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CYTOCHROME P4502E1 OR MENADIONE -INDUCED OXIDATIVE STRESS, CYTOTOXICITY, AND APOPTOSIS TO HEP G2 CELLS

by

Qi Chen

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1997

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

Cytochrome P4502E1 Or Menadione-Induced Oxidative Stress, Cytotoxicity, And Apoptosis To Hep G2 Cells

by

Qi Chen

Adviser: Professor Arthur I. Cederbaum

Menadione generates reactive oxygen intermediates and causes oxidative injury. Preincubation of Hep G2 cells with low, non-toxic concentrations of menadione increased the viability of the cells against toxic doses of menadione or H_2O_2. Menadione activated NF–κB, and this activation was prevented by antioxidants or salicylate. Transfection with an expression vector containing cDNA encoding mouse IκBβ resulted in increased toxicity by menadione. An increased level of glutathione (GSH) was observed after menadione pretreatment; this increase was blocked by salicylate, thereby linking the GSH increase to activation of NF-κB by menadione. This study suggests that menadione pretreatment protects Hep G2 cells from oxidative injury through an NF–κB related mechanism, which may involve, in part, increased production of GSH.

Arachidonic acid produced a concentration- and time-dependent toxicity to previously established E9 cells, which express cytochrome P4502E1 (CYP2E1), but little or no toxicity was found with control MV5 cells, which

were infected with retrovirus lacking CYP2E1 cDNA. In contrast to arachidonic acid, oleic acid was not toxic to both subclones. The cytotoxicity of arachidonic acid appeared to involve lipid peroxidation, the toxicity was enhanced after depletion of cellular glutathione, and toxicity was prevented by the antioxidants. The CYP2E1-dependent arachidonic acid toxicity appeared to involve apoptosis. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), which prevented toxicity of arachidonic acid, also prevented the apoptosis, suggesting a role for lipid peroxidation to apoptosis. Transfection with a plasmid containing *bcl-2* cDNA resulted in complete protection against the CYP2E1-dependent arachidonic acid toxicity. This study reproduced several of the key features associated with ethanol hepatotoxicity in the intragastric infusion model of ethanol treatment.

New Hep G2 subclones were established by transfecting Hep G2 cells with various vectors and limited dilution screening. Experiments demonstrated a growth inhibition effect and a cytotoxic effect of CYP2E1 in E47 and E43 cells, which express CYP2E1 at levels 4-10 times higher compared to the previously established E9 cells. These effects occur in the absence of externally added toxin or agent and therefore are directly due to high levels of expression of CYP2E1 itself. The slow growth may be a result of mitochondrial damage, the need to maintain cellular GSH level, and lower level of intracellular ATP content. The cytotoxicity is apoptotic in nature, and is initiated by the depletion of GSH by CYP2E1-related oxidative stress and elevated lipid peroxidation. The direct toxicity of overexpressed

CYP2E1 is probably a reflection of the ability of this isoform to produce reactive oxygen species (ROI) even in the absence of substrate. It would be anticipated that substrates which are oxidized by CYP2E1 to reactive metabolites, e.g. ethanol, acetaminophen, and CCl_4, or PUFA which produces elevated lipid peroxidation in the presence of CYP2E1, would show strong toxicity in the E47 and E43 cells. Preliminary studies showed that these agents are indeed strongly toxic in the E47 cells even without the necessity of depleting cellular GSH.

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Finally, I wish to thank Lei Yu and Xavier Roux for their understanding and support.

ABBREVIATIONS

4-HNE	4-hydroxy-2-nonenal
4-MP	4-methylpyrazole
A14 or A15	Hep G2 subclones transduced with vector containing antisense *bcl-2* cDNA
B27 or B28	transduced Hep G2 subclones overexpressing Bcl-2
BSO	buthionine sulfoximine
C34 or C37	Hep G2 subclones transduced with pCI-*neo* vector lacking any cDNA insert
CYP2E1	cytochrome P4502E1
DPPD	diphenylphenylenediamine
DTT	DL-dithiothreitol
E43 or E47	transduced Hep G2 subclones expressing CYP2E1 by transfection
E9	transduced Hep G2 subclone expressing CYP2E1 by retroviral infection
EMSA	electrophoretic mobility shift assay
FITC	fluorescein isothiocyanate
G 418	Geneticin®, O-2-amino-2,7-dideoxyl-Δ-glycero-α-Δ-glucoheptopyranosyl[1->4]-O-3-deoxy-4C-methyl-3-[methyl-amino]-β-L-arabinopyranosyl-Δ-streptamine, disulfate salt
GSH	glutathione, reduced form

- GSSG — glutathione, oxidized form
- *i*NOS — inducible nitric oxide synthetase
- LDH — lactate dehydrogenase
- Me_2SO — $(CH_3)_2SO$
- MDA — malondialdehyde
- MEM — minimum essential medium
- MN — menadione
- MTT — 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
- MV5 — Hep G2 subclones infected by retrovirus lacking CYP2E1 cDNA
- NAC — *N*-acetylcysteine
- *neo* — neomycin resistance gene
- NaSal — Sodium Salicylate
- PAGE — polyacrylamide gel electrophoresis
- PBS — phosphate-buffered saline
- PDTC — pyrrolidine dithiocarbamate
- PKC — protein kinase C
- PMA(TPA) — phorbol 12-myristate 13-acetate
- PMSF — phenylmethylsulfonyl fluoride
- PNP — *p*-nitrophenol

- PUFA — polyunsaturated fatty acid
- ROI — reactive oxygen intermediate
- SOD — superoxide dismutase
- TMPD — tetramethyl-*p*-phenylenediamine
- TNF-α — tumor necrosis factor-alpha
- Trolox — 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
- *TUNEL* — terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling
- VitE — vitamin E

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INTRODUCTION

The overall goal of the current project is to study the cytotoxicity of cytochrome P4502E1 (CYP2E1, CYP is the standard abbreviation for cytochrome P450) and menadione on hepatocellular carcinoma Hep G2 cell lines. Both CYP2E1 and menadione induce oxidative stress to Hep G2 cells and cause cytotoxicity; Hep G2 cells respond to the oxidative stress by upregulating one of their self-defense systems, increasing glutathione production. CYP2E1 toxicity was studied with the Hep G2 subclones transduced to express human CYP2E1 (Dai *et al.*, 1993; reviewed by Chen and Cederbaum, 1997a). Arachidonic acid, as a representative polyunsaturated fatty acid (PUFA), was added to the system to promote lipid peroxidation which induces apoptosis (Chen *et al.*, 1997; Chen and Cederbaum, 1997c). New Hep G2 subclones expressing higher level of CYP2E1 were also established to explore the direct toxicity of CYP2E1 itself to Hep G2 cells (manuscript in preparation). To study to the effect of oxidative stress on NF-κB activation and upregulation of self-defense mechanism, menadione, as well as H_2O_2, cytotoxicity to Hep G2 was studied and served as a model for further exploring the complexity of cytotoxicity involving CYP2E1. Menadione is toxic by a mechanism involving redox cycling and is independent of CYP2E1. Menadione toxicity was studied with regular Hep G2 cells, which are lacking CYP2E1 (Chen and Cederbaum,

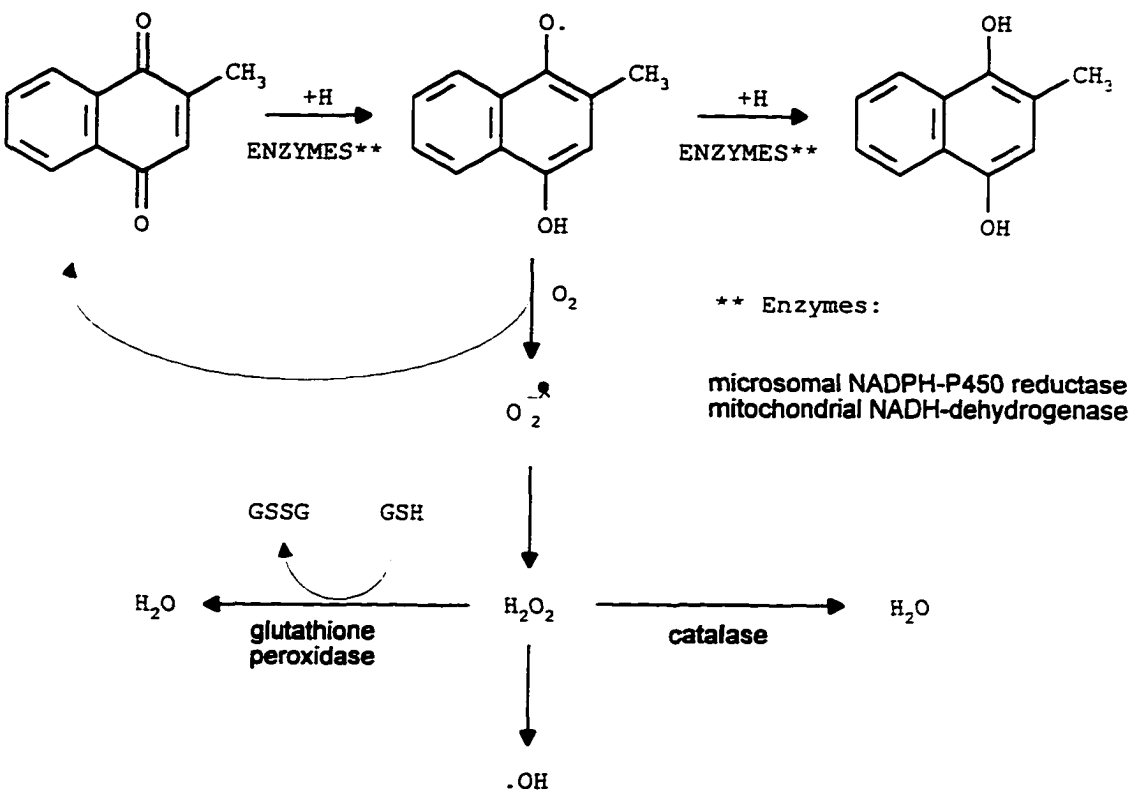
1997b). In this model, the role of NF-κB activation by oxidative stress in protecting cells from further oxidative damage and increased glutathione production were studied.

Reactive Oxygen Intermediates

Partially reduced intermediates formed from the initial univalent reduction of O_2 are reactive and toxic in biological systems. These intermediates include superoxide anion, hydrogen peroxide, and hydroxyl radical (Buechter, 1988). Generally, enzymes such as cytochrome oxidase do not allow these intermediates to dissociate from them to become free radicals in the living system. However, some of these intermediates dissociate from enzymes or autooxidizable chemicals and cause extensive cellular damage (Fridovich, 1982; Ingraham, 1985; Paine, 1978). The reduction of molecular oxygen includes four electron transfer steps. Superoxide radical is generated by accepting one electron from a donor; hydrogen peroxide is generated by accepting a second electron; hydroxyl radical is generated by accepting the third electron; and water is the final product after accepting all four electrons. In addition to oxygen, a number of exogenous compounds, such as menadione and a number of other redox cycling agents can be metabolized to reactive species, which may also result in oxidative injury. As summarized in Scheme I, menadione (vitamin K3, 2-methyl-1,4-naphthoquinone) could be reduced by NADPH-P450 reductase, NADH dehydrogenase, and other flavoproteins *in vivo* to generate the menadione semiquinone radical (Ernster *et al.*, 1962). This radical itself may directly

react with other cellular constituents to cause injury. It may also recycle back to menadione by rapid reaction with molecular oxygen to yield superoxide radical. The cytotoxicity of menadione appears to be associated with superoxide generation, protein thiol oxidation and alteration in the Ca^{2+} homeostasis (DiMonte *et al.*, 1984a; 1984b). Menadione may also cause toxicity due to depletion of GSH by conjugate formation, e.g. naphthoquinones such as menadione react readily with GSH to form glutathione conjugates; up to 20 % of the GSH in hepatocytes may be consumed by its direct reaction with menadione to form 2-methyl-3-glutathionyl-1,4-naphthoquinone (Wefers and Sies, 1983; Smith *et al.*, 1985).

Scheme 1



To protect themselves from oxidative injury, organisms have evolved protective systems to convert the ROI to less reactive components. These systems include several enzymes, such as superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione reductase, and cellular antioxidants, such as glutathione (GSH), vitamin C, and vitamin E. SOD (eukaryotes contain two types of SOD, a cytosolic Cu/Zn-SOD and a mitochondrial Mn-SOD) acts as a protective protein by converting superoxide to H_2O_2. Catalase usually localized in peroxisomes converts H_2O_2 to water and oxygen. Glutathione peroxidase removes the H_2O_2 by utilizing GSH (which is oxidized to GSSG and reduced back to GSH by glutathione reductase).

Glutathione is an important component of the anti-oxidative defense system of mammalian cells. GSH may function as a direct radical scavenger and for the removal of H_2O_2 and lipid hydroperoxides by glutathione peroxidases. To protect themselves against oxidative stress, cells can upregulate their antioxidant defense systems, e.g. many cells respond to oxidative stress by increasing production of GSH. Menadione and 2,3-dimethoxy-1,4-naphthoquinone, redox cycling quinones, have been shown to increase the activity of γ-glutamylcysteine synthetase, the rate limiting enzyme in GSH synthesis (Shi et at., 1994; Ochi, 1996). Incubation with L-DOPA or dopamine induced a rise in GSH level in mesencephalic cell cultures, which resulted in a protective effect against a toxic agent, *t*-butyl-hydroperoxide (Han *et al.*, 1996).

Alcohol and Cytochrome P4502E1

Alcohol is a widely consumed organic compound. Liver is the primary organ which metabolizes ethanol and plays a very important role in the ethanol oxidation (Diluzio, 1964; 1968; Lieber, 1988; 1990; Lieber and DeCarli, 1968; 1970). Liver alcohol dehydrogenase oxidizes the ethanol to acetaldehyde, which is subsequently oxidized to acetate. Acetaldehyde can directly damage the targeted tissue and cause acute toxic effects and symptoms (Goedde and Agarwal, 1989).

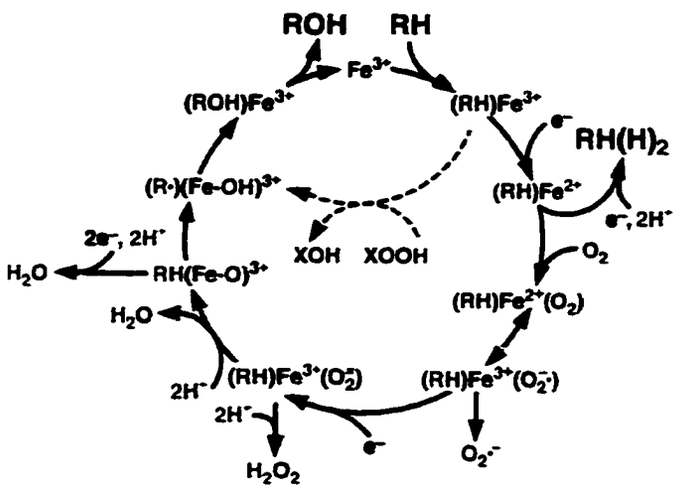
Ethanol metabolism may also involve the cytochrome P450 enzymes. Cytochrome P450 was first identified as a CO-binding pigment in the liver with an absorbance peak around 450 nm (Klingenberg, 1958; Garfinkle, 1958). The pigment was further characterized as a heme protein (Omura and Sato, 1961; 1964a; 1964b). Many cytochrome P450 isozymes have been identified thereafter (Gonzalez, 1989; Guengerich, 1987). There are around 150 known isozymes which are classified into 27 families, 10 of which exist in mammals (Gonzalez, 1992; Nebert *et al.*, 1991). The 10 mammalian cytochrome P450 families, containing 18 subfamilies, have been categorized into two genres: those involved in synthesis of steroids and bile acids including families 7, 11, 17, 19, 21, and 27; and those involved in metabolism of xenobiotics including families 1, 2, 3, and 4. CYP2E1, the ethanol-induced form, has received specific attention because of its inducibility by acute and chronic alcohol ingestion and its ability of oxidizing ethanol and activating a number of hepatotoxins in the liver. These hepatotoxins, after activation by CYP2E1, cause severe liver injury,

which may lead to a variety of alcohol-related diseases under different circumstances. These hepatotoxins include industrial solvents such as benzene (Johansson and Ingelman-Sundberg, 1988) and carbon tetrachloride (Johansson and Ingelman-Sundberg, 1985; Guengerich, 1991), drugs such as acetaminophen (Morgan *et al.*, 1983) and isoniazid, carcinogens and procarcinogens such as nitrosamines (McCoy and Koop, 1988; Sohn *et al.*, 1991; Yang *et al.*, 1990), as well as ethanol itself (Koop and Coon, 1986). The toxic effects of ethanol may come from its metabolite acetaldehyde, direct radical intermediates of ethanol such as the 1-hydroxylethyl radical (CH3CH•OH), other ROI such as superoxide and H_2O_2 generated from the reactions of the cytochrome P450 cycle, or initiation of lipid peroxidation when PUFA is present.

Substrates such as ethanol or acetaminophen will bind to the oxidized form of cytochrome P450. In a microsomal system, cytochrome P450, with or without substrate binding, can accept electrons either from P450 reductase, which accepts electrons from NADPH or from cytochrome b_5, which accepts electrons from NADH through cytochrome b_5 reductase. Molecular oxygen binds to ferrous cytochrome P450 with a K_d of ~ 10^{-6} M. The $[Fe^{2+}\text{-}O_2]$ complex has a resonance form of $[Fe^{3+}\text{-}O_2^-]$ complex in which an electron is transferred from the iron to the oxygen. The latter decays to produce a ferric cytochrome P450-substrate intermediate and releases a superoxide radical. The decay of oxygenated cytochrome P450 appears to be a major source of superoxide radical, which will form a hydroxyl radical in the presence of iron. Scheme II (Coon *et al.*, 1992) outlines the catalytic

mechanism of cytochrome P450. Importantly, relative to other cytochrome P450 isoforms, CYP2E1 is relatively uncoupled with high rates of O_2 uptake and NADPH consumption (Ingelman-Sundberg and Johansson, 1984). Studies from several laboratories have shown that microsomes from ethanol-treated rats are more reactive than pair-fed controls in generating superoxide and H_2O_2, and in interacting with iron to yield potent oxidizing agents such as •OH and initiators of lipid peroxidation. This increased activity was shown to be due to induction of CYP2E1 (reviewed in Cederbaum 1987; 1989; 1991; 1994).

