation, which subsequently promotes apoptosis and cell toxicity when the cells are enriched with polyunsaturated fatty acids such as arachidonic acid. The Hep G2-MV2E1-9 cells appear to be a valuable model to study interactions between CYP2E1, polyunsaturated fatty acids, reactive radicals, and the consequence of these interactions on cell viability and to reproduce several of the key features associated with ethanol hepatotoxicity in the intra gastric infusion model of ethanol treatment.

There is current interest in the role of oxidative stress and generation of reactive radical species in the mechanism(s) by which ethanol is toxic to the liver and other tissues (1). Induction of CYP2E11 by ethanol appears to be one of the central pathways by which ethanol is believed to generate a state of oxidative stress. Micromes isolated from rats treated chronically with ethanol display increased production of superoxide radical, H2O2, hydroxyl radical, and enhanced lipid peroxidation (2–8). Ethanol oxidation to the 1-hydroxyethyl radical is also elevated after ethanol consumption (9, 10). Increased formation of reactive radical species and lipid peroxides after chronic ethanol treatment are prevented by anti-CYP2E1 IgG and by chemical inhibitors of CYP2E1, thus linking these increases to induction of CYP2E1 (4, 11, 12).

The importance of dietary fat in alcoholic liver disease in humans is supported by epidemiological correlations which suggest that susceptibility to alcohol is related to different types of dietary fat (13, 14). A major advance in ethanol hepatotoxicity studies has been the development of the intragastric infusion model of ethanol feeding, which leads to more significant liver injury than the classical liquid diets (15–20). Liver injury occurs in this model when the rats consume diets containing polyunsaturated fatty acid (PUFA) but not saturated fatty acid. In these models, large increases in lipid peroxidation have been shown to correlate with CYP2E1 levels (15, 16, 18, 20–22). The general hypothesis to account for the liver injury with this model is that elevated production of reactive radical species occurs due to induction of CYP2E1, and this results in lipid peroxidation when the diet is supplemented with PUFA (15, 16, 18, 20–22).

In attempts to directly demonstrate that overexpression of CYP2E1 can result in hepatotoxicity of various agents, a Hep G2 cell line that constitutively expresses the human CYP2E1 was recently established (23). Electron spin resonance spectroscopy showed that microsomes from Hep G2-MV2E1-9 cells that express CYP2E1 produced superoxide radicals at rates about 10-fold greater than those from Hep G2-MV-5 cells that do not express CYP2E1; rates of H2O2 production were about 3-fold greater with the Hep G2-MV2E1-9 microsomes. Rates of microsomal lipid peroxidation were also greater with the Hep G2-MV2E1-9 cells (23). Ethanol and acetaminophen were shown to be toxic to Hep G2-MV2E1-9 cells but not Hep G2-MV-5 cells (24, 25). This model appears to be useful in efforts to

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¶ To whom correspondence should be addressed: Mount Sinai School of Medicine, Dept. of Biochemistry, Box 1020, One Gustave L. Levy Place, New York, NY 10029. Tel.: 212-241-7285; Fax: 212-996-7214; E-mail: ACEDERB@SMTPLINK.MSSM.EDU.

1The abbreviations used are: CYP2E1, cytochrome P4502E1; BSO, buthionine sulfoximine; DPPD, diphenylphenylenediamine; FITC, fluorescein isothiocyanate; E9, Hep G2-MV2E1-9 cells expressing CYP2E1; GSH, glutathione, reduced form; 4-HNE, 4-hydroxy-2-nonenal; LDH, lactate dehydrogenase; MDA, malondialdehyde; MEM, minimum essential medium; 4-MP, 4-methylpyrazole; MTT, [3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PUFA, polyunsaturated fatty acid; trolox, 6-hydroxy-2,5,7,8-tetramethylethylidene-2-carboxylic acid; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; PBS, phosphate-buffered saline.
establish a CYP2E1-dependent hepatotoxicity system and to evaluate the role of oxidative stress in the toxicity of compounds metabolized by CYP2E1.

Increased lipid peroxidation has been implicated as being associated with apoptosis, or programmed cell death. Direct exposure of various cell types to oxidants such as hydrogen peroxide or lipid hydroperoxides can directly induce apoptosis; in many experimental models pretreatment of the cells with antioxidants has been shown to protect against this form of cell death (26–29). The prototypic regulator of mammalian apoptosis is the proto-oncogene bcl-2 (30). The functions of bcl-2 have been suggested to include acting as an antioxidant (31), modulating some aspects of nuclear transport (32), intervention in calcium signaling (33), and associating with several other proteins (34). Overexpression of bcl-2 leads to protection for many cell types against apoptosis induced by exposure to a wide variety of adverse conditions and stimuli, including lipid peroxidation, suggesting that bcl-2 controls a distal step in a signal transduction pathway leading to apoptosis (35–43).