Scheme II



Oxidative Stress and NF-κB activation

ROI such as superoxide, H_2O_2, and •OH, produced as normal byproducts of cellular metabolism, clearly have effects on multiple cell types and organisms (Davies *et al.*, 1994). Bacteria such as *Escherichia coli* can develop resistance to normally lethal concentrations of H_2O_2 by induction of a series of defense and repair enzymes. Those genes are induced by pretreatment of H_2O_2 (Demple and Halbrook, 1983). Adaptive responses to the oxidative stress of H_2O_2 in yeast *Saccharomyces cerevisciae* strain RZ53 could increase the viability against a higher dose of H_2O_2 (Davies *et al.*, 1994). It has also been indicated that eukaryotic cells are capable of transient adaptive responses to oxidative stress (Gupta and Bhattacharjee, 1988; Laval, 1988; Lu *et al.*, 1993; Spitz *et al.*, 1995). Preincubation of mammalian cell lines, such as Chinese hamster ovary fibroblast CHO cells, embryonic mouse fibroblast C3H 10T½ cells, Chinese hamster lung fibroblast V79 cells, and rat liver epithelial Clone 9 cells, with relatively low doses of H_2O_2 have also been found to increase the resistance of those cells to H_2O_2 (Wiese *et al.*, 1995).

NF-κB was first characterized in mature B and plasma cells as a nuclear protein binding specifically to a 10-bp sequence (GGGGYNNCCY) in the κ intronic enhancer (Sen and Baltimore, 1986a; 1986b). Subsequent studies revealed that NF-κB exists virtually in all cells, and activation of NF-κB leads to regulation of a variety of genes indicating a significantly broader role for the NF-κB. NF-κB is a multisubunit transcription factor and can

rapidly activate the expression of genes involved in inflammatory, immune, and acute phase responses. Target genes regulated by NF-κB include: immuno-regulatory cytokines such as TNF-α, IL-2, IL-6, β-IFN and GM-CSF; MHC class I antigens and IL-2 cytokine receptor; and acute phase protein. In most cells, NF-κB is present in a non-DNA-binding cytosolic complex which contains p50, p65, and a third inhibitory subunit IκB. Treatment of cells with various conditions leads to the dissociation of the cytoplasmic complex and the translocation of free NF-κB to the nucleus (Baeuerle *et al.*, 1988). Therefore, NF-κB serves as a signal transducer by carrying information from external agents directly to the nucleus. NF-κB is classically recognized as a heterodimer consisting of p50 (NFKB1) and p65 (RelA) subunits; however, recent studies have show that they are members of a larger family, known as the Rel family. In addition to p50 and p65, the Rel family also includes p52/p100 (NFKB2), p105 (p50 precursor), Rel-B, the oncogene v-*rel* and the corresponding protooncogene c-*rel*, and the *Drosophila* morphogen *dorsal*. NF-κB can also be loosely described as a homo- or heterodimer of Rel proteins (Kopp and Ghosh, 1995). A conserved, N-terminal, 300-amino-acid segment, termed the Rel homology (RH) domain, is responsible for DNA binding, dimerization, activation and NF-κB/IκB interaction.

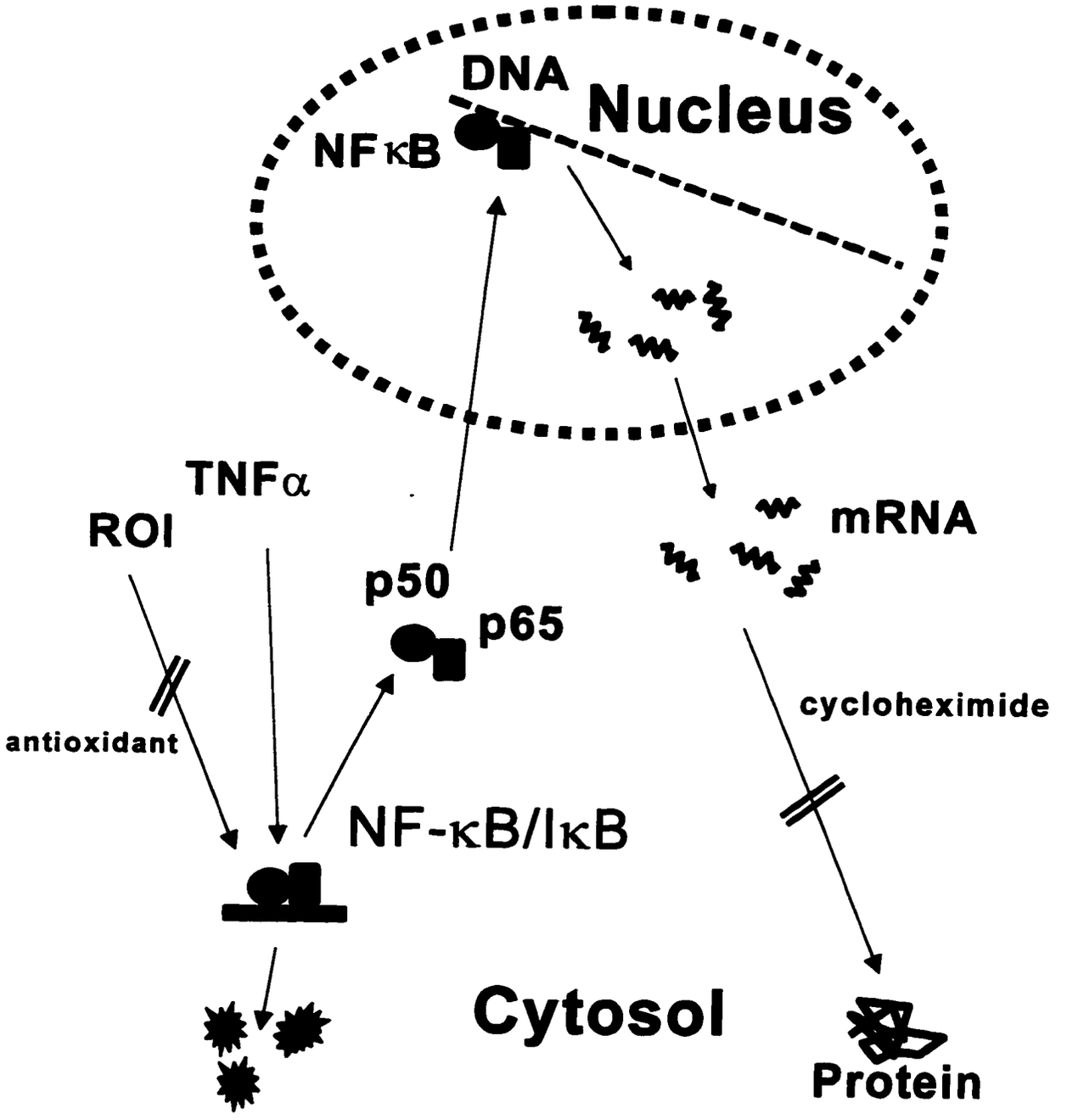
IκB binds to NF-κB, and therefore retains NF-kB in the cytoplasm in an inactive form (Baeuerle and Baltimore, 1988a, 1988b). IκB is a member of

another group of proteins. These proteins contain multiple conserved ankyrin repeats, which are thought to interact with the Rel domain of NF-κB (Davis *et al.*, 1991; Haskill *et al.*, 1991; Franzoso *et al.*, 1992). Currently, the IκB family consists of IκBα/MAD-3, IκBβ, IκBγ, and Bcl-3. The mechanism of NF-κB activation and its molecular details are yet to be clarified. Studies have suggested roles for phosphorylation and proteolysis in the rapid degradation of IκB subunit. Among the IκB family, IκBα, a 36-38 kDa protein, is mainly responsive to transient activation of NF-κB by some agents such as PMA and TNF-α; the rapid phosphorylation followed by degradation of IκBα leads to activation of NF-κB, which can also activate the expression of IκBα (autoregulatory feedback loop). The up-regulated IκBα is responsible for shutting down the NF-κB response. (reviewed by Baeuerle and Henkel, 1994; Kopp and Ghosh, 1994). IκBβ, a 45 kDa protein, appeared to be responsible for persistent activation of NF-kB, since IκBβ is not up-regulated upon induction of NF-κB. LPS and IL-1 lead to the loss of both IκBα and IκBβ proteins, while PMA and TNFα affect only IκBα.

The phorbol ester 12-myristate 13-acetate (PMA), an activator of protein kinase C (PKC), may activate NF-κB. PKC reacts with cytoplasmic or partially-purified NF-κB/IκB complex eventually causing activation of DNA-binding by NF-κB. Furthermore, treatment of IκB with PKC blocks

the inhibitory effect of IκB. Scheme III shows the suggested NF-κB activation pathway.

Scheme III



Lipid peroxidation and Apoptosis

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 (Nanji and French, 1985; 1986). 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 (French, 1992; Takahashi *et al.*, 1992; Tsukamoto and French, 1993; French *et al.*, 1993; Nanji *et al.*, 1993; Tsukamoto *et al.*, 1995). Liver injury occurs in this model when the rats consume diets containing PUFA but not saturated fatty acid. In these models, large increases in lipid peroxidation have been shown to correlate with CYP2E1 levels (French, 1992; Takahashi *et al.*, 1992; Nanji *et al.* 1993; Moromoto *et al.*, 1993; Kukielka and Cederbaum, 1994; Tsukamoto *et al.*, 1995). 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 (French, 1992; Takahashi *et al.*, 1992; Nanji *et al.* 1993; Moromoto *et al.*, 1993; Kukielka and Cederbaum, 1994; Tsukamoto *et al.*, 1995).

In attempts to directly demonstrate that overexpression of CYP2E1 can result in hepatotoxicity of various agents, a Hep G2 cell line which constitutively expresses the human CYP2E1 was recently established (Dai *et*

al., 1993). ESR spectroscopy showed that microsomes from E9 cells which express CYP2E1 produced superoxide radicals at rates about 10-fold greater than those from MV5 cells which do not express CYP2E1; rates of H_2O_2 production were about 3-fold greater with the E9 microsomes. Rates of microsomal lipid peroxidation were also greater with the E9 cells (Dai *et al.*, 1993). Ethanol and acetaminophen were shown to be toxic to E9 cells but not MV-5 cells (Dai and Cederbaum, 1995; Wu and Cederbaum, 1996). This model appears to be useful in efforts to establish a CYP2E1-dependent hepatotoxicity system and to evaluate the role of oxidative stress in the toxicity of compounds metabolized by CYP2E1, as well as to study the mechanism of *in vivo* CYP2E1 degradation.

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 (Talley *et al.*, 1995; Nobel *et al.*, 1995; Lin *et al.*, 1995; Weltin *et al.*, 1996). The morphological changes associated with apoptosis include condensation of nucleoplasm and cytoplasm, blebbing of cytoplasmic membrane, and fragmentation of cell into apoptotic bodies that are rapidly phagocytosed by neighboring cells (Kerr, 1971; Wyllie *et al.*, 1980). The biochemical markers of apoptosis include DNA fragmentation into nucleosomal fragments (Willie, 1980), activation of the ICE-family proteases (Schlegel *et al.*, 1996; Duan *et al.*, 1996; Wang *et al.*, 1996), and cleavage of various substrates of

ICE-family proteases, including PARP (Tewari *et al.*, 1995; Nicholson *et al.*, 1995), SREBPs (Wang et al, 1995, 1996), nuclear lamin (Lazebnik *et al.*, 1995), and U1 associated 70 kDa protein (Casciola-Rosen *et al.*, 1994). The prototypic regulator of mammalian apoptosis is the protooncogene *bcl-2* (Reed, 1994). Overexpression of *bcl-2* prevents mammalian cells from undergoing apoptosis induced by a variety of stimuli (reviewed by Reed, 1994). The functions of *bcl-2* have been suggested to include acting as an antioxidant (Hochenbery *et al.*, 1993), modulating some aspects of nuclear transport (Meikrantz *et al.*, 1994), intervention in calcium signaling (Baffy *et al.*, 1993), and associating with several other proteins (Sato *et al.*, 1994). The Bcl-2 protein is located primarily on the outer membrane of mitochondria (Monaghan *et al.*, 1992; Krajewski *et al.*, 1993; de Jong *et al.*, 1994), suggesting the active involvement of mitochondria in the development of apoptosis.

MATERIALS AND METHODS

Cells and Chemicals

Hep G2 cells (Aden *et al.*, 1979), and its transduced subclones, A14, A15, B27, B28, C34, C37, E9, E43, E47, and MV5 cells, were cultured in Minimum Essential Medium (MEM), supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin and 2 mM glutamine in a humidified atmosphere in 5% CO_2 at 37°C. Most reagents were purchased from Sigma Chemical Co., St. Louis, MO. Specific reagents are described below. The Hep G2 subclones mentioned above will be described in Results section, their phenotypes are listed below:

E43, E47	express CYP2E1
A14, A15	no detectable Bcl-2 expression
B27, B28	express Bcl-2
C34, C37	control subclones transfected with pCI-neo plasmid
E9	express CYP2E1, previously established (Dai *et al.*, 1993)
MV5	control subclone for E9

Establishment of Hep G2 subclones overexpressing CYP2E1

Human CYP2E1 cDNA, excised from a plasmid p91023(B)-2E1 (kindly provided by Dr. F. J. Gonzalez, National Cancer Institute, Bethesda, MD),

was inserted into the Eco RI restriction site of pCI-*neo* expression vector (Promega, Madison, WI) in the sense and antisense orientation (confirmed by restriction mapping) to form the plasmids pCI-2E1 and pCI-as-2E1. Transfections of Hep G2 cells were carried out by utilizing the LipofectAMINE reagent (Life Technologies Inc., Gaithersburg, MD) as described by Hawley-Nelson (1993). Eighteen hours after transfection, fresh MEM containing 0.8 mg/ml G 418 (Boehringer Mannheim Co., Indianapolis, IN) was added and the cells were incubated for an additional 2 days. The cells were harvested by trypsinization for G 418 selection and Western blot analysis. About 1.5 X 10^6 transfected Hep G2 cells were seeded into 100 mm culture dishes with MEM containing 0.8 mg/ml G 418. Seven days later, survivors were harvested by trypsinization and seeded into 96-well tissue culture plates at average densities of 0.5, 1, and 2 cell/well (limited dilution). Culture medium was changed weekly. Monoclones were formed in about 3 weeks. Colonies were grown to large scale, and subjected to Western blot analysis and *p*-nitrophenol (PNP) oxidation activity assay (described below). Positive clones were subjected to another two rounds of limited dilution screening to create stable cell lines.

Establishment of Hep G2 subclones overexpressing *bcl-2*

A full-length human *bcl-2* cDNA, excised from pSFFV-bcl-2 expression vector (kindly provided by Dr. George Acs and Dr. Beatriz Pogo, Mount Sinai School of Medicine, New York, NY), was inserted into the Eco RI restriction site of pCI-*neo* expression vector in the sense and antisense orientations (confirmed by restriction mapping) to form the expression

vectors pCI-bcl-2 and pCI-as-bcl-2. These vectors were used for transfections of Hep G2 cells using the LipofectAMINE reagent as described above. After transfection, G 418 selection and limited dilution screening, stable new clones were analyzed by immunoblotting using anti-Bcl-2 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Transduction of Hep G2 Cells with IκBβ cDNA plasmid

The full-length mouse IκBβ cDNA (Thompson *et al.*, 1995), excised from pBSK-IκBβ plasmid (kindly provided by Dr. Sankar Ghosh, Yale University School of Medicine, New Haven, CT) was inserted into the Not I restriction site of pCI-*neo* expression vector, in the sense orientation to form pCI-IκBβ. Transfection of Hep G2 cells was carried out by utilizing the LipofectAMINE reagent. Hep G2 cells were grown to 80-90% confluence, harvested by trypsinization, and 1.5 X 10^6 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 serum-free MEM) and solution B (100 μl of LipofectAmine reagent in 800 μl 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 6.4 ml MEM, added to the culture dish containing the Hep G2 cells, followed by incubation for 5 h at 37°C in a CO_2 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 4 days. The cells were collected by

trypsinization and used for Western blot analysis and for menadione toxicity studies.

Transient Transfection with vectors containing CYP2E1 or *bcl-2* cDNA

Transfection of Hep G2 cells or Hep G2 subclones with expression vectors was carried out with the LipofectAMINE reagent. 1.5 X 10^6 cells were seeded into a 100 mm culture dish and grown until 50-70% confluence (about one day of culture). Cells were rinsed with serum free MEM before transfection. 5-15 μg of plasmid DNA (determined with preliminary experiment for optimal conditions) and 100 μl of LipofectAMINE reagent were used to transfect the cells. The cells were collected by trypsinization and used for Western blot analysis and for studies of cell growth and cytotoxicity. The optimized conditions for the transfections in a 6-well culture plate are: for each well of transfection, use 2 μg of desired plasmid DNA, e.g. pCI-2E1 or pCI-*neo* plasmid plus 15 μl LipofectAMINE for Hep G2 cells, plasmid plus 12 μl LipofectAMINE for E9 cells, pCI-as-2E1 or pCI-*neo* plasmid plus 10 μl LipofectAmine for E47 cells, and pCI-2E1 plasmid plus 15 μl LipofectAMINE for A14, B28, or C34 cells. The transfection conditions for 100 mm culture dish are same except with 7 times of DNA and LipofectAMINE.

Western Blot Analysis

Cell lysates were produced by vortexing and boiling 1 X 10^6 cells in 0.2 ml SDS-PAGE running buffer. A 20 μl aliquot of pretreated cell sample was

resolved on SDS polyacrylamide gels (12% gel for CYP2E1 and IκBβ proteins, and 8% gel for Bcl-2 protein, stable current of 25 mA/gel) and transblotted onto nitrocellulose sheets (Bio-Rad Laboratories, Hercules, CA), at a stable current of 125 mA for 1 h, for Western blot analysis (Laemmli, 1970; Towbin *et al.*, 1979). Rabbit anti-human CYP2E1 polyclonal antibody (provided by Dr. J. M. Lasker, Mount Sinai School of Medicine, New York, NY), mouse anti-human Bcl-2 monoclonal antibody, or rabbit-anti-mouse IκBβ polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used as the primary antibody for detecting CYP2E1, Bcl-2, or IκBβ, respectively, followed by treatment with alkaline phosphatase conjugated to goat anti-rabbit IgG or goat anti-mouse IgG (Bio-Rad Laboratories, Hercules, CA) as the second antibody. Staining intensity was developed with the NBT-BCIP mixture (Promega, Madison, WI).

PNP Oxidation Assay

Cells were washed once with phosphate-buffered saline (pH 7.4) and harvested by scraping and subsequent sonication using a Heat Systems-Ultrasonics Model W-375 Sonicator™ (45s, duty cycle 25%, output control 40%). Microsomes were prepared by differential centrifugation (7,000g X 10 min to remove cell debris and large subcellular particles with the suppernatant then centrifuged at 100,000g X 45 min to bring down the microsomal fraction) and resuspended in PBS buffer containing 20% glycerol. Oxidation of PNP was determined using 100 μg microsomal protein in a 100 μl-reaction-system containing PBS, 0.4 mM PNP and 1 mM

NADPH. All reactions, carried out in duplicate, were initiated with NADPH, incubated at 37°C, and stopped after 60 min with 30 µl 20% TCA. Absorbance of the final product of the reaction was measured at 586 nm, and activity determined using an extinction coefficient of 9.4 $mM^{-1}cm^{-1}$ (Clejan *et al.*, 1989).

Electrophoretic Mobility Shift Assay

Nuclear extracts were isolated by a method modified from Dignam *et al.* (1983). Briefly, cells were pretreated with various reagents for 45 minutes. 1 X 10^7 cells were harvested and washed once with PBS and twice with buffer A (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT). The cell pellet was suspended in 200 µl of buffer A + 0.1% Nonidet P-40, and incubated on ice for 15 min with brief mixing. After centrifugation for 10 min at 4°C, nuclear pellet was washed once with buffer A, suspended in 15 µl of buffer C (20 mM HEPES pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT), incubated for 15 min on ice, mixed briefly, and centrifuged for 10 min at 4°C. This supernatant was diluted with 75 µl of modified buffer D (20 mM HEPES pH 7.9, 20% glycerol, 0.05 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) and stored at -70°C. Protein concentration was determined by the Bio-Rad DC-20 Protein Assay. A double-stranded oligonucleotide containing a tandem repeat of the consensus sequence of the NF-κB DNA binding site, -GGGGACTTTCC-, was used as a probe. To determine the sequence specificity of the DNA-protein interaction, twenty times more of non-

radioactive oligonucleotide, as well as an oligonucleotide containing mutations in the NF-kB consensus sequence, were added to compete with the NF-kB probe. The sequences of the oligonucleotides were:

5'-GATCCAA**GGGGACTTTCC**ATGGATCCAA**GGGGACTTTCC**ATG-3' (wild type)

3'-GTT**CCCCTGAAAGG**TACCTAGGTT**CCCCTGAAAGG**TACCTAG-5'

5'-GATCCAA**GCTCACTTTCC**ATGGATCCAA**GCTCACTTTCC**ATG-3' (mutated)

3'-GTT**CGAGTGAAAGG**TACCTAGGTT**CGAGTGAAAGG**TACCTAG-5'

Probes were end-labeled by T4 polynucleotide kinase (Life Technologies, Inc., Gaithersburg, MD) with [γ-32P]ATP (DuPont NEN, Boston, MA). Briefly, in a 1.5 ml microcentrifuge tube, 5 μl 5X polynucleotide kinase buffer (5X = 300 mM Tris, pH 7.5, 50 mM $MgCl_2$, 75 mM β-mercaptoethanol and 1.65 μM ATP), 5 ng oligonucleotide, 100 μCi [γ-32P]ATP, 5 units T4 kinase and H_2O were mixed in a 25 μl reaction volume, and incubated 45 min at 37°C. The reaction was terminated by adding 50 μl of 50 mM Tris-HCl pH 7.5. The labeled oligonucleotide was purified on a ProbeQuant G-50 Micro Column (Pharmacia LKB Biotechnology, Uppsala, Sweden). Electrophoretic mobility shift assay was performed at room temperature for 20 minutes in a total 25 μl reaction volume containing 5 μl 5X incubation buffer (5X = 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5 mM DTT, 5 mM EDTA, 20% glycerol and 0.4 mg/ml sonicated salmon sperm DNA), 8-12 μg nuclear extract, and 5 X 10^4 cpm of labeled oligonucleotide, followed by polyacrylamide gel electrophoresis for DNA (150 V, 2-3 h). The

dried gels were analyzed after autoradiography by a PhosphorImager system (Molecular Dynamics).

TUNEL Analysis

Apoptosis in individual cells was assessed by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (*TUNEL*) techniques as described by Gavrieli *et al.* (1992) and Portera-Cailliau *et al.* (1994) with modifications. Briefly, 5 X 10^5 E9 or MV-5 cells were plated onto each well of 6-well culture plates. After incubation with or without arachidonic acid, cells were washed twice with PBS + 1% BSA at 4°C, adjusted to a concentration of 0.2 X 10^7 per 0.2 ml PBS buffer, and fixed with 0.1 ml freshly prepared 4% paraformaldehyde solution (in PBS, pH 7.4) for 30 min at room temperature. Cells were washed twice with PBS + 1% BSA, and resuspended in 0.1 ml of permeabilization solution (0.1% Triton® X-100 in 0.1 % sodium citrate) for 2 min on ice, followed by washing twice with PBS + 1% BSA. Cells were then resuspended in 50 µl *TUNEL* reaction mixture or label solution (without terminal transferase) as negative control, incubated for 60 min at 37°C in a humidified atmosphere in the dark, followed by washing twice in PBS + 1% BSA. Cells were analyzed by flow cytometry (EPICS® Profile Analyzer, Coulter Corporation).

DNA Agarose Gel Electrophoresis

DNA fragmentation was determined as a biochemical index of apoptosis (Harmom *et al.*, 1979; Vedeckis and Bradshaw, 1983; Caron-Leslie and Cidlowski, 1991; Compton, 1992).. Cells were scraped off the 6-well culture

plates (Corning Co., Corning, NY) with culture medium, and were centrifuged at 1,200 rpm X 10 min. The cell pellets were resuspended in 1 ml lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM EDTA, 100 μg/ml proteinase K, and 0.5% SDS) and incubated for 1 h at 50 °C. After lysis, samples were extracted with 1-2 ml phenol (neutralized with TE buffer, pH 7.5), followed by extraction with 1 ml chloroform:isoamyl alcohol (24:1) mixed solution. The aqueous supernatants were precipitated with 2.5 volumes ice-cold ethanol plus 10% volume of 3 M sodium acetate (pH 5.2) at -20°C overnight. After centrifugation at 13,000g X 10 min, the pellets were air-dried, resuspended with 50 μl TE buffer (10 mM Tris-HCl pH 7.5 and 1mM EDTA) supplemented with 0.1 μg/ml RNase A, and electrophoretically separated on a 1.5% agarose gel in 0.5 X TBE buffer containing 1 μg/ml ethidium bromide at 50 V for 3 h. Pictures of the gels were taken by UV transillumination.

Cytotoxicity Measurement: MTT Assay

Cytotoxicity was primarily measured by the MTT assay (Mosmann, 1983). Tetrazolium salts such as MTT are metabolized by mitochondrial dehydrogenases to form a blue formazan dye and are therefore useful for the assay of mitochondrial function and as a measurement of cytotoxicity. Approximately 1.0-1.5 X 10^4 cells were plated onto each well of a 24-well plate (Corning Co., Corning, NY), and incubated in 5% CO_2 at 37 °C for 24 h. In some samples, 0.1 mM BSO was then added to the culture medium for a designated preincubation time, typically 48 h. The culture medium was

removed and cell viability was evaluated by the MTT assay, which was performed using the Cell Titer 96 Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI). Briefly, 15% volume of dye solution was added to each well for a one-hour incubation at 37°C. An equal volume of solubilization/stop solution was then added to each well for an additional 4-24 h incubation. The absorbance of the reaction solution at 570 nm was recorded. The absorbance at 630 nm was used as reference. The net A_{570}-A_{630} was taken as the index of cell viability. The net absorbance from the wells of cells cultured with control medium was taken as the 100% viability value. The percent viability of the treated cells was calculated by the formula: $(A_{570}-A_{630})_{sample}/(A_{570}-A_{630})_{control}$ X 100.

Cytotoxicity Measurement: LDH Assay

Leakage of lactate dehydrogenase (LDH) was measured as another index of cytotoxicity. Approximately 1-2 X 10^6 cells were plated onto each well of a 6-well plate in MEM with or without additional testing reagents and incubated for the designated time. At the end of this period, media were collected to measure LDH activity (referred to as LDH_{out}). Cells were harvested by scraping, washed with PBS, suspended in 1 ml of PBS and sonicated using a Heat Systems-Ultrasonics Model W-375 Sonicator™ (5s, duty cycle 25%, output control 40%). The LDH activity of the total cell lysate was measured (referred to as LDH_{in}). Lactate Dehydrogenase Assay Kit LD-L20 (Sigma Chemical Co., St. Louis, MO) was used for the quantitative kinetic determination of LDH activity. This reagent contains 50 mM lactate plus 7 mM NAD^+ in a pH 8.9 buffer system. To determine the

LDH activity, 50-200 µl aliquots of cell tissue culture medium or of cell lysates were added to the LDH assay system, and the increase in absorbance at 340 nm due to NADH formation was recorded. The cytotoxicity index was expressed as the ratio of LDH_{out}/LDH_{in}.

GSH Assay

5 X 10^6 cells were subcultured into a 10 mm culture dish overnight before testing reagents was added. After designated period of incubation, the cells were harvested by scraping. Cells cultured in normal MEM were scraped and considered as the time zero sample. Cells were washed with PBS and resuspended in PBS and sonicated using a Heat Systems-Ultrasonics Model W-375 Sonicator™ (10s, duty cycle 25%, output control 40%). After protein assay, cell lysate equivalent to 2 mg protein was used to measure the content of intracellular GSH by the Glutathione Assay Kit (Calbiochem-Novabiochem Co., La Jolla, CA). Briefly, an initial sample volume of 200 µl was incubated with 50 µl of reagent R1 (solution of 0.12 M of a patented chromogenic reagent in 0.2 N HCl) and thoroughly mixed. 50 µl of solution R2 was added and thoroughly mixed, followed by incubation for 30 min at 37°C. The final absorbance at 400 nm was measured. Reduced glutathione was used to prepare a standard curve. The intracellular GSH value was standardized against the protein concentration of the mixture.

Lipid Peroxidation Assay

Malondialdehyde (MDA) and 4-hydroxyalkenals, 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 (Esterbauer, 1990). Lipid peroxidation in cells was monitored by measuring total MDA and 4-HNE production utilizing the lipid peroxidation assay kit, LPO-586 (Calbiochem-Novabiochem Co., La Jolla, CA). Briefly, 10 X 10^6 cells were subcultured into ten to thirty 10-mm-culture-dishes overnight before testing reagents were added. After the designated period of incubation with or without test reagent, the cells were harvested by scraping. Cells cultured in normal MEM were scraped and considered as the control. Cells were washed with PBS and resuspended in PBS and sonicated using a Heat Systems-Ultrasonics Model W-375 Sonicator™ (10s, duty cycle 25%, output control 40%). The pellets were resuspended in 20 mM Tris-HCl buffer, pH 7.4, lysed by sonication, and centrifuged at 5,000g X 5 min. The protein content of the cell lysates was determined with the DC-20 Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) followed by the LPO-586 assay.

ATP Assay

The intracellular ATP level was assayed using a kit (366-A) purchased from Sigma Chemical Co., St. Louis, MO. The measurement is based on the reactions catalyzed by the enzymes phosphoglyceric acid phosphokinase, which catalyzes conversion of 3-phosphoglycerate to 1,3-diphosphoglycerate by consuming ATP, and glyceraldehyde 3-phosphate dehydrogenase, which catalyzes reduction of 1,3-diphosphoglycerate to glyceraldehyde-3-phosphate. The overall reactions consume one ATP molecule and one NADH. The ATP concentration was estimated by the consumption of

NADH monitored as the decrease in absorbance at 340 nm using extinction coefficient of 6.22 $mM^{-1}cm^{-1}$.

Mitochondrial O_2 Consumption

An Oxygen Monitor (YSI Model 53, Yellow Springs International Co., Inc., Yellow Springs, Ohio) was used to measure the oxygen consumption with various substrates as described by Anja Krippner *et al.* (1996) with minor modification. Transduced Hep G2 cells were harvested by trypsinization, washed, and resuspended in respiration buffer (0.25 M sucrose, 0.1% bovine serum albumin, 10 mM $MgCl_2$, 10 mM HEPES, 5 mM KH_2PO_4, pH 7.2) at a final concentration of 3 X 10^7 cells/ml. One milliliter of the suspension was added into a chamber containing 2.0 ml of air-saturated respiration buffer plus 1mM ADP which was prewarmed to 37°C. The cells were permeabilized with digitonin added to a final concentration of 0.005%, and the sequential substrates and inhibitors were added in the following order and final concentrations: 5 mM malate + 5 mM pyruvate; 100 nM rotenone; 5 mM succina

RESULTS

Project I. Menadione Toxicity and NF-κB activation

Menadione Cytotoxicity to Hep G2 Cells

Oxidative stress generated by menadione semiquinone, superoxide, H_2O_2, •OH, and the elimination of GSH were considered as primary events involved in menadione cytotoxicity. 5 X 10^4 monolayer Hep G2 cells in each 24-well plate were used for menadione cytotoxicity assays, as measured with the MTT assay. The data were expressed as average values obtained from 3-5 wells. Under these conditions, most cells were killed after an overnight incubation in the presence of 25-50 μM menadione (Fig. 1). For most subsequent experiments, incubation times of 18 h and menadione concentrations of 15-20 μM were chosen as routine incubation conditions (the LD_{50} of an 18 hour incubation was about 18-19 μM). The cytotoxicity by menadione was also dependent on the cell number (data not show). Menadione cytotoxicity was validated by assays of LDH leakage and morphology. The cytotoxicity by menadione was prevented by addition of *N*-acetylcysteine (Fig. 2) or by iron chelators such as α,α-bipyridyl (data not shown). *N*-acetylcysteine may directly react with menadione or menadione-derived radicals.

Preincubation of Hep G2 Cells with Menadione Protects against Cytotoxicity of H_2O_2 or Menadione

Glucose oxidase/glucose was used to generate H_2O_2 in the culture medium

(which contains 1 mg/ml glucose) to avoid the rapid decomposition of H_2O_2 which occurs in the complete medium and to avoid addition of high bolus concentrations of the oxidant. Glucose plus glucose oxidase was also toxic to the Hep G2 cells in a dose and time dependent manner (data not shown). To study whether menadione was able to activate cell defense systems, Hep G2 cells were preincubated with lower doses of menadione (from 1 μM to 5 μM) which by themselves were not toxic. Preincubation of Hep G2 cells with these lower doses of menadione increased the viability of Hep G2 cells and protected them against a toxic dose of H_2O_2 (generated from glucose oxidase/glucose system) compared to cells incubated with culture medium lacking menadione (Fig. 3). The protective effect of preincubation with menadione was related to the concentration of menadione, with maximum protection occurring at 3-5 μM menadione (Fig. 5), and was dependent on the length of preincubation time with menadione, becoming maximal at 45 min (Fig. 6). Besides protecting against toxicity of H_2O_2, the pretreatment with menadione also resulted in protection against menadione toxicity (Fig. 4). Protection by pretreatment with menadione was also observed by utilizing the LDH release assay instead of the MTT assay to determine cytotoxicity (Fig. 7).

Menadione Activation of the Transcription Factor NF-κB in Hep G2 Cells

Oxidative stress generated by H_2O_2 can activate the transcription factor NF-κB (30-32). Since H_2O_2 is generated during the metabolism of menadione, it appeared reasonable to determine whether NF-κB was activated during or

after the preincubation with menadione. Activation of NF-κB was measured by an electrophoretic mobility shift assay. Pretreatment of Hep G2 cells with 3 μM menadione led to the activation of NF-κB as shown in Fig. 8, lane 3 compared to lane 1; lane 2 is from the nuclear extract prepared from Hep G2 cells incubated with 50 ng PMA, a known activator of NF-κB (33). When 20 times unlabeled oligonucleotide containing the consensus sequence of NF-κB binding sites was added to the EMSA reaction mixtures, the DNA binding and mobility shift ability of nuclear extracts from PMA or menadione activated Hep G2 cells was inhibited (Fig 8, lanes 5 and 6). However 20 times unlabeled oligonucleotide containing mutations in the NF-κB binding site did not affect the EMSA assay (Fig. 8, lanes 8 and 9). The activation of NF-κB by menadione was time-dependent and could be observed as early as 10 min after menadione addition (Fig. 9). This time period (10 min) was earlier than the time in which maximal protection against H_2O_2 toxicity by menadione pretreatment occurs (45 min). NF-κB contains p50 and p65 subunits; treatment of nuclear extracts with antibodies against p50 and p65 can result either in prevention of binding to the probe or in a "supershift" of the NF-κB–oligonucleotide complex. Incubation of the nuclear extract from menadione-treated Hep G2 cells with anti-p50 IgG (Santa Cruz Biotechnology, Santa Crus, CA) prevented binding to the oligonucleotide probe, whereas treatment with anti-p65 IgG (Upstate Biotechnology, Lake Placid, NY) resulted in a supershift of the complex (Fig. 10). Preimmune IgG had no effect. Salicylate inhibited the activation

of NF-κB in Jurkat cells (Kopp and Ghosh, 1994). We therefore studied whether salicylate could inhibit the activation of NF-κB by menadione in the Hep G2 cells. Indeed, when 10 mM salicylate was added together with menadione to the Hep G2 cells, the activation of NF-κB was inhibited (Fig. 11, lane 2 compared to lane 1).