The goal of the current study was to evaluate the cytotoxicity effects of arachidonic acid, a representative PUFA, to Hep G2 cells expressing CYP2E1 and to compare these effects to control cells not expressing CYP2E1. The effect of antioxidants and of bcl-2 on arachidonic acid toxicity and whether the toxicity was apoptotic in nature was also determined. It was hoped that this Hep G2 cell model might be a direct system that can establish linkage between CYP2E1, PUFA, oxidative stress, and cytotoxicity and thus mimic in a simple culture system the conditions believed to be representative of the gastric infusion model of ethanol toxicity.

MATERIALS AND METHODS

Cells and Chemicals—Hep G2-MV2E1-9 and Hep G2-MV-5 cells (23), human hepatocellular carcinoma Hep G2 sublines, were cultured in minimum essential medium (MEM), supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 2% glutamine in a humidified atmosphere in 5% CO2 at 37 °C. Hep G2-MV2E1-9 cells contain a copy of the human CYP2E1 cDNA and constitutively express CYP2E1. Hep G2-MV-5 cells are the appropriate controls for Hep G2-MV2E1-9 cells as they contain only viral vector lacking expression vector (Promega), in the sense and antisense (as) orientation of the CYP2E1 cDNA. Most reagents were from Sigma. Specific reagents are described below.

Cytotoxicity Measurement: MTT Assay—Cytotoxicity of PUFA was primarily measured by the MTT assay (44). Tetrazolium salts such as MTT are metabolized by mitochondrial dehydrogenases to form a blue formazan dye and are therefore useful for the measurement of cytotoxicity. Approximately 2.0–2.5 × 105 cells, suspended in MEM containing 2 mM 4-methylpyrazole (added to stabilize CYP2E1 against degradation), were plated onto each well of a 24-well plate (Corning Co.) and incubated for 24 h at 37 °C. After 24 h, the cells were treated with arachidonic acid, cells were scraped off the 6-well culture plates with 1 ml of lysis buffer (Corning Co.) and incubated for 24 h at 0 °C. After lysis, samples were extracted with 2 ml of phenol (neutralized with TE buffer, pH 7.5) followed by extraction with 1 ml of chloroform/isooamyl alcohol (24:1). The aqueous supernatants were precipitated with 2.5 volumes of ice-cold ethanol plus 10% volume of 3 mM sodium acetate, pH 5.2, at −20 °C overnight. After centrifugation at 13,000 × g for 10 min, the pellets were air-dried, resuspended with 50 μl of TE buffer, pH 7.5, supplemented with 0.1 μg/μl RNase A, and electrophoretically separated on a 1.5% agarose gel in 0.5 × TBE buffer containing 1 μg/ml ethidium bromide and 100 V for 2 h. Pictures of the gels were taken by UV transillumination.

Transduction of Hep G2-MV2E1-9 Cells—The full-length human bcl-2 cDNA, excised from pSFFV-bcl-2 expression vector (kindly provided by Dr. F. J. Gonzalez, National Cancer Institute, Bethesda, Maryland), was inserted into the EcoRI restriction site of pCI-neo expression vector (Promega), in the sense and antisense (as) orientations to form pCI-bcl-2 or pCI-as-bcl-2, and pCI-2E1 or pCI-as-2E1, respectively. Transfection of Hep G2-MV2E1-9 cells was carried out utilizing the LipofectAMINE reagent (Life Technologies, Inc.) as described by Hawley-Nelson et al. (51). Hep G2-MV2E1-9 cells were grown to 80–90% confluence and harvested by trypsinization, and 1.5 × 106 cells were seeded into a 100-mm culture dish and grown until 50–70% confluence. Cells were rinsed with serum-free MEM before transfection. Solution A (15 μg of the appropriate plasmid DNA in 800 μl of serum-free MEM) and solution B (100 μl of LipofectAMINE reagent in 800 μl of serum-free MEM) were gently mixed and incubated at room temperature for 30 min to form a DNA-liposome complex. The complex was diluted with 8 ml of MEM, added to the Petri dish containing the Hep G2-MV2E1-9 cells, followed by incubation for 5 h at 37 °C in a CO2 incubator. 8 ml of MEM with 20% fetal calf serum was then added to each culture dish. After 18 h of incubation, fresh MEM was added, and the cells were incubated for an additional 2 days. The cells were collected by trypsinization and used for Western blot analysis and for stimulation with arachidonic acid.