Salicylate Potentiation of Menadione Cytotoxicity

As shown in Fig. 11, salicylate prevented activation of NF-κB by menadione in Hep G2 cells. If activation of NF-κB was important in the mechanism by which menadione protected Hep G2 cells against H_2O_2 or menadione cytotoxicity, it would be anticipated that salicylate would prevent the protection by menadione, and perhaps might even potentiate the cytotoxicity. Indeed, this proved to be the case; results in Fig. 12 show that in the absence of salicylate, menadione at a concentration of 10 μM was not toxic to Hep G2 cells. However, in the presence of salicylate (2.5-20 mM), menadione toxicity was clearly observed. A menadione dose-dependent curve of cytotoxicity is shown in Fig. 13; concentrations of 2.5-7.5 μM menadione were not toxic to the Hep G2 cells in the absence of salicylate, but striking toxicity was found in the presence of salicylate. Salicylate not only increased the toxicity of menadione, but also potentiated the toxicity of H_2O_2. A time course for the potentiation of 10 μM menadione and 100 μM H_2O_2 toxicity by salicylate is shown in Fig. 14. In other experiments, we observed that aspirin (acetyl salicylic acid) had the same actions as salicylate (data not shown). Salicylate, in the absence of menadione, was not toxic to

the Hep G2 cells. While salicylate may have other actions, e.g. antioxidant properties, such properties would be expected to decrease, rather than enhance, the toxicity of menadione.

Effect of Antioxidants on NF-κB Activation by Menadione and Protection against Cytotoxicity by Menadione Preincubation

It has been suggested that ROI act as an intermediate during the activation of NF-κB (Roederer *et al.*, 1990; Staal *et al.*, 1990; Schreck and Baeuerle, 1991). The effects of several antioxidants on NF-κB activation by menadione was evaluated. The antioxidants *N*-acetylcysteine, PDTC, thiourea, or uric acid were added during the pretreatment period with menadione and subsequently removed by washing the cells prior to the addition of a toxic concentration of menadione. As shown in Fig. 11, preincubation of Hep G2 cells with 3 μM menadione for 45 min, in the presence of *N*-acetylcysteine, PDTC, thiourea, or uric acid, did not lead to activation of NF-κB (Fig. 11, lanes 4 to 7 compared to lane 1). When Hep G2 cells were preincubated with 3 μM menadione in the presence of these antioxidants, the protective effect of menadione produced by this preincubation was no longer observed (Table I). Although antioxidants may have non-specific effects on cellular metabolism and viability, the results with four different antioxidants are suggestive that ROI derived from menadione metabolism may play a role in the activation of NF-κB by menadione and that when NF-κB activation is prevented by these antioxidants, there is a loss of the protective effect produced by menadione

pretreatment.

Depletion of PKC by PMA Eliminates Protective Effect of Menadione Preincubation

Prolonged treatment of cells with the active phorbol ester, PMA, is known to down-regulate protein kinase C (PKC) (Rodriguez-Pena *et al.*, 1984), and to inhibit the PKC-dependent NF-κB activation (Ghosh and Baltimore, 1990). Hep G2 cells were treated with medium or with 100 ng/ml PMA for 24 hours followed by a 45 min preincubation with 3 μM menadione or medium, and then exposure to 18 μM menadione. After treatment with PMA, preincubation with 3 μM menadione (a concentration that enables the cells to become more resistant to menadione and H_2O_2 cytotoxicity) did not increase the resistance of Hep G2 cells to the higher dose of menadione (Fig. 15). Analogous to the results with salicylate, Hep G2 cells became more sensitive to menadione after 24 hours of PMA treatment (Fig. 16). It is interesting to speculate that down-regulation of PKC eventually suppresses the activation of NF-κB by ROI generated from menadione metabolism, and thereby eliminates the possible protective effect which results from NF-κB activation. Short term treatment with PMA activates NF-κB (Fig. 8, lane2); this could lead to protection of the Hep G2 cells against a toxic concentration of menadione, analogous to menadione pretreatment. This proved to be the case as treatment of the cells with 25 ng/ml PMA for 25 min increased the resistance to menadione (Fig. 17).

Effect of Cycloheximide, a Protein Synthesis Inhibitor, on Protection of Menadione Cytotoxicity by Menadione Preincubation

Activation of NF-κB, a transcription factor, should result in activation of target genes, followed by synthesis of enzymes or factors which may play a role in protection against toxicity of H_2O_2 or menadione. We therefore evaluated whether protein synthesis was necessary for the protection against menadione cytotoxicity produced by the menadione pretreatment. When the protein synthesis inhibitor cycloheximide was present during the preincubation with menadione, a protective effect against menadione cytotoxicity was not observed (Table I). Since the immediate activation of NF-κB is not a protein synthesis-dependent event (14, 15), the presence of a protein synthesis inhibitor should not alter the activation of NF-κB. In the presence of menadione plus cycloheximide, NF-κB activation was observed to a comparable extent to that in the presence of menadione alone (Fig. 11, lane 3 compared to lane 1), most likely due to the fact that cycloheximide itself is an activator of NF-κB in certain cell lines, including the Hep G2 cells. The fact that cycloheximide blocks the protective effect of menadione preincubation, but does not prevent NF-κB activation suggests that certain proteins or factors may be synthesized during or after the preincubation with menadione, which produce the actual protection.

Effect of IκBβ on Menadione Toxicity

IκB binds to NF-κB, preventing its translocation into the nucleus, thereby preventing NF-κB modulation of transcription. To further implicate a role

for NF-κB in the protection afforded by preincubation with low, non-toxic concentrations of menadione, the Hep G2 cells were transfected with an expression vector containing mouse IκBβ cDNA. The level of IκBβ overexpression in Hep G2 cells is shown the Western blot of Fig. 18. Menadione toxicity was increased by the transfection with IκBβ about 2½-fold compared to control transfection with pCI plasmid (Fig. 19).

Intracellular GSH Level after Menadione Preincubation

GSH is a tripeptide whose nucleophilic and reducing properties play a central role in metabolic pathways as well as in the antioxidant system of most aerobic cells. Since *N*-acetylcysteine protected Hep G2 cells from menadione cytotoxicity (Fig. 2), GSH may play an important role in protecting against this cytotoxicity. Depletion of GSH could therefore enhance the menadione cytotoxicity. Treatment of the Hep G2 cells with 0.1 mM BSO overnight, a condition that depletes the cellular GSH, resulted in an increase in the menadione cytotoxicity (Fig. 20). To evaluate possible factors responsible for the protective effect induced by menadione preincubation, the Hep G2 cells were treated with 3 μM menadione or medium for 45 min followed by removal of the menadione and continued incubation in normal medium. As shown in Fig. 21, the GSH level of the cells which were pretreated with normal medium was unchanged for 8 hours after the medium change. However, the Hep G2 cells pretreated with 3 μM menadione showed an increased intracellular GSH level during incubation in the absence of menadione. When 10 mM sodium salicylate (which by itself

had no effect on the GSH level) was present together with 3 μM menadione during the preincubation, an increased GSH level was not observed. This links the increased GSH level to a salicylate-sensitive reaction, suggesting a possible role for NF-κB activation in the pathway leading to the elevated levels of GSH and to protection against menadione cytotoxicity.

Project II. Arachidonic Acid Induced Cytotoxicity and Apoptosis in Hep G2 Cells Expressing CYP2E1

Arachidonic Acid Cytotoxicity in Hep G2 Cells Expressing CYP2E1

It has been show 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, E9 and MV5 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 caused 43-72% (mean of 62%) loss of viability to E9 cells, whereas no significant loss of viability (0-13%, mean of 4%) was found with MV5 cells. Compared to arachidonic acid, oleic acid (18:1) showed no significant toxicity to the E9 cells even at concentrations (0.05 mM) in which arachidonic acid was highly cytotoxic (Table II). Arachidonic acid toxicity was also evaluated by morphology and by the LDH leakage assay. As shown in Fig. 22, arachidonic acid caused a 3-fold increase of LDH leakage (in terms of LDH_{out}/LDH_{in} ratio) with E9 cells 24 h after removal of arachidonic acid. Only a small increase (30%) in LDH leakage was found with MV5 cells. In contrast to arachidonic acid, preloading cells with oleic acid did not result in increased LDH leakage by the E9 (and MV5) cells (Fig. 22). Arachidonic acid caused substantial morphological changes when added to the E9 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. 23). No such changes in morphology were evident when arachidonic acid was added to the MV5 cells (Fig. 23).

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. 24. 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 E9 cells but not to MV5 cells. At a concentration of 0.05 mM, arachidonic acid caused more than 80% loss of viability of E9cells; some toxicity was also observed in MV5 cells although it was significantly lower than that in the E9 cells. As shown in Fig. 25, 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 MV5 cells over the same incubation period. At 36 h after preloading, viability of E9 cells was lowered by 73% by the arachidonic acid treatment, whereas viability of MV5 cells was decreased 26%.

Role of CYP2E1 in Arachidonic Acid Cytotoxicity

Inasmuch as the only apparent difference between E9 and MV5 cells is the expression of CYP2E1 in the former, it appears that the greater toxicity caused by arachidonic acid in E9 cells is due to the presence of CYP2E1 in

these cells. To validate the role of CYP2E1 in the elevated arachidonic acid cytotoxicity in E9 cells, a plasmid, pCI-as-2E1, containing cDNA encoding antisense CYP2E1 was transfected into E9 cells to block CYP2E1 production. Alternatively, a plasmid (pCI-2E1) containing human CYP2E1 cDNA was used to enrich transiently the CYP2E1 content of the E9 cells. Western blot analyses of the CYP2E1 content after transfection with the CYP2E1 sense and antisense plasmid indicated that the expression of CYP2E1 was decreased by about 80% with pCI-as-2E1 as compared to control transfection with pCI, whereas expression of CYP2E1 was elevated about 3-fold after transfection with pCI-2E1 (Fig. 31, 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 E9 (Fig. 32 pCI curve, compared to Fig. 24 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. 32) was similar to the toxicity curve found for the MV5 cells (Fig. 24, 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 to the control pCI transfection (Fig. 32). Thus, arachidonic acid toxicity is dependent upon CYP2E1 expression under these reaction conditions and at these concentrations of arachidonic acid.

Fatty acids can be metabolized by cytochrome P450 (Sharma *et al.*, 1988; Dirven *et al.*, 1991;Castle *et al.*, 1995; Guengrich *et al.*, 1995) CYP2E1

catalyzes ω-1 hydroxylation of arachidonic acid to a variety of complex products (Capdevila *et al.*, 1992; Laethem *et al.*, 1993; Fukuda *et al.*, 1994). Since CYP2E1 is a "loosely-coupled" protein, i.e., it can generate ROI such as superoxide and H_2O_2 in the presence or absence of a metabolic substrate (Gorsky *et al.* 1984; Ekstrom and Ingelman-Sundberg, 1989). It was interesting to evaluate two possible mechanisms for CYP2E1 in promoting the toxicity of arachidonic acid; i.e. CYP2E1 directly oxidized arachidonic acid to reactive metabolites which produced the toxicity (equation 1) or CYP2E1 generated superoxide and H_2O_2 which then reacted with arachidonic acid to produce toxicity (equation 2). The latter possibility would not require direct oxidation of arachidonic acid by CYP2E1.

CPY2E1 + arachidonic acid → metabolites → Cytotoxicity (equation 1)

CYP2E1 + NADPH → Reactive oxygen Intermediates (equation 2)

↓ Arachidonic acid

Lipid peroxidation → Cytotoxicity

4-MP is a ligand of CYP2E1 and an effective inhibitor of CYP2E1-catalyzed oxidation of substrates (Feierman and Cederbaum, 1986). 4-MP was shown to completely prevent the toxicity of acetaminophen and ethanol to the E9 cells, indicating that the toxicity of ethanol or acetaminophen required metabolism of these agents by CYP2E1 (Dai and Cederbaum, 1995a; Wu and Cederbaum, 1996). 4-MP was added to the culture medium of E9 cells during both the 24 h loading period with arachidonic acid and 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. 33), however, this protection was much less than the complete protection afforded by 4-MP against toxicity of acetaminophen and ethanol (Dai and Cederbaum, 1995a; Wu and Cederbaum, 1996). As will be discussed below, other CYP2E1 ligands and competitive substrates such as Me_2SO or ethanol did not significantly protect the E9 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 E9 cells.

Enhanced Lipid Peroxidation Induced by Arachidonic Acid in E9 Cells

The mechanism for arachidonic acid toxicity suggested in equation 2 implicates a central role for lipid peroxidation in the toxicity. Lipid peroxidation of E9 and MV5 cells was assessed by measuring production of the lipid peroxidation end products MDA and 4-HNE. As shown in Figs. 26 and 27, arachidonic acid induced lipid peroxidation in E9 cells in a concentration-dependent manner; enhanced formation of MDA and 4-HNE was observed in both cell lysate (Fig. 26) and in the culture medium from the cells (Fig. 27). Arachidonic acid (up to 0.03 mM) caused little or no lipid peroxidation in MV5 cells. The significant difference in lipid peroxidation between the two subclones suggests that expression 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 produce by arachidonic acid.

Effect of Antioxidants on Arachidonic Acid Cytotoxicity

To further characterize 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 III, 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 E9 cells. Me_2SO (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 E9 is due to 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 III).

Role of GSH in Arachidonic Acid Toxicity to Hep G2 Cells

Treatment with 0.1 mM BSO for 24 h caused GSH depletion in both MV5 and E9 cells. 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 cause about a two to three-fold increase in toxicity by arachidonic acid in both Hep G2 cell lines (Fig. 24). However, the BSO treatment did not potentiate the toxicity of oleic acid to the E9 cells (Table II). Since GSH depletion potentiated the cytotoxicity of arachidonic acid, GSH status appears to be critical in protecting the cells against the oxidative stress initiated by arachidonic acid. We therefore

evaluated the GSH level before and after treating the E9 cells with the arachidonic acid. As shown in Fig. 28, the intracellular GSH level in the E9 cells was gradually increased after the addition of arachidonic acid. The increased GSH level reached a peak (40-120% higher compared to control) around 8 h after arachidonic acid addition. The increased intracellular GSH appeared to be due to increased synthesis of GSH, since when BSO was added together with arachidonic acid to the E9 cells, a much less pronounced increase in GSH was observed (data not shown).

Redox cycling quinones cause an activation of NF-κB and subsequent induction of anti-oxidative protein(s) or factor(s), such as GSH (Shi *et al.* 1994; Ochi, 1996; Chen and Cederbaum; 1997c). To test the hypothesis that the elevated GSH levels produced by arachidonic acid was an initial response to the CYP2E1 catalyzed oxidative stress, we examined the ability of antioxidants, such as trolox (a vitamin E derivative) and ascorbate, to modulate the elevation in GSH in the E9 system. As shown in Fig. 29, trolox and ascorbate, which had no effect on control GSH levels, strongly prevented the increase of GSH in E9 cells induced by arachidonic acid.

The ability of menadione to elevate GSH levels in Hep G2 cells was shown to correlate with activation of NF–κB and to be prevented by sodium salicylate (Project I; Chen and Cederbaum, 1997c). The increase in GSH produced by arachidonic acid treatment in E9 cells was strongly prevented by sodium salicylate as well (Fig. 29). If the increase in GSH levels is produced as an initial protective response to arachidonic acid, it would be

anticipated that by preventing this increase, the E9 cells would be more sensitive to cytotoxicity induced by arachidonic acid. Indeed, this proved to be the case as sodium salicylate, at a concentration which strongly prevented the increase in GSH produced by arachidonic acid, potentiated the cytotoxicity produced by arachidonic acid (Fig. 30). The increased cytotoxicity found in the presence of salicylate plus arachidonic acid could be partially prevented by trolox, suggesting that the toxicity is related to oxidative stress.

in situ DNA Nick End Labeling of E9 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 E9 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 (Harman *et al.*, 1979; Vadeckis and Brashaw, 1983; Caron-Leslie and Cidlowski, 1991; Compton, 1992). After 24 h of arachidonic acid preloading, E9 and MV5 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 E9 (mean, 0.41-1.02) and MV5 (mean, 0.5-0.74) cells

(Fig. 34). Arachidonic acid enhanced the FITC-labeling with both cell lines; however, the intensity of FITC-labeling in the E9 cells preincubated with 0.03 mM arachidonic acid (mean, 12.11-13.34) was significantly higher than that of MV5 (mean, 2.12-2.84). Results from several TUNEL experiments are summarized in Figs. 35 and 36; at an arachidonic acid concentration of 0.03 mM, the intensity of FITC-labeling was about five-fold greater with the E9cells compared to MV5 cells. A second incubation time of 8 h was chosen for these experiments since too many E9 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 E9 cells (and MV5 cells) was effectively inhibited by trolox (histogram in Fig. 34; quantitation in Fig. 36). These results suggest that enhanced lipid peroxidation caused by arachidonic acid preincubation induced apoptosis and cytotoxicity in E9 cells.