Western Blot Analysis—Cell lysis was achieved by sonication (5 s, duty cycle 25%, output control 40%), followed by centrifugation at 5,000 × g for 5 min. The supernatant was collected and protein determined with the DC-20 Protein Assay Kit (Bio-Rad). Protein (50 μg for each sample) was resolved on a 10% SDS-polyacrylamide gel and transferred onto nitrocellulose sheets (Bio-Rad) for Western blot analysis (52, 53). Rabbit anti-human CYP2E1 polyclonal antibody (provided by...
Arachidonic Acid Cytotoxicity

**TABLE I**

Comparison of the effect of arachidonic acid and oleic acid on the viability of Hep G2-MV2E1-9 cells

| Addition          | % viability Without BSO | % viability With BSO |
|-------------------|-------------------------|----------------------|
| 0.02 mM oleic acid| 89.9 ± 10.9             | 96.7 ± 3.9           |
| 0.02 mM arachidonic acid | 72.9 ± 1.8               | 47.9 ± 4.7           |
| 0.05 mM oleic acid | 89.2 ± 9.1              | 98.2 ± 3.3           |
| 0.05 mM arachidonic acid | 17.2 ± 5.8               | 0.0 ± 0.2            |

Dr. J. M. Lasker, Mt. Sinai School of Medicine and mouse anti-human-bcl-2 monoclonal antibody (Boehringer Mannheim) were used as the primary antibodies followed by treatment with alkaline phosphatase either conjugated to goat anti-rabbit IgG (Bio-Rad) or to rabbit anti-mouse IgG (Boehringer Mannheim) as the second antibody. Staining intensity was developed with the NBT-BCIP mixture (Promega).

_Lipid Peroxidation Assay—Malondialdehyde (MDA) and 4-hydroxy-alkenals, such as 4-hydroxy-2-nonenal (4-HNE), end products derived from peroxidation of PUFA and related esters, provide a convenient index as a measure for lipid peroxidation (54). Lipid peroxidation in Hep G2-MV2E1-9 and Hep G2-MV-5 cells was monitored by measuring total MDA and 4-HNE production, utilizing the lipid peroxidation assay kit, LPO-586 (Calbiochem). Briefly, after incubating the cells with varying concentrations of arachidonic acid, the tissue culture medium from the first and second 24-h incubation period was collected and assayed. The cells were collected by scraping and centrifugation. The pellets were resuspended in 20 mM Tris-HCl, pH 7.4, buffer, lysed by sonication, and centrifuged at 5,000 × g for 5 min. The protein content of the cell lysates was determined (Bio-Rad DC-20 Protein Assay Kit) followed by the LPO-586 assay.

**RESULTS**

_Arachidonic Acid Cytotoxicity in Hep G2-MV2E1-9 Cells—_It has been shown that dietary fat composition and subsequent elevated lipid peroxidation are related to the severity of alcohol-induced liver injury in the intragastric feeding rat model. To evaluate a role of PUFA in alcohol-related toxicity, Hep G2-MV2E1-9 and Hep G2-MV-5 cells were loaded with arachidonic acid (20:4) for 24 h, the medium was removed, and the cells were rinsed and continuously incubated at 37 °C for an additional 24 h in normal MEM. Cell viability was then assessed by the MTT assay. Pretreatment with 0.03 mM arachidonic acid (20:4) for 24 h, the medium was removed, and the cells were rinsed and continuously incubated at 37 °C for an additional 24 h in normal MEM. Cell viability was then assessed by the MTT assay. Pretreatment with 0.03 mM arachidonic acid caused 43–72% (mean of 62%) loss of viability to Hep G2-MV2E1-9 cells as many cells were detached and floated to the top of the culture dish; cells were shrunken and dispersed and a monolayer was not formed (Fig. 2). No such changes in morphology were evident when arachidonic acid was added to the Hep G2-MV-5 cells (Fig. 2).

To characterize the cytotoxicity produced by arachidonic acid, time course and dose-dependent experiments were conducted. The cytotoxic effect of preloading with various concentrations of arachidonic acid is shown in Fig. 3A. At concentrations of 0.005 or 0.01 mM, there was no significant toxicity by arachidonic acid in either cell line. At 0.02–0.03 mM, arachidonic acid caused significant toxicity to Hep G2-MV2E1-9 cells but not to Hep G2-MV-5 cells. At a concentration of 0.05 mM, arachidonic acid caused more than 80% loss of viability in Hep G2-MV2E1-9 cells; some toxicity was also observed in Hep G2-MV-5 cells although it was significantly lower than that in the Hep G2-MV2E1-9 cells. As shown in Fig. 3B, some toxicity by arachidonic acid could be observed immediately after the initial 24-h preincubation period, and this toxicity became more pronounced during the second incubation period after removal of the arachidonic acid. No significant cytotoxicity was observed in Hep G2-MV-5 cells over the same incubation period. At 36 h after preloading, viability of Hep G2-MV2E1-9 cells was lowered by 73% by the arachidonic acid treatment.
Removal of GSH on the arachidonic acid toxicity was evaluated since GSH is known to protect cells against the toxicity of numerous agents. The effect of depletion of GSH in both cell lines (24). Since GSH appears to be important in protecting the Hep G2-MV2E1-9 cells against arachidonic acid-induced toxicity.

Treatment with 0.1 mM BSO caused an approximate 90% depletion of GSH in both cell lines (24). Since GSH is known to protect cells against the toxicity of numerous agents, the effect of removal of GSH on the arachidonic acid toxicity was evaluated. In the presence of 0.1 mM BSO, arachidonic acid was more toxic to both cell lines. BSO treatment caused about a 2–3-fold increase in toxicity by arachidonic acid in both Hep G2 cell lines (Fig. 3A). However, the BSO treatment did not potentiate the toxicity of oleic acid to the Hep G2-MV2E1-9 cells (Table 1). Since GSH depletion potentiated the cytotoxicity of arachidonic acid, GSH appears to be important in protecting the Hep G2 cells against arachidonic acid-induced toxicity.

Role of CYP2E1 in Arachidonic Acid Cytotoxicity—Inasmuch as the only apparent difference between Hep G2-MV2E1-9 and Hep G2-MV-5 cells is the expression of CYP2E1 in the former, but not the latter, it would appear that the greater toxicity caused by arachidonic acid in Hep G2-MV2E1-9 cells is due to the presence of CYP2E1 in these cells. To validate the role of CYP2E1 in the elevated arachidonic acid cytotoxicity in Hep G2-MV2E1-9 cells, a plasmid, pCI-as-2E1, containing cDNA encoding antisense CYP2E1 was transfected into Hep G2-MV2E1-9 cells to block CYP2E1 production. Alternatively, a plasmid (pCI-2E1) containing human CYP2E1 cDNA was used to enrich the CYP2E1 content of the Hep G2-MV2E1-9 cells. Western blot analyses of the CYP2E1 content after transfection with the CYP2E1 sense and antisense plasmids indicated that the expression of CYP2E1 was decreased by about 80–90% with pCI-as-2E1 as compared with control transfection with pCI, whereas expression of CYP2E1 was elevated about 3-fold after transfection with pCI-2E1 (Fig. 4, lanes 1, 3, and 5). Arachidonic acid toxicity in the cells transfected with control plasmid was very similar to that found previously with the non-transfected Hep G2-MV2E1-9 (Fig. 5A, pCI curve, compared with Fig. 3A E9 without BSO curve). Transfection with pCI-as-2E1 partially prevented the arachidonic acid toxicity; in fact, the arachidonic acid toxicity curve in the presence of pCI-as-2E1 (Fig. 5A) was similar to the toxicity curve found for the Hep G2-MV-5 cells (Fig. 3A, without BSO curve). This suggests that transfection with pCI-as-2E1 largely protected against the CYP2E1-dependent arachidonic acid toxicity. Transfection with pCI-2E1 plasmid increased the toxicity by arachidonic acid compared with the control pCI transfection (Fig. 5A). Thus, arachidonic acid toxicity is dependent upon CYP2E1 expression under these reaction conditions and at these concentrations of arachidonic acid.

Pafatty acids can be metabolized by cytochrome P450 (55–58); CYP2E1 catalyzes ω-1 hydroxylation of arachidonic acid to a variety of complex products (59–61). Since CYP2E1 is a loosely coupled cytochrome P450, i.e. can generate reactive oxygen species such as superoxide and H2O2 in the absence of a metabolic substrate (4, 62), it was necessary to evaluate two possible roles for CYP2E1 in promoting the toxicity of arachidonic acid, i.e. CYP2E1 directly oxidized arachidonic acid to reactive metabolites that produced the toxicity (Equation 1) or CYP2E1 generated superoxide and H2O2 which then reacted with arachidonic acid to produce toxicity (Equation 2). The latter possibility would not require direct oxidation of arachidonic acid by CYP2E1.

\[
\text{CYP2E1 + arachidonic acid \rightarrow metabolites \rightarrow cytotoxicity} \quad (\text{Eq. 1})
\]

\[
\text{CYP2E1 + NADPH \rightarrow reactive oxygen species} \quad \text{\downarrow \ arachidonic acid} \quad (\text{Eq. 2})
\]

\[
\text{lipid peroxidation \rightarrow cytotoxicity}
\]
4-Methylpyrazole (4-MP) is a ligand of CYP2E1 and an effective inhibitor of CYP2E1-catalyzed oxidation of substrates (63). 4-MP was shown to completely prevent the toxicity of acetaminophen and ethanol to the Hep G2-MV2E1-9 cells, indicating that this toxicity required metabolism of these agents by CYP2E1 (24, 25). 4-MP was added to the culture medium of Hep G2-MV2E1-9 cells during the 24-h loading period with arachidonic acid and during the 24-h post-loading period. 4-MP did not protect against the cytotoxicity produced by arachidonic acid in the absence of BSO; a small protection was observed against the enhanced toxicity found in the presence of BSO (Fig. 5B); however, this protection was much less than the complete protection afforded by 4-MP against toxicity of acetaminophen and ethanol (24, 25). As will be discussed below, other CYP2E1 ligands and competitive substrates such as Me$_3$SO or ethanol did not significantly protect the Hep G2-MV2E1-9 cells against arachidonic acid toxicity. These results suggest that direct metabolism of arachidonic acid by CYP2E1 may not play an important role in arachidonic acid toxicity in Hep G2-MV2E1-9 cells.