Apoptosis in E9 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 E9 cells is apoptotic in nature. To study this further, DNA fragmentation within E9 cells was determined. E9 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. 37, lanes 3 to 5, 7 to 9, 11 to 13 compared to lanes 2, 6, 10). However, 8 h after preloading, 0.03 and 0.04 mM arachidonic acid caused DNA fragmentation in the E9 cells (Fig. 37, lanes 16 and 17 compared to lane 14 - no arachidonic acid added). Eight hours after the initial 24-h preloading with 0.03 mM arachidonic acid, MV5 cells did not show a significant DNA ladder (Fig. 38, lane 6, compared to E9 cells shown in lane 7). The DNA fragmentation in E9 cells was completely blocked by 0.1 mM trolox (Fig. 38, lanes 8 and 9, compared to lane 7).

bcl-2 Protects E9 Cells against Arachidonic Acid Toxicity

bcl-2 has been shown to be protective against apoptosis in several reaction systems (35-43). E9 cells contained a low level of *bcl-2*, as shown by Western blot analysis (Fig. 31, pCI-*neo* lane). To determine the effect of *bcl-2* on the arachidonic acid toxicity, we transfected E9 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 E9 cells produced a much higher level of *bcl-2* (Fig. 31, *bcl-2* lane) compared to the pCI transfected cells (Fig. 31, pCI-*neo* lane). After 24 h of arachidonic acid (0.02-0.04 mM) preloading and 24 h of additional incubation, pCI transfected E9 cells displayed similar cytotoxicity (20-50%) as did the non-transfected E9 cells (Fig. 39, pCI-*neo* curve, compared to Fig. 24, E9 minus BSO curve). Under the same conditions, pCI-bcl-2 transfectants showed only marginal toxicity (less than 10%) by arachidonic acid. pCI-as-bcl-2

transfected E9 cells showed a somewhat greater toxicity compared to pCI transfectants (Fig. 39); very little *bcl-2* was detected in the cells after transfection with the antisense plasmid (Fig. 31, as *bcl-2* lane). These results suggest that *bcl-2* modifies the sensitivity of E9 cells to arachidonic acid. Fig. 31 shows that CYP2E1 levels were similar in the cells transfected with plasmids pCI, pCI-bcl-2, and pCI-as-bcl-2.

Project III. CYP2E1 Toxicity on Hep G2 Cells

Establishment of Hep G2-CI2E1-47 (E47) and Hep G2-CI2E1-43 (E43) Cell Lines Overexpressing Human CYP2E1

Hep G2-CI2E1-47 (E47) and Hep G2-CI2E1-43 (E43) clones were selected from Hep G2 cells transfected with a pCI-2E1 plasmid. After G418 selection, three-time limited dilution screening, and a half-year of continuous tissue culture, the two clones appear to be stable with respect to expression of CYP2E1 and microsomal PNP oxidation activity. Western blot analysis of cell extracts from E47 or E43 cells showed a clear band at a molecular weight of about 54 kDa, which is identical to the band generated by human liver microsomes (data not shown) and to cell lysate from a previously established E9 subclone (Fig. 40). Expression of CYP2E1 in the new subclones was about 10-fold higher than that found with previously established E9 subclone (quantified by densitomytry of Fig. 40). The constitutive expression of CYP2E1 in E47 and E43 cells is promoted by the human cytomegalovirus (CMV) immediate-early enhancer/promoter (CMV enhancer/promoter). For the purpose of comparative study, we also established two control cell clones, Hep G2-CI-34 (C34) and Hep G2-CI-37 (C37), through pCI-*neo* plasmid transfection. The C34 and C37 cells, similar to the parental Hep G2 cells, do not express detectable CYP2E1 (Fig. 40).

Enzymatic activities of E47, E43, C34, and C37 cells were determined by the ability of their microsomes to carry out a typical CYP2E1-dependent reaction, *p*-nitrophenol oxidation. As shown in Table IV, the average PNP

oxidation activities of the microsomes prepared from E47, E43, C34, and C37 cells were 343, 186, 5, and 3 pmol/min/mg microsomal protein, respectively. Although there is considerable variability in the CYP2E1 content of human liver microsomes, the PNP oxidation activities of E47 and E43 microsomes are lower, but within a reasonable range of that found with human liver microsomes (0.4 - 1 nmol/min/mg microsomal protein). However, the PNP activity by microsomes from the E47 and E43 cells was 4 to 8-fold greater than that by microsomes from the previously established Hep G2-MV2E1-9 cells (40-60 pmol/min/mg microsomal protein) (Dai *et al.*, 1993).

Growth Inhibition Effect of CYP2E1 on Hep G2 cells

The expression of CYP2E1 caused an apparent decrease in the growth curve for the Hep G2 cells. As shown in Fig. 43, the C34 and C37 cells grow at a similar rate as the parental Hep G2 cells, whereas the increase in cell numbers with time are lower with the E47 and E43 cells. The doubling-time for E47 or E43 cells is about 30 or 28 hr, respectively, which is longer than control C34 or C37 cells (21 hr) or normal Hep G2 cells (20 hr) (Table IV). However, cell morphology of E47 or E43 cells appears to be normal and similar to C34 or C37 cells, or parental Hep G2 cells (Fig. 44). In addition, no significant LDH leakage was observed with E47 cells (Fig. 45). Thus the overexpression of CYP2E1 appears to decrease cell growth, but the cells remain viable.

To validate that the decrease in cell growth is a reflection of the expression of CYP2E1 rather than clonal variation, the effect of transfecting Hep G2 cells with plasmid containing CYP2E1 cDNA (pCI-2E1) and control plasmid (pCI-*neo*) was determined. The growth rate of the Hep G2 cells transfected with pCI-2E1 plasmid was slower than the cells transfected with pCI-*neo*. For example, after plating the same number of cells, the number of cells after 7 days was 4.5 X 10^6 for the pCI-*neo* transfectant, and 1.37 X 10^6 for the pCI-2E1 transfectant; after 12 days, the number of cells was 2.44 X 10^7 for pCI-*neo* transfectant, and 3.3 X 10^6 for the pCI-2E1 transfectant.

GSH Level in E47 and C34 Cells

GSH is among the most important intracellular antioxidants. ROI generated from CYP2E1 or other sources are removed either by direct reaction with GSH or by the glutathione peroxidase reaction. The intracellular GSH level is effectively maintained by recycling GSSG back to GSH and by *de novo* synthesis. BSO is an effective inhibitor of γ-glutamylcysteine synthetase, the rate-limiting enzyme for glutathione synthesis. The intracellular GSH level of E47 and C34 cells with or without BSO treatment was evaluated. In the absence of BSO treatment, the E47 cells had a slightly higher level of GSH than the Hep G2 cells or C34 cells (Fig. 46). As expected, BSO-treatment resulted in decreasing levels of GSH. However, BSO-treatment caused an accelerated decline of intracellular GSH in E47 cells as compared to C34 cells (Fig. 46). This may be due to increased ROI formation in the E47 cells.

Cytotoxicity and Apoptosis in Hep G2 Cells Expressing CYP2E1

As discussed above, the E47 and E43 cells appear fully viable in the absence of BSO treatment. However, after treatment with BSO for two days, substantial morphological changes of E47 cells were observed (Fig. 44). Many E47 cells were detached and floated to the top of the culture medium, cells were shrunken and dispersed and a monolayer was not formed. No such changes in morphology were evident when BSO was added to culture medium of C34 cells (Fig. 44). Moreover, LDH leakage was observed after BSO-treatment of the E47 cells, but not after adding BSO to the C34 cells (Fig. 45) or Hep G2 cells (data not shown). The cytotoxicity in the E47 cells caused by CYP2E1 was quantified with an MTT assay. As shown in Fig. 47, about 40-50% of the Hep G2 cells expressing CYP2E1 (E47 and E43) died after two days of BSO treatment, whereas no loss in viability was observed with the C34 or C37 cells or Hep G2 cells. A time course for the decrease in MTT reduction is shown in Fig. 48. Loss of cell viability upon BSO treatment of the E47 cells was evident after two days of culture, and became much more pronounced with increasing time of culture. A lower MTT reading (A_{570}-A_{630}) was also observed for the E47 cells in the absence of BSO, as compared to the C34 cells with or without BSO treatment (Fig. 48); this is probably a reflection of the decrease in cell growth of the E47 cells rather than a significant loss of viability, since cell morphology was intact and LDH leakage was minimal (Figs. 44 and 45). The effect of BSO treatment on viability of Hep G2 cells transiently transfected with vector containing CYP2E1 cDNA or control vector pCI-*neo* was determined with

the MTT assay. There was a 3-fold decrease (percentage-wise) in cell viability when Hep G2 cells were transfected with pCI-2E1 compared to pCI-as-2E1 (Fig 49). These results suggested that when GSH levels are lowered in the CYP2E1-expressing cells, there is a dramatic loss of cell viability, whereas no such loss in viability occurs in the control cells not expressing CYP2E1.

The extent of DNA fragmentation, a biochemical hall mark of apoptotic cell death, was determined to evaluate the morphological observation of apoptosis in Hep G2 subclones (Chen *et al.*, 1997). Induction of apoptosis in Hep G2 cells expressing CYP2E1 treated for 2 days with 0.1 mM BSO was analyzed by an agarose gel electrophoresis assay. 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. As shown in Fig. 50, a "DNA ladder" composed of DNA fragments at an interval of 200 bp was produced by the DNA purified from BSO-treated E47 cells; C34 cells with or without BSO treatment, and E47 cells not treated with BSO, did not produce a DNA ladder. The cytotoxicity induced in E47 cells appears to be apoptotic, and is only induced after GSH was depleted.

Role of CYP2E1 in Growth Inhibition and Cytotoxicity of E47 Cells

The only apparent difference between E47 and C34 cells, as well as between pCI-2E1 and pCI transfectants, is the expression of CYP2E1 in the former. It appears that the growth inhibition effect and cytotoxicity or apoptosis

(after BSO-treatment) in Hep G2 cells containing CYP2E1 is due to the presence of CYP2E1 in these cells. To validate this hypothesis, a plasmid, pCI-as-2E1, containing cDNA encoding antisense CYP2E1 was transfected into E47 cells to inhibit CYP2E1 production. Western blot analyses of the CYP2E1 content after transfection with the pCI-as-2E1 plasmid indicated that the expression of CYP2E1 was decreased about 70% with pCI-as-2E1 as compared to control transfection with pCI-*neo* plasmid (Fig. 41). pCI-as-2E1 transfected E47 cells grew faster than pCI-*neo* transfected cells as measured by cell counting 7 days after transfection (Fig. 51). Viability in the cells transfected with pCI-as-2E1 plasmid and treated with BSO for two days was higher (about 65% viable) than that in cells transfected with the control vector pCI-*neo* (less than 25% viable) (Fig. 52). Thus, the observed growth inhibition effect in the absence of BSO treatment and toxicity after BSO-treatment in Hep G2 cells are dependent upon CYP2E1 expression under these experimental conditions.

4-MP and Me_2SO are ligands for CYP2E1, and have been shown to stablize CYP2E1 in rat hepatocyte cultures (Feierman and Cederbaum, 1986) and in the Hep G2 cells (Carroccio *et al.*, 1994). After culture in 4-MP or DMSO containing medium for 2 days, E47 cells contain an increased level of CYP2E1 compared to E47 cells cultured in normal MEM (Fig. 42). Cells cultured in MEM plus 4-MP or Me_2SO showed an enhanced loss of viability after treatment with BSO as compared to E47 cells, suggesting a correlation between the expression level of CYP2E1 and cytotoxicity (Fig. 53).

Evidence of Lipid Peroxidation in HepG2 Cells Expressing CYP2E1

One consequence of ROI formation may be lipid peroxidation. Lipid peroxidation of E47 and C34 cells was assessed by measuring production of lipid peroxidation end products, MDA and 4-HNE. As shown in Table V, there is no detectable lipid peroxidation in C34 cells; some lipid peroxidation could be observed in the E47 cells during the regular cell culture. BSO-treatment did not induce lipid peroxidation in C34 cells. However, lipid peroxidation was elevated in the E47 cells after BSO-treatment for 2 days. The significant difference in lipid peroxidation between the two cell subclones suggests that overexpression of CYP2E1 caused lipid peroxidation, possibly through the CYP2E1-induced generation of ROI. Subsequent studies were carried out to evaluate whether the enhanced lipid peroxidation was responsible for the cytotoxicity found when GSH was depleted from the E47 cells.

Effect of Antioxidants on CYP2E1 Toxicity

To further characterize the nature of the CYP2E1 cytotoxicity, several antioxidants were added to the culture medium and their effect on the cytotoxicity produced upon GSH depletion was determined. As shown in Fig. 54, Vitamin E, Trolox, and Vitamin C were protective against CYP2E1 cytotoxicity to the E47 cells. Vitamin E also blocked the DNA fragmentation induced in E47 cells upon GSH depletion (Fig. 50) and prevented the enhanced lipid peroxidation (Table V). Furthermore, these antioxidants also moderately increased the growth rate of E47 cells (data not shown). These results suggest that the CYP2E1 induced growth inhibition

and apoptotic cytotoxicity are related to lipid peroxidation and development of a state of oxidative stress.

bcl-2 Protects Hep G2 Cells against CYP2E1 Toxicity

Hep G2 cells contain a low level of bcl-2, as shown by Western blot analysis (Fig. 55). To determine the effect of *bcl-2* on the CYP2E1 toxicity, two Hep G2 subclones, Hep G2-CIBcl-27 (B27) and Hep G2-CIBcl-28 (B28), were established, after transfection of Hep G2 cells with the expression vector pCI-bcl-2, which contains a full length human *bcl-2* cDNA. Two other Hep G2 subclones, Hep G2-CIA-14 (A14) and Hep G2-CIA-15 (A15), were obtained from the transfection with vector pCI-as-bcl-2 which contains an antisense *bcl-2* cDNA. As shown in Fig. 55, Hep G2 and C34 cell lysates produced very little endogenous Bcl-2 protein; B27 and B28 cells produced a significantly higher level of Bcl-2; while A14 and A15 cells Bcl-2 expression was not detected.

To study the effect of *bcl-2* on CYP2E1 toxicity, B28, A14, and C34 cells were transfected with the same amount (1 μg plasmid DNA/1 X 10^5 cells) of pCI-2E1 plasmid. Four days after transfection, B28-CYP2E1, A14-CYP2E1, and C34-CYP2E1 transfectants were subjected to BSO treatment. As shown in Fig 57, B28-CYP2E1 cells were relatively resistant to BSO-treatment; C34-CYP2E1 cells showed a similar loss of cell viability as previously found with E47 and E43 cells; A14-CYP2E1 cells were the most sensitive to BSO-treatment. On an agarose gel electrophoresis experiment, B28 transfectant did not produce a "DNA ladder" as observed in C34 and A14 transfectants

(Fig 58), or E47 cells (Fig. 50). As shown in Fig. 56, the expression levels of CYP2E1 in the three transfectants were similar. Thus, the apparent difference among the three transfectants, which explains the varying loss of viability, was the Bcl-2 level. The transfection experiment suggests that Bcl-2 is protective against the CYP2E1 cytotoxicity, which is consistent with the observation that CYP2E1 cytotoxicity is apoptotic in nature.

Intracellular ATP Concentrations of Hep G2 cells

The observed growth inhibition effect of CYP2E1 to the Hep G2 cells does not seem to reflect a loss of cell viability due to ROI-induced-damage. Normally cultured E47 cells appear to be morphologically similar to either Hep G2 or C34 cells, LDH leakage is not observed, and a low level of lipid peroxidation was observed in E47 cells in the absence of 0.1 mM BSO. We explored other possible mechanisms to explain the slow growth. ATP serves as an essential energy source for most intracellular synthetic reactions, and is necessary for cellular repair of damaged macromolecules, and to maintain adequate levels of GSH. Decreased levels of ATP could contribute to the slow growth rate of the Hep G2 cells overexpressing CYP2E1. Indeed, the ATP level in E47 cells (0.48 ± 0.01 μmol per 1 X 10^6 cells) was about 30% lower than that in C34 cells (0.70 ± 0.02 μmol per 1 X 10^6 cells).

Mitochondrial Damage in Hep G2 Cells Overexpressing CYP2E1

The lower content of ATP in the E47 cells may reflect antioxidative reactions, which consume ATP, and/or a decreased rate of production of ATP by mitochondria. Mitochondrial electron flow is transported through

four multisubunit complexes, designated Complex I to IV, which reside in the inner mitochondrial membrane. Complex I and II accept electrons from NADH and succinate, respectively, then pass the electrons to Complex III via ubiquinone, and then to Complex IV mediated by cytochrome *c*. The respiratory function of the mitochondria from C34 or E47 cells was assayed by measuring oxygen consumption after the sequential addition of substrates, which are specific for each electron transport pathway, to the respiration buffer containing cells treated with 0.005% digitonin, a condition which permeabilizes the cellular membrane and mitochondrial outer membrane. As shown in Table VI, the oxygen consumption rate with pyruvate-malate, which produces NADH and donates electrons to Complex I is significantly lower with mitochondria of E47 cells as compared to mitochondria of the C34 cells. However oxygen consumption with succinate or ascorbate-TMPD, which donate electrons through Complex II or Complex IV, respectively, is the same with mitochondria from E47 cells and C34 cells. After BSO-treatment, the oxygen consumption rates with substrates donating electrons through all three complexes are lower with E47 mitochondria compared to C34 mitochondria (BSO did not change the oxygen consumption rate of C34 mitochondria).

Vitamin E, which prevented lipid peroxidation and apoptosis in the BSO-treated E47 cells, completely protected against the damage to Complex I in normal E47 cells and all three complexes in BSO-treated E47 cells (Table VI). These results suggest that ROI generated by CYP2E1 caused damage in

E47 cells, and that the presence of GSH limited this damage mainly to Complex I.

DISCUSSION

Adaptive responses to the oxidative stress of H_2O_2 in yeast *Saccharomyces cerevisciae* strain RZ53 could increase the viability against a higher dose of H_2O_2 (Davies *et al.*, 1995). It has been previously shown that preincubation of mammalian cells with low doses of H_2O_2 or L-DOPA could protect these cells from the cytotoxicity of higher doses of H_2O_2 or t-butyl-hydroperoxide (Wiese *et al.*, 1995; Han *et al.*, 1996). However the mechanism for this protection is still unknown. In **Project I**, we evaluated a possible protective effect of preincubation of lower, non-toxic, doses of menadione on oxidative injury produced by toxic doses of menadione and H_2O_2. A major goal was to investigate the possible role of NF-κB activation in the protection mechanism produced by menadione preincubation.

Preincubation of Hep G2 cells with a low dose of menadione (3 μM) was found to protect these cells from oxidative injury caused by the subsequent addition of a higher toxic dose of menadione or H_2O_2 generated by the glucose oxidase - glucose system. When anti-oxidants, such as *N*-acetylcysteine, pyrrolidine dithiocarbamate, thiourea, or uric acid, were present together with menadione during the preincubation period, no protective effect by menadione is observed. This suggests that it is not menadione itself, but rather the production of reactive oxygen intermediates generated by the metabolism of menadione which is responsible for the eventual protective effect. *N*-acetylcysteine, a nucleophile, reacts with a

variety of reactive species and helps to maintain cellular GSH levels. Thiourea and uric acid react with hydroxyl radical-like species. Pyrrolidine dithiocarbamate, a metal chelator, would prevent iron-catalyzed formation of potent oxidizing species.

Using the electrophoretic mobility shift assay, menadione was shown to activate NF-κB in Hep G2 cells. The NF-κB specific binding in EMSA was confirmed by adding excess unlabeled NF-κB oligonucleotide, which blocked the specific binding, and excess mutated NF-κB oligonucleotide, which did not block the specific binding, to the EMSA reactions, and by the ability of anti-p50 IgG to block binding to the oligonucleotide probe, while anti-p65 IgG produced a supershift of NF-κB-probe complex. These results suggest the presence of p50 and p65 subunits in the NF-κB complex activated by menadione. The activation of NF-κB by menadione may be related to the oxidative stress generated from the metabolism of menadione as the same anti-oxidants that blocked the protective effect produced by preincubation with menadione (Table I) also inhibited the activation of NF-κB by menadione. Concentrations of menadione which activated NF-κB were also effective concentrations for protection against cytotoxicity of H_2O_2 or menadione. Nevertheless, the activation of NF-κB by menadione was a relatively rapid response, evident as early as 10 minutes after treatment. The time needed to activate NF-κB by menadione was shorter than the menadione preincubation time when maximum protection was

observed. This suggests that formation of some protein(s) or factor(s) first needs to be induced before the cells achieve the ability to resist oxidative stress.

Sodium salicylate has been shown to inhibit the NF-κB activation in some cell lines (Kopp and Ghosh, 1994). Sodium salicylate inhibited NF-κB activation by menadione in Hep G2 cells. Associated with this prevention of NF-κB activation was a potentiation of the cytotoxicity of menadione and H_2O_2. Concentrations of menadione or H_2O_2 which were not toxic to the cells in the absence of salicylate were toxic in the presence of salicylate. This raises the possibility that by inhibiting NF-κB activation, salicylate eliminated the protective effect generated by menadione-induced low level oxidative stress. In a similar manner, overexpression of IκBβ in Hep G2 cells also increased the toxicity of menadione. Active phorbol esters such as PMA are known for their ability to activate protein kinase C which phosphorates IκB and then activates NF-κB. Prolonged culture in PMA containing medium down regulates protein kinase C activity (Rodriguez-Pena and Rozengurt, 1984), which results in inhibition of PKC-dependent NF-κB activation. When Hep G2 cells were maintained in culture medium containing 100 ng/ml of PMA for 24 hours, those cells became more sensitive to menadione than the control cells which were maintained in normal medium. Menadione preincubation failed to increase the viability of PMA-treated cells in the menadione cytotoxicity assay. However, short-term treatment with PMA, which activates protein kinase C and subsequently NF-

κB, protected against menadione cytotoxicity. The results with salicylate, IκBβ and PMA suggest a role of NF-κB in the protection by menadione preincubation.

NF-κB activation, which includes the release of IκB and translocation of NF-κB into the nucleus, is not sufficient to cause the protection observed after menadione pretreatment. Activation of NF-κB regulates the expression of certain genes which contain NF-κB binding sequences in their upstream regulation regions (Lenardo *et al.*, 1987; Atchison and Perry, 1987). It has been suggested that genes involved with oxidative stress might be induced after activation of NF-κB. Cycloheximide could also activate NF-κB in Hep G2 cells, but it did not increase the viability of these cells in response to menadione. When cycloheximide was present together with menadione during preincubation, there was no protective effect against menadione cytotoxicity. These data indicate that some protein(s) must be synthesized in response to the activation of NF-κB caused by the pretreatment with menadione and it is likely that such protein(s) are responsible for protecting the cells from oxidative injury.

Menadione preincubation was found to significantly increase the intracellular GSH level of Hep G2 cells. This increase may play an important role in the mechanism of the protective effect of menadione preincubation, especially since menadione cytotoxicity was elevated when GSH was depleted after treatment of the cells with buthionione sulfoximine. Shi *et al.*,

(1994), found an increased GSH concentration in bovine pulmonary artery endothelial cells after the cells were treated with 2,3-dimethoxy-1,4-naphthoquinone and menadione, while Ochi (1996) showed that menadione increased GSH levels in Chinese hamster V79 cells. The increase in GSH levels in Hep G2 cells by menadione pretreatment may be one consequence of the activation of NF-κB by menadione since salicylate inhibits the GSH increase induced by menadione treatment under conditions in which salicylate potentiates menadione cytotoxicity in the Hep G2 cells. Menadione is metabolized to ROI, which are toxic to the cells when produced in high amounts which overwhelm cellular defensive mechanisms. At lower concentration, menadione may induce cellular defense, partially by increasing GSH levels. This protective effect would be eliminated in the presence of salicylate if the protection is a NF-κB dependent event. The salicylate potentiation of menadione cytotoxicity is consistent with the suggestion that menadione protects the Hep G2 cells against higher menadione cytotoxicity through a NF-κB dependent pathway.

The NF-κB family is activated by a variety of stimuli, such as cytokines, viruses, UV light, as well as oxidative stress (reviewed by Beg and Baldwin, 1993; Gilmore and Morin, 1993). Based on the observation that induction of NF-κB is an early response to oxidative stress, the NF-κB signaling pathway appears to be a natural protective mechanism against injury. Certain proteins related to oxidative stress, such as Mn-SOD (18, 19), DT-diaphorase (20), and iNOS (21, 22), and Ferritin H (23) have been shown to

be induced after NF-κB activation. Results in the current study show that reactive oxygen intermediates generated from menadione metabolism can induce a protective mechanism against oxidative stress in Hep G2 cells. This protection mechanism appears to involve an NF-κB activation and may be due, in part, to elevation of cellular GSH levels.

The primary goal of **Project II** was to investigate the toxicity of arachidonic acid in a Hep G2 subclone in which the major or the only significant cytochrome P450 isoform is CYP2E1. Induction of CYP2E1 and the formation of reactive intermediates, including reactive metabolites, reactive oxygen intermediates, and lipid peroxidation derivatives, appear 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 isozymes, CYP2E1 displays high NADPH oxidase activity, is loosely coupled and is more reactive in oxidizing ethanol to the 1-hydroxyethyl radical (Gorsky *et al.*, 1984; Reinke *et al.*, 1987; Ekstrom and Ingelman-Sundberg, 1989; Albano *et al.*, 1991; Castillo *et al.*, 1992). 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 (Thurman, 1973; Boveris *et al.*, 1983; Ekstrom *et al.*, 1986; Dicker and Cederbaum, 1988; Puntarulo and Cederbaum, 1988; Ekstrom and Ingleman-Sundberg, 1989; Rashba-Step *et al.*, 1993;). Correlation between induction of CYP2E1, lipid peroxidation and ethanol-induced liver injury

have been reported with the continuous intragastric infusion model of ethanol feeding (French, 1992; Takahashi *et al.*, 1992; Moromoto, *et al.*, 1993). 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 (Nanji, 1993). In order 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 subclone E9, which was transduced with human CYP2E1 cDNA by using a retrovirus shuttle vector (Dai *et al.*, 1993). An advantage of the E9 model is the stable, constitutive expression of CYP2E1, in contrast to the rapid decline of the isoform in primary cultured hepatocytes. Experiments were carried out to evaluate: whether arachidonic acid, a representative PUFA, is more toxic to cells expressing CYP2E1 compared to 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.

E9 cells expressing CYP2E1 and MV5 cells which 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 E9 cells, while

significantly lower or no cytotoxicity was found in the control MV5 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 E9 cells. Three lines of experiments are supportive for a role for lipid peroxidation in the PUFA toxicity to E9 cells. Depletion of GSH by BSO treatment increased arachidonic acid toxicity to the E9 (and the MV5) cells. GSH is known to protect cells against oxidative stress and damage caused by lipid peroxidation (Monks and Lau, 1989; Meister, 1994). Formation of characteristic end products of lipid peroxidation, malondialdehyde and 4-hydroxy-2-nonenal, was strikingly elevated in the E9 cell extracts and in the culture medium from the E9 cells after addition of arachidonic acid, whereas only a small increase in these lipid aldehydes was found with MV5 cells. A variety of antioxidants which prevent lipid peroxidation including α-tocopherol phosphate, trolox, ascorbate, propylgallate, DPPD, and the iron chelator, desferrioxamine, were effective in preventing the toxicity by arachidonic acid to the Hep G2-MV2E1-9 cells. Me_2SO and ethanol, besides being substrates or ligands for CYP2E1, are also effective hydroxyl radical scavenging agents. These compounds afforded little protection against the arachidonic acid toxicity, suggesting that either hydroxyl radical-like species were not involved in the PUFA toxicity (e.g. hydroxyl radical scavengers do

not prevent microsomal lipid peroxidation (Aust *et al.*, 1985; Puntarulo and Cederbaum, 1986) or that secondary radicals produced from the interaction of Me_2SO (methyl radical) or ethanol (1-hydroxyethyl radical) were themselves toxic.

It is not likely that the enhanced PUFA toxicity to E9 cells is mediated via arachidonic acid metabolism to eicosaenoid products since there should be no difference in cyclooxgenase pathways between the E9 and the MV5 cells. In addition, aspirin did not significantly protect against the PUFA toxicity while transfection with a plasmid containing antisense CYP2E1 cDNA lowered the PUFA toxicity. The significant difference between the E9 and MV5 cells is the expression of CYP2E1 in the former and not in the latter.

Some toxicity by higher concentrations of arachidonic acid was also observed with the Hep G2-MV-5 cells, which 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. 24); b) small increases in malondialdehyde and 4-hydroxynonenal were produced upon incubating the MV5 cells with 0.03 mM arachidonic acid (Fig. 26 and 27); and c) the small increase in FITC labeling found when arachidonic acid was incubated with the MV5 cells, analogous to the large increase found with the CYP2E1-expressing cells, was prevented by the antioxidant trolox (Fig. 34, 35 and 36). Most likely, reactive oxygen species are being produced from other cellular sources than cytochrome P450 mixed function oxidase

activity, e.g. mitochondria may be the predominant source of reactive oxygen species under many conditions. There are numerous studies in the literature showing that enrichment of hepatocytes or tumor cells with arachidonic acid or other PUFAs results in lipid peroxidation and cellular toxicity which can be prevented by antioxidants such as vitamin E or DPPD (Canuto *et al.*, 1991, 1995; Mikkelsen *et al.*, 1993, 1994; Sugihara *et al.*, 1994; Furuno, 1996), analogous to the toxicity produced by high concentrations of arachidonic acid to the MV5 cells.

Human CYP2E1 has been shown to metabolize arachidonic acid to ω-1-hydroxy-arachidonic acid (Capdevila *et al.*, 1992; Laethem *et al.*, 1993; Fukuda *et al.*, 1994). A CYP2E1 inhibitor, 4-MP, did not prevent the toxicity of arachidonic acid at concentrations which prevented toxicity of ethanol, CCl_4, and acetaminophen (Dai and Cederbaum, 1995a; Wu and Cederbaum, 1996). CYP2E1 substrates, such as ethanol and Me_2SO also did not effectively inhibit the arachidonic acid toxicity (Table III). These results suggest that the direct metabolism of arachidonic acid to potentially toxic products by CYP2E1 does not contribute significantly to the PUFA toxicity. To validate the role of CYP2E1 in the enhanced PUFA toxicity to the E9 cells, transfection experiments with plasmids containing CYP2E1 cDNA in the sense and antisense orientations were carried out. Compared to transfection with the control plasmid, transfection with sense CYP2E1 cDNA increased arachidonic acid toxicity, while transfection with antisense CYP2E1 cDNA decreased PUFA toxicity to the level observed with MV5

cells. Isolated microsomes from E9 cells have been shown to produce superoxide radical and H_2O_2 at elevated rates compared to MV5 microsomes (Dai *et al.*, 1993). Low concentrations of ferric-ATP effectively catalyzed lipid peroxidation with E9 microsomes but not with MV5 microsomes (Dai *et al.*, 1993). Taken as a whole, these results suggest that elevated production of reactive oxygen intermediates due to the presence of CYP2E1 in E9 cells can result in the formation of potent oxidants which can initiate lipid peroxidation if sufficient levels of PUFA are available.

Since CYP2E1 is a "loosely-coupled" enzyme (Gorsky *et al.*, 1984; Ekstrom and Ingelman-Sundberg, 1989), formation of reactive oxygen intermediates occurs even in the absence of added substrates. In fact, formation of superoxide and H_2O_2 by microsomes from the E9 cells was not altered by the addition of substrates and ligands of CYP2E1, including ethanol and 4-MP (Dai *et al.*, 1993), which probably explains why these agents did not protect against arachidonic acid toxicity.

DNA fragmentation assessed by DNA agarose gel electrophoresis and the TUNEL method showed that the toxicity induced by arachidonic acid in the E9 cells involved apoptosis. Trolox, a vitamin E analog and a lipid peroxidation inhibitor, prevented E9 cells from apoptosis and cytotoxicity induced by arachidonic acid, suggesting that lipid peroxidation played a role in the developing apoptosis and in the cytotoxicity. Intracellular reactive oxygen species and elevated levels of lipid peroxidation have been implicated as being associated with apoptosis (Hockenbery *et al.*, 1993;

Busciglio and Yankner, 1995; Peled-Kamar *et al.*, 1995). Our results suggest that enrichment of the polyunsaturated fatty acid levels in biological membranes of E9 cells is critical for development of apoptosis induced by CYP2E1-dependent oxidative stress. *bcl-2* inhibits many types of apoptotic cell death, although the mechanism is not completely clear (Hochenbery, 1992; Merino *et al.*, 1994; Ryan *et al.*, 1994; Memon *et al.*, 1995; Reap *et al.*, 1995; Sinicrope *et al.*, 1995; Amstrong *et al.*, 1996; Jacobsen *et al.*, 1996; Okazawa *et al.*, 1996). Bcl-2 is localized to intracellular sites of reactive oxygen species generation including mitochondria, endoplasmic reticulum, and nuclear membranes (Hockenbery *et al.*, 1993; Korsmeyer dt al., 1993, 1995; Nguyen *et al.*, 1994). When E9 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. 31). Transfection with the *bcl-2* sense or antisense plasmid did not affect expression of CYP2E1 as compared to transfection with control plasmid (Fig. 31).

In summary, a series of experiments have been carried out to demonstrate that arachidonic acid is toxic to cells expressing CYP2E1 but not to cells not expressing CYP2E1. The PUFA toxicity is associated with increased lipid peroxidation and can be diminished by antioxidants which 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 to 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 preloaded with PUFA. These results indicate that enrichment of cells expressing CYP2E1 with PUFA results in cytotoxicity. The E9 model appears 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.

In addition to cytotoxicity of arachidonic acid, experiments were also carried out to show that arachidonic acid initially increased the GSH production, possibly through NF-κB activation, correlating to the study of menadione activation of NF-κB. CYP2E1 induces elevated production of ROI, which results in enhanced lipid peroxidation when arachidonic acid, a representative polyunsaturated fatty acid, is also present. Since cytotoxicity by arachidonic acid required a two-day treatment period, we evaluated whether the polyunsaturated fatty acid-induced oxidative stress might also trigger an initial self-defense response by the cells in order to protect against toxicity. Indeed, in the E9 cells, one important antioxidant, the GSH content, is up-regulated as an early response to the arachidonic acid-induced oxidative stress. Without such up-regulation of GSH production, the

intracellular GSH level should decline, since the increased oxidative stress after arachidonic acid addition would consume GSH. The increased level of GSH suggests that GSH synthesis may be elevated by addition of arachidonic acid to the E9 cells. Another possible mechanism for the increase could be an increased turnover of GSSG to GSH as catalyzed by glutathione reductase. The experiment with BSO suggests increased synthesis of GSH as the primary mechanism responsible for elevated GSH levels as BSO affects γ-glutamylcysteine synthetase, but not glutathione reductase; BSO effectively inhibited the increase in GSH induced by arachidonic acid. It is of interest that γ-glutamylcysteine synthetase has NF-κB responsive elements in its promoter (Yao *et al.*, 1995), and oxidative stress can result in activation of NF-κB (reviewed in 20, 21). Sodium salicylate, which inhibits NF-κB activation in a variety of systems (Kopp and Ghosh, 1994), did prevent the increase in GSH produced by arachidonic acid, suggesting that the increase in GSH produced by arachidonic acid involves activation of NF-κB.