Enhanced Lipid Peroxidation Induced by Arachidonic Acid in Hep G2-MV2E1-9 Cells—The possible mechanism for arachidonic acid toxicity suggested in Equation 2 directly implicates lipid peroxidation as playing a central role in the toxicity. Lipid peroxidation of Hep G2-MV2E1-9 and Hep G2-MV-5 cells was assessed by measuring production of the lipid peroxidation end products MDA and 4-HNE. As shown in Fig. 6, arachidonic acid induced lipid peroxidation in Hep G2-MV2E1-9 cells in a concentration-dependent manner; enhanced formation of MDA and 4-HNE was observed in both cell lysate (Fig. 6A) and in the culture medium from the cells (Fig. 6B). Arachidonic acid (up to 0.03 mM) caused little or no lipid peroxidation in Hep G2-MV-5 cells. The significant difference in lipid peroxidation between the two cell sublines suggests that overexpression of CYP2E1 enhanced the PUFA-induced lipid peroxidation. Subsequent studies were carried out to evaluate whether the enhanced
lipid peroxidation was responsible for the cytotoxicity and cell damage produced by arachidonic acid.

**Effect of Antioxidants on Arachidonic Acid Cytotoxicity**—To characterize further the nature of arachidonic acid cytotoxicity, several antioxidants were added to the culture medium, and their effect on arachidonic acid toxicity was determined. As shown in Table II, ascorbic acid, the iron chelator desferrioxamine, and several typical inhibitors of lipid peroxidation, such as trolox, α-tocopherol phosphate, propylgallate, and DPPD, produced efficient protection against 0.03 mM arachidonic acid toxicity in the Hep G2-MV2E1-9 cells. Me₂SO (5–50 mM) and ethanol (25–160 mM) as ligands for CYP2E1 and as hydroxyl radical scavengers failed to prevent arachidonic acid toxicity. These results suggest that the arachidonic acid toxicity in Hep G2-MV2E1-9 is due to, at least in part, the enhanced lipid peroxidation. Aspirin, an inhibitor of the cyclooxygenase pathway for arachidonic acid metabolism, did not protect against the toxicity of arachidonic acid (Table II).

**In Situ DNA Nick End Labeling of Hep G2-MV2E1-9 Cells**—Two distinct modes of cell death, apoptosis and necrosis, can be distinguished based on differences in morphological, biochemical, and molecular changes of dying cells. Experiments were carried out to determine whether apoptotic cell death occurs in arachidonic acid-induced cytotoxicity to Hep G2-MV2E1-9 cells. In general, cells undergoing apoptosis display a characteristic pattern of structural changes in nucleus and cytoplasm, including rapid blebbing of the plasma membrane and nuclear disintegration. The nuclear collapse is associated with extensive damage to chromatin and DNA cleavage into oligonucleosomal length DNA fragments (47–50). After 24 h of arachidonic acid preloading, Hep G2-MV2E1-9 and Hep G2-MV-5 cells were placed in normal MEM for an additional 8 h of incubation. Cells were then harvested for in situ DNA nick end labeling as determined by the TUNEL method. In the absence of arachidonic acid, the intensity of FITC labeling was similar for the Hep G2-MV2E1-9 (mean, 0.41–1.02) and Hep G2-MV-5 (mean, 2.12–2.84) cells (Fig. 7). Arachidonic acid enhanced the FITC labeling with both cell lines; however, the intensity of FITC labeling in the Hep G2-MV2E1-9 cells preincubated with 0.03 mM arachidonic acid (mean, 12.11–13.34) was significantly higher than that of Hep G2-MV-5 (mean, 2.12–2.84). Results from several TUNEL experiments are summarized in Fig. 8; at an arachidonic acid concentration of 0.03 mM, the intensity of FITC labeling was about 5-fold greater with the Hep G2-MV2E1-9 cells compared with Hep G2-MV-5 cells. A second incubation time of 8 h was chosen for these experiments since too many Hep G2-MV2E1-9 cells lost viability after the typical 24-h second incubation period. Since antioxidants prevent arachidonic acid toxicity, 0.1 mM trolox was added during the first incubation with arachidonic acid and to the medium after removal of arachidonic acid. The TUNEL labeling of Hep G2-MV2E1-9 cells (and Hep G2-MV-5 cells) was effectively inhibi-

**TABLE II**

**Effect of anti-oxidative agents on the cytotoxicity produced by arachidonic acid to Hep G2-M2E1-9 cells**

Hep G2-MV2E1–9 cells were incubated with or without 0.03 mM arachidonic acid for 24 h in the presence of the indicated additions. The respective media were removed and replaced by MEM supplied with the corresponding agents but without arachidonic acid, and a second incubation for 24 h was carried out in the absence of arachidonic acid. Cell viability was evaluated by the MTT assay. Net absorbance refers to absorbance at 570 nm minus absorbance at 630 nm, and percent viability refers to net absorbance of arachidonic acid treated, divided by net absorbance of control, times 100.

| Addition                  | Net absorbance (A₅₇₀ – A₆₃₀) | Viability % |
|---------------------------|------------------------------|-------------|
| Control                   | Control Arachidonic acid     |             |
| None                      | 0.294 ± 0.013                | 40          |
| Me₂SO 20 mM               | 0.317 ± 0.025                | 50          |
| Ethanol 50 mM             | 0.334 ± 0.010                | 54          |
| DPPD 0.005 mM             | 0.277 ± 0.022                | 89          |
| Propylgallate 0.025 mM    | 0.308 ± 0.003                | 70          |
| Trolox 0.02 mM            | 0.332 ± 0.010                | 75          |
| α-Tocopherol phosphate 0.02 mM | 0.352 ± 0.020              | 89          |
| Ascorbic acid 1 mM        | 0.277 ± 0.016                | 90          |
| Desferrioxamine 0.3 mM    | 0.218 ± 0.006                | 90          |
| Aspirin 1 mM              | 0.257 ± 0.004                | 52          |

**FIG. 7.** Histograms of in situ DNA nick end labeling of Hep G2-MV2E1-9 and Hep G2-MV-5 cells. Cells were preincubated in MEM with or without 0.03 mM arachidonic acid and with or without 0.1 mM trolox for 24 h. After an additional 8 h of culture in medium without arachidonic acid, cells were harvested for TUNEL analysis. At least 3000 cells from each group were measured by flow cytometry. Overlaid histograms are presented to show the differential FITC labeling of cells treated with (solid line) or without (dashed or fine line) arachidonic acid.
These results suggest that enhanced lipid peroxidation caused by arachidonic acid preincubation induced apoptosis and cytotoxicity in Hep G2-MV2E1-9 cells.

**Apoptosis in Hep G2-MV2E1-9 Cells**—Apoptotic cells often produce a unique ladder composed of nucleotide fragments at an interval of 200 base pairs, which can be visualized by DNA-agarose electrophoresis. The TUNEL in situ labeling suggested that arachidonic acid toxicity in Hep G2-MV2E1-9 cells is apoptotic in nature. To study this further, DNA fragmentation within Hep G2-MV2E1-9 cells was determined. Hep G2-MV2E1-9 cells were harvested at various times after arachidonic acid incubation (6, 12, and 24 h) and 8 h after removal of arachidonic acid. Total DNA was purified for the agarose gel electrophoresis assay. During the 24-h preloading period, 0.02–0.04 mM arachidonic acid did not induce significant DNA fragmentation (Fig. 9A, lanes 3–5, 7–9, and 11–13 compared with lanes 2, 6, and 10). However, 8 h after preloading, 0.03 and 0.04 mM arachidonic acid caused DNA fragmentation in the Hep G2-MV2E1-9 cells (Fig. 9A, lanes 16 and 17 compared with lane 14, no arachidonic acid added). Eight hours after the initial 24-h preloading with 0.03 mM arachidonic acid, Hep G2-MV2E1-9 cells did not show a significant DNA ladder (Fig. 9B, lane 6, compared with Hep G2-MV2E1-9 cells shown in lane 7). The DNA fragmentation in Hep G2-MV2E1-9 cells was completely blocked by 0.1 mM trolox (Fig. 9B, lanes 8 and 9, compared with lane 7).

**bcl-2 Protects Hep G2-MV2E1-9 Cells against Arachidonic Acid Toxicity**—bcl-2 has been shown to be protective against apoptosis in several reaction systems (35–43). Hep G2-MV2E1-9 cells contained a low level of bcl-2, as shown by

![Graph A](image1.png)

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**Graph A**

1. **Y-axis:** Mean of FITC labeling
2. **X-axis:** Arachidonic acid concentration (mM)
3. **Legend:**
   - E9
   - MV5
   - MV5 with trolox
   - DMV5 with trolox

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**Graph B**

1. **Y-axis:** Mean of FITC labeling
2. **X-axis:** Arachidonic acid concentration (mM)
3. **Legend:**
   - E9
   - E9 with trolox
   - MV5
   - DMV5 with trolox