The increase in GSH produced by arachidonic acid in the E9 cells appears to involve an initial response to the oxidative stress induced by the fatty acid in the CYP2E1-expressing cells. The elevation in GSH was prevented by antioxidants such as trolox and ascorbate. Preventing the increase in GSH with salicylate potentiated the toxicity of arachidonic acid, which further suggests that the elevation in GSH is an initial response by the cells for protection against the PUFA-induced toxicity. Since the arachidonic acid

toxicity requires two days to develop, it is likely that only when the initial adaptive response is overwhelmed by prolonged oxidative stress does cytotoxicity and apoptosis occur. It is important to note that preventing the increase in GSH does not always result in potentiation of toxicity, e.g. trolox and ascorbate effectively prevent the increase in GSH, however, unlike salicylate, these agents were fully protective against the arachidonic acid cytotoxicity and apoptosis. Clearly, several antioxidants are available to protect cells against oxidative stress.

The primary goal of **Project III** was to investigate the toxicity of CYP2E1 itself in the absence of added toxin in Hep G2 cells which have been transduced to express CYP2E1. Induction of CYP2E1, formation of ROI, elevation of lipid peroxidation, and cytotoxic damage are features observed in alcohol-induced hepatotoxicity, but direct linkage between these events has been different to evaluate. Previous studies have shown that agents, such as acetaminophen, ethanol, and CCl_4, are metabolized into toxic products which cause cytotoxicity, and this metabolism and toxicity is increased when CYP2E1 is induced (Nordmann *et al.*, 1992). In the intragastric infusion model of ethanol feeding, liver injury occurred when the rats consumed diets containing PUFA but not saturated fatty acid; injury was associated with striking elevation of CYP2E1 (Tsukamoto *et al.*, 1986, 1995; French, 1992; Takahashi *et al.*, 1992; Nanji *et al.*, 1993; Tsukamoto and French, 1993). In previous studies and Project II, significant cytotoxicity was observed when the E9 cells were treated with acetaminophen (Dai and Cederbaum, 1995) or ethanol (Wu and Cederbaum, 1996) or were preloaded

with a representative PUFA, arachidonic acid, but not a control fatty acid, oleic acid (Chen *et al.*, 1997). In these models, elevated lipid peroxidation was evident and appeared to correlate with CYP2E1 levels. Microsomes from the transduced Hep G2 cells expressing CYP2E1 produced higher amount of ROI compared to microsomes from the control cells which do not express detectable CYP2E1 (Dai *et al.*, 1993). However, there is no evidence of direct toxicity observed in these cells in the absence of added agents such as ethanol, acetaminophen, CCl_4, or PUFA. No toxicity was observed even when *de novo* GSH synthesis is inhibited; the only effect consistently found was a slightly slower rate of growth in long time culture. Possible explanations are that CYP2E1 itself is not directly toxic in the absence of added toxin which requires metabolism by CYP2E1, or the CYP2E1 expression level in the E9 cells is still relatively low. The significance of Project III is that the newly established Hep G2 subclones E47 and E43 express CYP2E1 at much higher levels compared to the E9 cells. The CYP2E1 content in E47 and E43 cells is about 10 times higher than that in the E9 cells as estimated by Western blot analysis, and microsomal PNP-oxidation activity of E47 or E43 cells is about 4 to 7 times higher that of microsomes from the E9 cells. Under these conditions of enhanced expression, direct effects of CYP2E1 on the rate of cell growth and cellular viability can readily be observed in the absence of any added agent.

The E47 and E43 cells grew at a slower rate than the control C37 or C34 cells, or parental Hep G2 cells, as the average doubling time for the

CYP2E1-expressing cells was about 28-30 h compared to the 20-21 h of doubling time for Hep G2 cells not expressing CYP2E1. Despite this slow rate of growth, the E47 or E43 cells remain viable as shown by morphology, lack of LDH leakage and by vital dye reduction. Maintenance of cellular viability in the presence of elevated CYP2E1 expression appears to reflect maintenance of cellular levels of GSH. Microsomes from the transduced Hep G2 cells expressing CYP2E1 produce more superoxide and H_2O_2 at elevated rates as compared to control microsomes even in the absence of CYP2E1 ligand or substrate (Dai *et al.*, 1993). This is an apparent reflection of the "loosely-coupled" nature of CYP2E1. We initially predicted a possible decline of GSH in the E47 cells, since GSH is a primary antioxidant to remove the ROI. However, the GSH level in E47 cells was not decreased compared to C34 cells or parental Hep G2 cells; indeed it was 5-15% higher than the controls. This suggests a possible up-regulation of GSH production and/or increased GSSG-GSH turnover. Oxidative stress generated by menadione (Project I) or arachidonic acid (Project II) initially increased GSH production, possibly due to the NF-κB activation. Since increased synthesis of GSH or turnover of GSH requires ATP, it is interesting to speculate that the slower rate of growth of the CYP2E1-expressing cells may reflect the increased demand for ATP for maintaining cellular GSH levels. Indeed, ATP levels were found to be about 30% lower in the CYP2E1-expressing cells.

If GSH is important in maintaining cellular viability, depletion of GSH would be expected to result in toxicity to the CYP2E1-expressing cells and not to the control cells. Depletion of GSH by treatment with BSO resulted in a striking loss of viability of the E47 cells, without any effect on control or parental Hep G2 cells. GSH levels declined more rapidly in the E47 cells after BSO addition than the control cells, clearly indicting that the CYP2E1-expressing cells are under elevated oxidative stress. Thus, the E47 model depicts two modes of CYP2E1 toxicity; a slower growth rate when cellular GSH levels are maintained, and a loss of cellular viability when cellular GSH levels are not maintained.

To evaluate a role for lipid peroxidation in these actions of CYP2E1, the production of MDA plus 4-HNE was determined. In the E47 cells, very low lipid peroxidation was found when GSH levels were maintained. After GSH depletion, an increased level of lipid peroxidation was found in the E47 cells, consistent with an elevated state of oxidative stress. No lipid peroxidation was observed in the C34 cells even after BSO treatment, consistent with the maintenance of GSH levels. The classical antioxidant and lipid peroxidation inhibitor, vitamin E, prevented the elevated lipid peroxidation (Table III), as well as cytotoxicity (Fig. 54) and apoptosis (Fig. 50). Other antioxidants such as ascorbate and trolox also prevent the CYP2E1-dependent cytotoxicity. These results suggest that elevated lipid peroxidation also plays an important role in the CYP2E1-dependent toxicity, and that enhanced lipid peroxidation can be observed even without

enrichment of the cellular membranes with PUFA when the content of CYP2E1 is high.

The role of CYP2E1 in growth inhibition and cytotoxicity is established by comparison between two CYP2E1 expression subclones (E47 and E43) and control cells (C34, C37 and parental Hep G2) (Fig. 47), and by comparison between Hep G2 cells transfected with vector containing CYP2E1 cDNA or control vector that does not contain CYP2E1 cDNA (Fig. 49). To provide further evidence for a direct role of CYP2E1, experiments were performed with a vector containing antisense CYP2E1 cDNA. In the pCI-as-2E1 transfected E47 cells, the CYP2E1 expression was 70% less than that of the original E47 cells. Viability of these cells in BSO-containing medium was about 3-fold higher than that of the E47 cells transfected with the control pCI-*neo* vector, and cell growth rate was about 2-fold faster. 4-MP and Me_2SO, which bind to and stabilize CYP2E1, increase the CYP2E1 content (Carroccio *et al.*, 1994). These compounds also increased the CYP2E1 content in the E47 cells, and the E47 cells treated with 4-MP or Me_2SO were more sensitive to the BSO treatment (Fig. 53). The role of ligands in CYP2E1 toxicity might be difficult to interpret, since besides increasing the content of CYP2E1 these compounds may cause other effects, e.g. change the rate of electron flow to CYP2E1 or become metabolized to reactive products. Me_2SO is a good scavenger of •OH, however, this should provide protection against toxicity and not exacerbation of the injury. Previous studies have shown that 4-MP, *in vitro*, did not alter microsomal production of superoxide and H_2O_2 (Dai *et al.*, 1993). Taken as a whole, these data are

consistent with the hypothesis that a higher level of CYP2E1 content is related to a greater extent of cytotoxicity.

Typical morphological changes associated with apoptosis, such as membrane blebbing, cytoplasmic shrinkage, as well as massive conversion into apoptotic bodies, were observed after treatment of E47 or E43 cells with BSO. DNA fragmentation assessed by DNA agarose gel electrophoresis and protection of cytotoxicity of CYP2E1 to Hep G2 cells by bcl-2 overexpression suggested that the toxicity induced by CYP2E1 in the Hep G2 cells involved apoptosis. Vitamin E prevented CYP2E1-dependent DNA fragmantation in Hep G2 cells, suggesting that lipid peroxidation played a role in the developing apoptosis and in the cytotoxicity. Intracellular ROI and elevated levels of lipid peroxidation have been implicated as being associated with apoptosis (Lin *et al.*, 1995; Nobel *et al.*, 1995; Tally *et al.*, 1995;Weltin *et al.*, 1996; Chen *et al.*, 1997). The elevated lipid peroxidation induced by CYP2E1 appears to be critical for the development of apoptosis. *bcl-2* inhibits many types of apoptotic cell death, although the mechanism is not completely clear (Hockenbery, 1992; Merino *et al.*, 1994; Ryan *et al.*, 1994; Memon *et al.*, 1995; Reap *et al.*, 1995; Sinicrope *et al.*, 1995; Amstrong *et al.*, 1996; Jacobsen *et al.*, 1996; Okazawa, 1996; Chen *et al.*, 1997). The B28 subclone, which overexpresses Bcl-2, was more resistant to the CYP2E1 toxicity, and in these cells apoptosis did not develop after 2 days of BSO treatment. Interestingly, in the A14 subclone, which does not express detectable Bcl-2, CYP2E1 transfection caused somewhat more toxicity compared to the C34 subclone, which expresses a low level of endogenous

Bcl-2. Meanwhile, levels of CYP2E1 in these cells were shown to be the same after transfection with same amount of CYP2E1 plasmid.

As discussed above, the growth inhibition effect of CYP2E1 to Hep G2 cells does not appear to relate to cytotoxicity. However, the ATP level in E47 subclone is about 35% lower compared to C34 subclone. The lower level of ATP may be a result of increased GSH production and GSH turnover related to the oxidative stress generated by CYP2E1. On the other hand, mitochondrial ATP production may also be altered. The mitochondrial complex I, which mediates the electron transfer from NADH, appeared to be affected as a consequence of the CYP2E1 expression. The oxygen consumption rate of E47 mitochondria with pyruvate/malate as substrate was about 35% lower than that of C34 mitochondria; complexes II, III, and IV did not appear to be damaged since oxygen consumption with succinate or ascorbate/TMPD was similar to that of the C34 subclone.

A very low level of lipid peroxidation was detected in the E47 cells in the absence of BSO treatment. Vitamin E completely restored the oxygen consumption rate with pyruvate/malate (Table VI), and also partially increased the growth rate of E47 cells. Therefore, it appears that the low level of lipid peroxidation which occurs in E47 cells is sufficient to couse specific damage to complex I of the mitochondrial respiratory chain; this damage probably contributes to the lower content of ATP, which subsequently decreases the growth rate of the cells. Maintaining intracellular GSH levels prevents widespread damage to the mitochondria. BSO

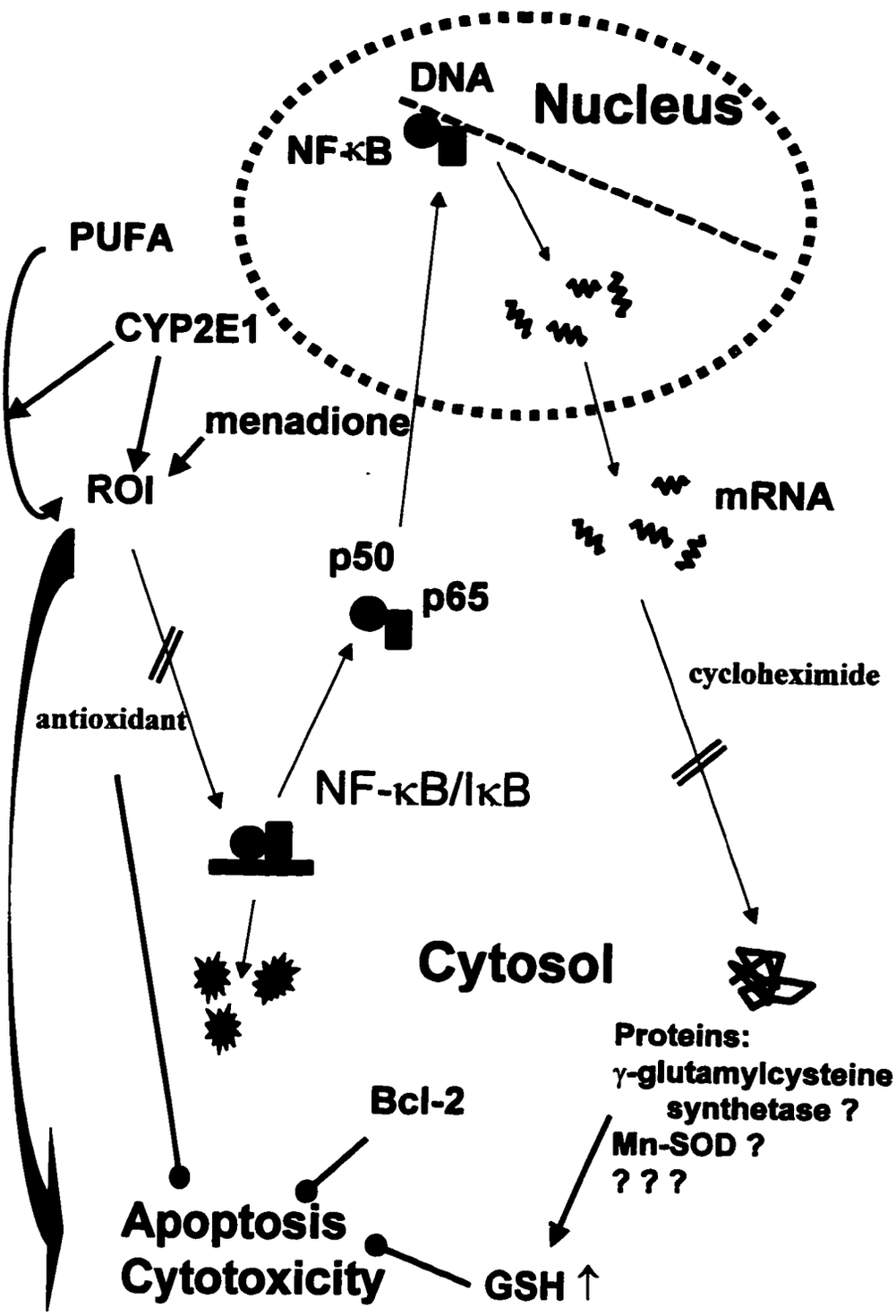
treatment caused a strong elevation in lipid peroxidation in E47 cells, and decreased oxygen consumption was observed with all substrates; the decrease in electron flow through all complexes was prevented by vitamin E. Why complex I is especially sensitive to CYP2E1-dependent lipid peroxidation, or whether there is a regulative effect on expression of complex I protein(s), is not known but could relate to the high concentration of iron-sulfur clusters. It is of interest that chronic ethanol consumption was previously shown to damage complex I (Cederbaum *et al.*, 1974).

In summary of Project III, experiments have been carried out which demonstrate a growth inhibition effect and a cytotoxic effect of CYP2E1 in transduced liver hepatoma cell lines. These effects occur in the absence of externally added toxin or agent and therefore are directly due to high levels of expression of CYP2E1 itself. The slow growth may be a result of mitochondrial damage, the need to maintain cellular GSH level, and lower level of intracellular ATP content. The cytotoxicity is apoptotic in nature, and is initiated by the depletion of GSH by CYP2E1-related oxidative stress and elevated lipid peroxidation. The direct toxicity of overexpressed CYP2E1 is probably a reflection of the ability of this isoform to produce ROI even in the absence of substrate. It would be anticipated that substrates, which are oxidized by CYP2E1 to reactive metabolites, e.g. ethanol, acetaminophen, and CCl4, or PUFA, which produces elevated lipid peroxidation in the presence of CYP2E1, would show strong toxicity in the E47 and E43 cells. Preliminary studies showed that these agents are indeed

strongly toxic in the E47 cells even without the necessity of depleting cellular GSH.

As summarized in Scheme IV, both CYP2E1 and menadione can induce oxidative stress and cause cytotoxicity to Hep G2 cells. Hep G2 cells respond to the oxidative stress initially by upregulation of one of their endogenous anti-oxidative systems, GSH. Increased GSH production is protective against ROI-toxicity. When oxidative stress is overwhelming, cytotoxicity and apoptosis start to develop in these cells.

Scheme IV



Tables

Table I

Effect of antioxidants on protective effect of menadione preincubation

Hep G2 cells were pretreated with either control MEM medium or 3 μM menadione for 45 min in the presence of indicated additions. Final concentrations of the additions were: cycloheximide 20 μg/ml; *N*-acetylcysteine 10 mM; pyrrolidine dithiocarbamate 50 μM; thiourea 5 mM; uric acid 0.5 mM. The cells were then exposed to 18 μM menadione for 18 h and viability was determined as described in Materials and Methods. Results refer to mean ± SD and are from 3 to 5 wells per experiment, and 3 separate experiments. Compared to control (-menadione), * $p < 0.0001$, ** $p > 0.05$.