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**Figure 9.** Apoptosis induced by arachidonic acid in Hep G2-MV2E1-9 cells. **A,** Hep G2-MV2E1-9 cells were cultured in control medium or medium supplemented with 0.02, 0.03, or 0.04 mM arachidonic acid for 6, 12, or 24 h. Cells were harvested at each of these points. An additional set of cells was incubated with arachidonic acid for 24 h, followed by a second incubation for 8 h in the absence of arachidonic acid. Lanes refer to the following: lanes 1, 2, and 3, no arachidonic acid added; lanes 4, 5, and 6, 0.02 mM arachidonic acid added and incubation for 6, 12, or 24 h, respectively; lanes 7, 8, and 9, 0.03 mM arachidonic acid added and incubation for 6, 12, or 24 h, respectively; lanes 10, 11, and 12, 0.04 mM arachidonic acid added and incubation for 6, 12, or 24 h, respectively; lanes 13, 14, and 15, 0.02 mM arachidonic acid and incubation for 6, 12, 24, and 24 plus 8 h, respectively; lanes 16 and 17, 0.03 mM arachidonic acid and incubation for 6, 12, 24, and 24 plus 8 h, respectively.

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**Figure 8.** Quantification of in situ DNA nick end labeling of Hep G2-MV2E1-9 and Hep G2-MV-5 cells. **A,** cells were cultured in control medium or medium containing arachidonic acid (0.01–0.03 mM) for 24 h, followed by culture with normal MEM for an additional 8-h incubation. **B,** the effect of 0.03 mM arachidonic acid in the absence or presence of 0.1 mM trolox in in situ DNA nick end labeling was determined. Cells were harvested for TUNEL analysis. At least 3000 cells from each group were measured by flow cytometry. The mean of FITC labeling was used as an index of DNA fragmentation.
isozymes, CYP2E1 displays high NADPH oxidase activity, is loosely coupled, and is more reactive in oxidizing ethanol to the 1-hydroxyethyl radical (4, 9–11, 62). Microsomes from ethanol-treated rats are more reactive than the controls in producing a variety of reactive oxygen intermediates by reactions sensitive to anti-CYP2E1 antibodies and to chemical inhibitors of CYP2E1 (2–8). Correlation between induction of CYP2E1, lipid peroxidation, and ethanol-induced liver injury has been reported with the continuous intragastric infusion model of ethanol feeding (16, 18, 22). The studies using the intragastric model of rat feeding indicated that a high content of polyunsaturated fatty acids would lead to enhanced CYP2E1-dependent lipid peroxidation and pathogenesis of alcoholic liver disease (20). To establish direct linkage between CYP2E1, PUFA toxicity, and the role of lipid peroxidation and oxidative stress, we utilized a previously established human hepatoma Hep G2 subline, Hep G2-MV2E1-9 clone, which was transduced with human CYP2E1 cDNA by using a retrovirus shuttle vector (23). An advantage of this model is the stable, constitutive expression of CYP2E1, in contrast to the rapid decline of the isozyme in primary cultured hepatocytes. Experiments were carried out to evaluate whether arachidonic acid, a representative PUFA, is more toxic to cells expressing CYP2E1 compared with control cells not expressing CYP2E1, whether the elevated toxicity is associated with enhanced lipid peroxidation, whether antioxidants can rescue the cells against PUFA cytotoxicity, whether the cytotoxicity is apoptotic in nature, and whether bcl-2 can protect the cells against the PUFA cytotoxicity.

Hep G2-MV2E1-9 cells expressing CYP2E1 and Hep G2-MV-5 cells that do not have detectable CYP2E1 expression were first incubated with arachidonic acid for 24 h, followed by removal of the PUFA, addition of fresh medium not containing added PUFA, and analysis for toxicity. Indices of toxicity included LDH leakage, morphology, and decreased vital dye reduction (MTT assay). Arachidonic acid (0.03 mM) induced cytotoxicity in Hep G2-MV2E1-9 cells, whereas significantly lower or no cytotoxicity was found in the control Hep G2-MV-5 cells. The cytotoxicity produced by arachidonic acid was concentration- and time-dependent. An important control is the observation that oleic acid was not toxic to the CYP2E1 expressing cells under conditions in which arachidonic acid was toxic, indicating that toxicity is not due to fatty acid metabolism per se but rather due to the presence of a PUFA. This suggests that lipid peroxidation plays a role in the arachidonic acid cytotoxicity to Hep G2-MV2E1-9 cells. Three lines of experiments are supportive for a role for lipid peroxidation in the PUFA toxicity to Hep G2-MV2E1-9 cells. Depletion of GSH by BSO treatment increased arachidonic acid toxicity to the Hep G2-MV2E1-9 (and the Hep G2-MV-5 cells). GSH is known to protect cells against oxidative stress and damage caused by lipid peroxidation (64, 65). Formation of characteristic end products of lipid peroxidation, malondialdehyde and 4-hydroxy-2-nonenal, was strikingly elevated in the Hep G2-MV2E1-9 cell extracts and in the culture medium from the Hep G2-MV2E1-9 cells after addition of arachidonic acid, whereas only a small increase in these lipid peroxidation products was observed with BSO treatment.

Western blot analysis (Fig. 4, pCI lane). To determine the effect of bcl-2 on the arachidonic acid toxicity, we transfected Hep G2-MV2E1-9 cells with pCI-bcl-2 plasmid, which contains cDNA encoding human bcl-2, with control pCI plasmids (the empty vector), and with pCI-as-bcl-2, which contains the bcl-2 cDNA in reversed orientation (the antisense cDNA). The pCI-bcl-2-transfected Hep G2-MV2E1-9 cells produced a much higher level of bcl-2 (Fig. 4, bcl-2 lane) compared with the pCI-transfected cells (Fig. 4, pCI lane). After 24 h of arachidonic acid (0.02–0.04 mM) preloading and 24 h of additional incubation, pCI-transfected Hep G2-MV2E1-9 cells displayed similar cytotoxicity (20–50%) as did the non-transfected Hep G2-MV2E1-9 cells (Fig. 10, pCI curve, compared with Fig. 3, E9 minus BSO curve). Under the same conditions, pCI-bcl-2 transfected Hep G2-MV2E1-9 cells showed only marginal toxicity (less than 10%) by arachidonic acid. pCI-as-bcl-2 transfected Hep G2-MV2E1-9 cells showed a somewhat greater toxicity compared with pCI transfected (Fig. 10); very little bcl-2 was detected in the cells after transfection with the antisense plasmid (Fig. 4, as bcl-2 lane). These results suggest that bcl-2 modifies the sensitivity of Hep G2-MV2E1-9 cells to arachidonic acid. Fig. 4 shows that CYP2E1 levels were similar in the cells transfected with plasmids pCI, pCI-bcl-2, and pCI-as-bcl-2.

**DISCUSSION**

The primary goal of the present study was to investigate the toxicity of arachidonic acid in a human liver cell line in which the major or the only significant cytochrome P450 isozyme is CYP2E1. Induction of CYP2E1 and the formation of reactive intermediates, including reactive metabolites, reactive oxygen species, lipid peroxidation derivatives appears to be one of the mechanisms that is receiving much current interest in studies evaluating how ethanol is hepatotoxic. It has been demonstrated that relative to several other cytochrome P450
The presence of added substrates. In fact, formation of superoxide and reactive oxygen intermediates occurs even in the absence of CYP2E1. This toxicity by higher concentrations of arachidonic acid was also observed with the Hep G2-MV-5 cells that do not express CYP2E1. This toxicity by higher concentrations of arachidonic acid most likely reflects a non-CYP2E1-mediated lipid peroxidation process since (a) toxicity was enhanced after BSO treatment to lower cellular GSH levels (Fig. 3A), (b) small increases in malondialdehyde and 4-hydroxynonenal were produced upon incubating the Hep G2-MV-5 cells with 0.03 mM arachidonic acid (Fig. 6); and (c) the small increase in FITC labeling found when arachidonic acid was incubated with the Hep G2-MV-5 cells, analogous to the large increase found with the CYP2E1-expressing cells, was prevented by the antioxidant trolox (Figs. 7 and 8). Most likely, reactive oxygen species are being produced from other cellular sources than cytochrome P450, such as mitochondrial membranes, endoplasmic reticulum, and nuclear membranes (31, 76–78). When Hep G2-MV-5 cells were transfected with bcl-2, they became resistant to the arachidonic acid toxicity, which is consistent with the protection by various antioxidants. Interestingly, the transfectants from plasmid containing antisense bcl-2 cDNA showed an increased toxicity by arachidonic acid, probably due to the suppression of the low level of endogenous bcl-2 in the Hep G2 cells (Fig. 4B). Transfection with the bcl-2 sense or antisense plasmid did not affect expression of CYP2E1 as compared with transfection with control plasmid (Fig. 4A).

In summary, experiments have been carried out that demonstrate that arachidonic acid is toxic to cells that express CYP2E1 but not to cells that do not express CYP2E1. The PUFA toxicity is associated with increased lipid peroxidation and can be diminished by antioxidants that prevent lipid peroxidation. The toxicity appears to be apoptotic in nature and can be prevented by overexpression of bcl-2. Since production of reactive oxygen intermediates is elevated with microsomes isolated from cells expressing CYP2E1 compared with controls, it is proposed that this elevated generation of reactive intermediates can initiate lipid peroxidation, which subsequently causes apoptosis and cellular damage, when the cells are pre-loaded with PUFA. These results indicate that enrichment of cells that express CYP2E1 with PUFA results in cytotoxicity. The Hep G2-MV-2E1-9 cells appear to be a useful model to study interactions between CYP2E1, PUFA, and free radicals and the consequences of these interactions on cell viability. They also appear to reproduce, in a simple cell culture model, several of the key features associated with ethanol hepatotoxicity in the intragastric infusion model of ethanol treatment.

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