	Percentage of Viable Cells	
Additions	- menadione	+ menadione
experiment I		
control	54 ± 3.8	85 ± 3.6 *
cycloheximide	44 ± 3.3	49 ± 4.5 **
N-acetylcysteine	55 ± 3.1	61 ± 4.5 **
experiment II		
control	28 ± 2.0	46 ± 2.7 *
PDTC	24 ± 2.0	28 ± 0.8 **
thiourea	26 ± 5.6	27 ± 5.1 **
uric acid	31 ± 6.6	26 ± 6.7 **

Table II

Comparison of the effect of arachidonic acid and oleic acid on the viability of E9 cells

E9 cells were incubated with 0.02 or 0.05 mM oleic acid or arachidonic acid for 24 h in the absence or presence of 0.1 mM BSO. The medium was removed and replaced by normal MEM medium and a second incubation for 24 h was carried out in the absence of fatty acid. Cell viability was then evaluated by the MTT assay. Compared to oleic acid pretreatment, * $p > 0.05$, ** $p < 0.001$.

	Percentage of Viable Cells	
Additions	- BSO	+ 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 **

Table III

Effect of Anti-oxidative Agents on the Cytotoxicity Produced by Arachidonic Acid to Hep G2-MV2E1-9 Cells

E9 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. Compared to %viability (40%) in the absense of additions, * $p > 0.05$, ** $p < 0.001$.

	Viability ($A_{570} - A_{630}$)		
Additions	-Arachidonic Acid	+Arachidonic Acid	%Viability
None	0.294 ± 0.013	0.117 ± 0.038	40
Me_2SO 20 mM	0.317 ± 0.025	0.158 ± 0.033	50 *
Ethanol 50 mM	0.334 ± 0.010	0.179 ± 0.035	54 *
DPPD 0.002 mM	0.277 ± 0.022	0.246 ± 0.021	89 **
Propylgallate 0.025 mM	0.308 ± 0.003	0.216 ± 0.005	70 **
Trolox 0.02 mM	0.332 ± 0.010	0.247 ± 0.004	75 **
VitE 0.02 mM	0.352 ± 0.020	0.305 ± 0.014	89 **
Ascorbic Acid 1 mM	0.277 ± 0.016	0.248 ± 0.014	90 **
Desferrioxamine 0.3 mM	0.218 ± 0.006	0.196 ± 0.008	90 **
Aspirin 1 mM	0.257 ± 0.004	0.132 ± 0.019	52 *

Table IV

Comparison of Hep G2 subclones

The oxidation of PNP by microsomes from parental Hep G2 cells and transduced Hep G2 subclones was determined as described under "Materials and Methods". The doubling time was estimated after 20 days of cell number counting using the formula: DT (h) = 20 X 24 / $\log_2$ [cell number]$_{20}$/[cell number]$_0$. DT refers to doubling time, and [cell number]$_{20}$ or [cell number]$_0$ refers to cell number at day 20 or day 0, respectively. Compared to control (parental Hep G2), * $p > 0.05$, ** $p < 0.01$.

Subclones	Oxidation of PNP (nmol/min/mg microsome)	Doubling Time (h)
Hep G2	0.005	20
C34	0.003 *	21 *
C37	0.005 *	21 *
E43	0.186 **	28 **
E47	0.343 **	30 **

Table V

Lipid peroxidation measurement

C34 and E47 cells were cultured in MEM with or without BSO or/and Vitamin E for two days. The lipid peroxidation levels in C34 and E47 cells were monitored by the production of lipid peroxidation end products, MDA and 4-HNE, as described under "Materials and Methods". Compared to C34 cell (without or with BSO respectively), * $p < 0.001$, ** $p > 0.1$.

Subclones	Additions	LPO (nmol 4-HNE+MDA/mg protein)
C34		0
C34	+ BSO	0
E47		0.031 *
E47	+ BSO	0.161 *
E47	+ VitE	0 **
E47	+ VitE & BSO	0.013 **

Table VI

Oxygen consumption rate of permeablized C34 and E47 cells

C34 and E47 cells were cultured in MEM with or without BSO and vitamin E for 2 days. Oxygen consumption was determined as described under "Materials and Methods". Numbers in parenthesis and *p*-values refer to the change in the rate of oxygen consumption by the E47 cells compared to the corresponding (-/+ BSO) C34 cells, * $p<0.01$, ** $p>0.05$, *** $p<0.05$.

		Oxygen consumption rates after substrate addition (n mole/million cells/min)		
Cells	Additions	Pyruvate-Malate	Succinate	Ascorbate/TMPD
C34		0.149	0.158	0.362
C34	BSO	0.186	0.178	0.347
E47		0.097(-35)*	0.152(-4)**	0.388(+7)**
E47	BSO	0.034(-82)*	0.029(-84)*	0.122(-65)*
E47	VitE	0.145(-3)**	0.156(-1)**	0.406(+12)**
E47	VitE+BSO	0.136(-27)***	0.162(-9)**	0.388(+12)**

Figures and Legends

Fig. 1

Menadione cytotoxicity to Hep G2 cells

5 X 10^4 cells/ml/well Hep G2 cells were plated onto a 24 well plate. Different concentrations of menadione were added to the culture medium for 18 h, followed by a viability assay (MTT assay) as described under "Materials and Methods".

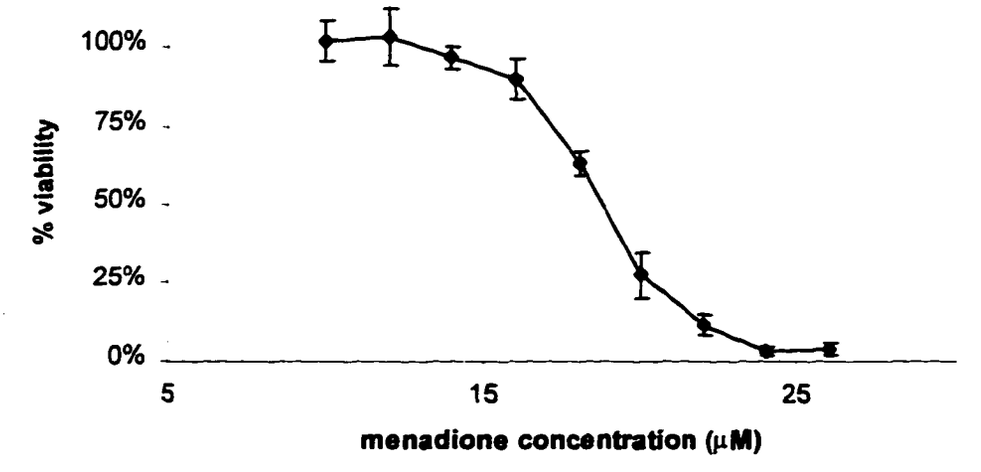


Fig. 2

Protection of menadione cytotoxicity by NAC

5 X 10^4 cells/ml/well Hep G2 cells were plated onto a 24 well plate. 5 mM NAC or control medium was added to the culture medium 15 min before 18 μM menadione. After 18 h of incubation, the percent viability was determined as described under "Materials and Methods". * $p < 0.001$

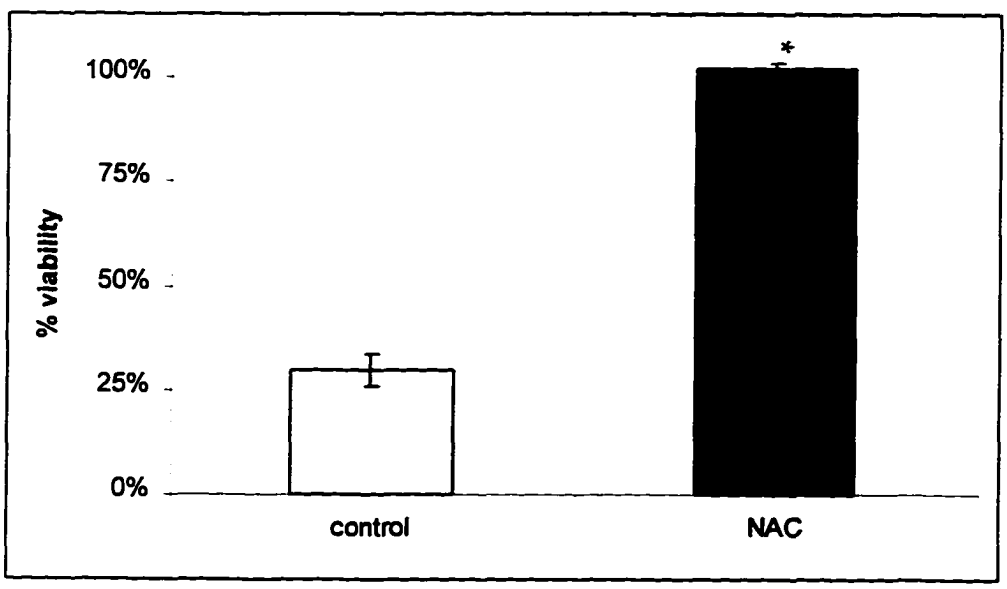


Fig. 3

Preincubation of Hep G2 cells with menadione protects against cytotoxicity of H_2O_2

Hep G2 cells were pretreated with control medium or 3 μM menadione for 45 min and then were exposed to H_2O_2 generated by 20, 25, 30 ng/ml glucose oxidase + 1 mg/ml glucose for 18 h followed by an MTT assay. * $p<0.001$.

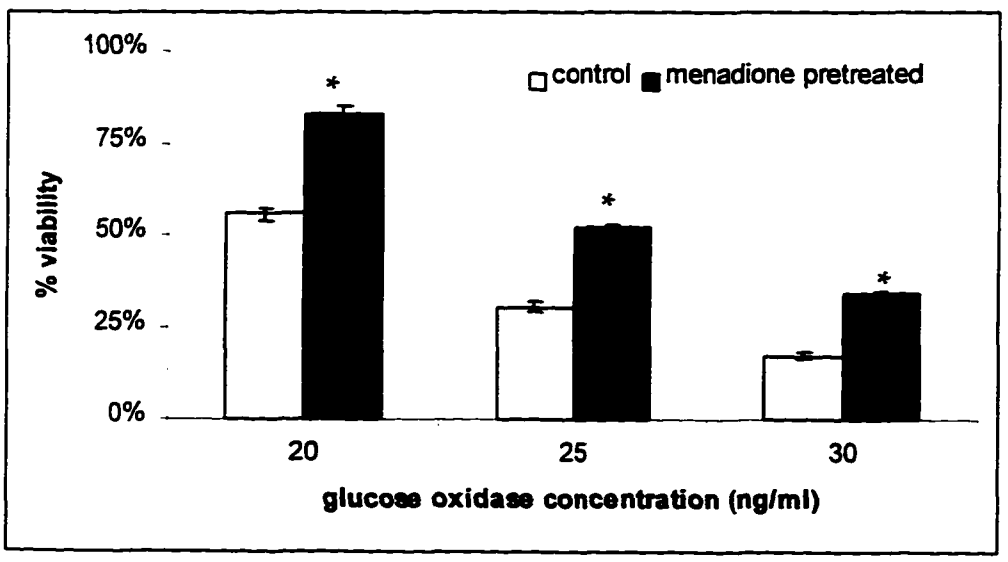


Fig. 4

Preincubation of Hep G2 cells with menadione protects against cytotoxicity of menadione

The cells were pretreated with control medium or 3 μM menadione for 45 min and then were exposed to 15, 18, or 20 μM menadione for 18 h, followed by an MTT assay as described under "Materials and Methods". * $p<0.01$.

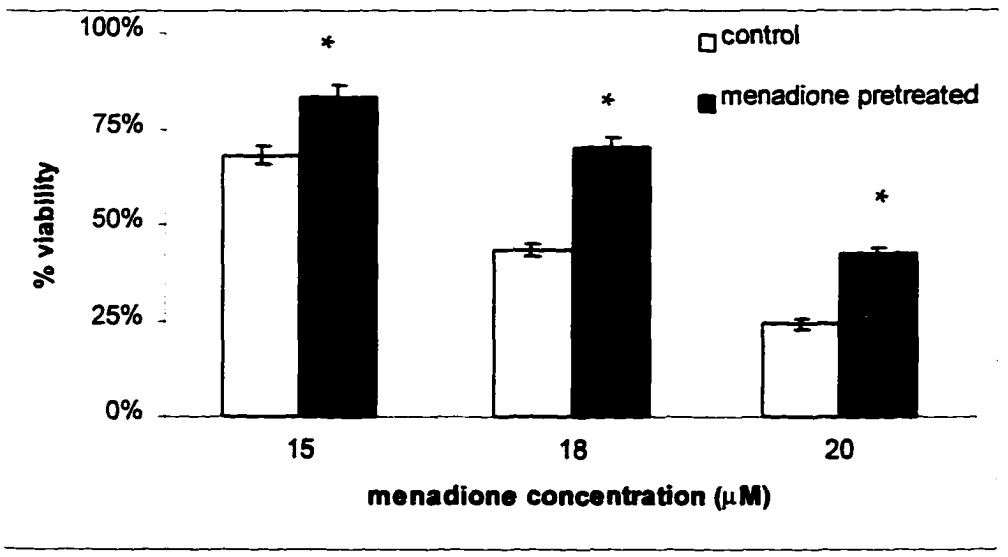


Fig. 5

Dose dependence of menadione preincubation

Hep G2 cells were pretreated with control medium and different concentrations of menadione for 45 min and then exposed to 25 ng/ml glucose oxidase (and 1 mg/ml glucose) for 18 h, followed by an MTT assay as described under "Materials and Methods". * $p < 0.001$.

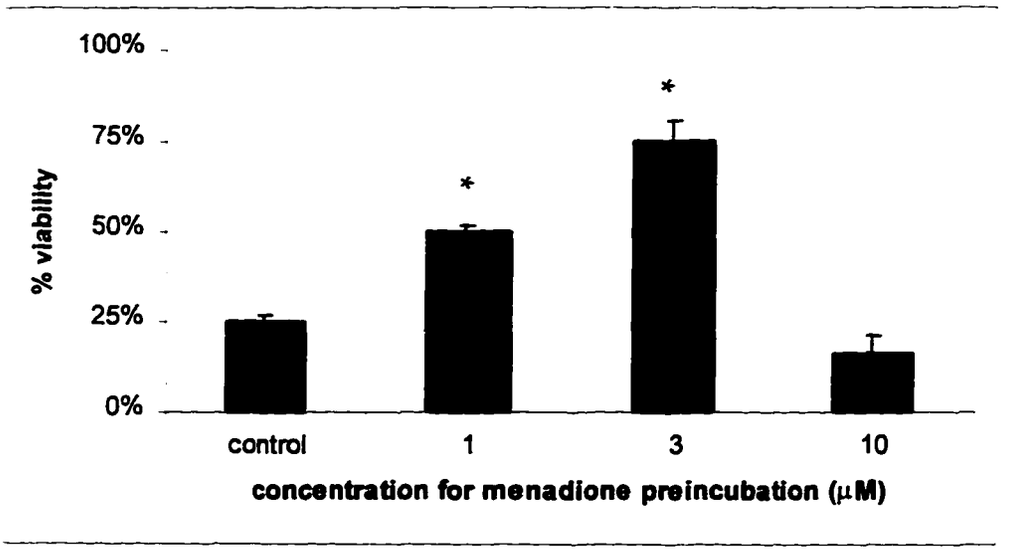


Fig. 6

Time course of menadione preincubation

Hep G2 cells were pretreated with 3 μM menadione for different times and then exposed to 25 ng/ml glucose oxidase (and 1 mg/ml glucose) for 18 h. The percent viability was determined as described under "Materials and Methods". * $p<0.01$, ** $p<0.05$

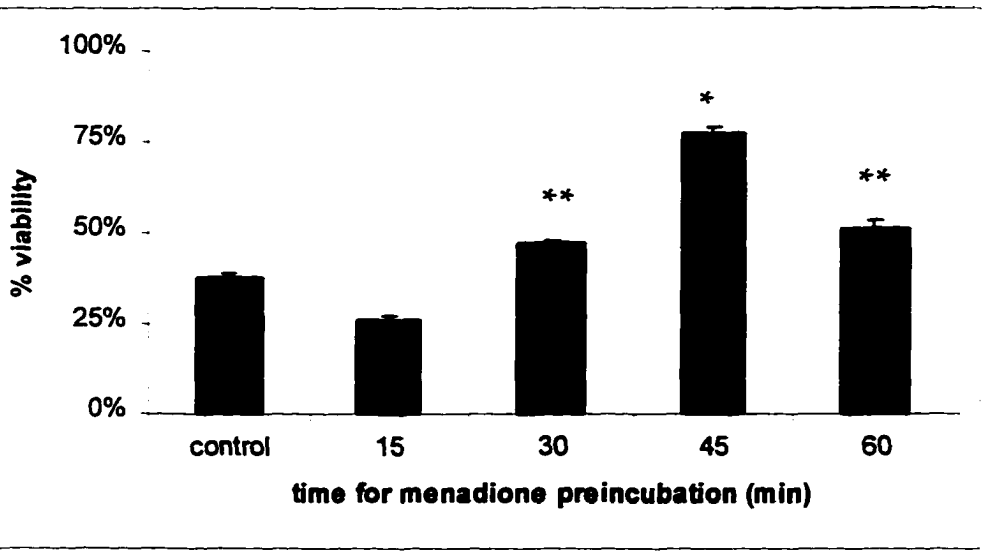


Fig. 7

Preincubation of Hep G2 cells with menadione protects against cytotoxicity of H_2O_2

Hep G2 cells were pretreated with control medium or 3 μM menadione for 45 min and then were exposed to H_2O_2 generated by 25 ng/ml glucose oxidase + 1 mg/ml glucose and followed by LDH release assay as described under "Materials and Methods". * p <0.001

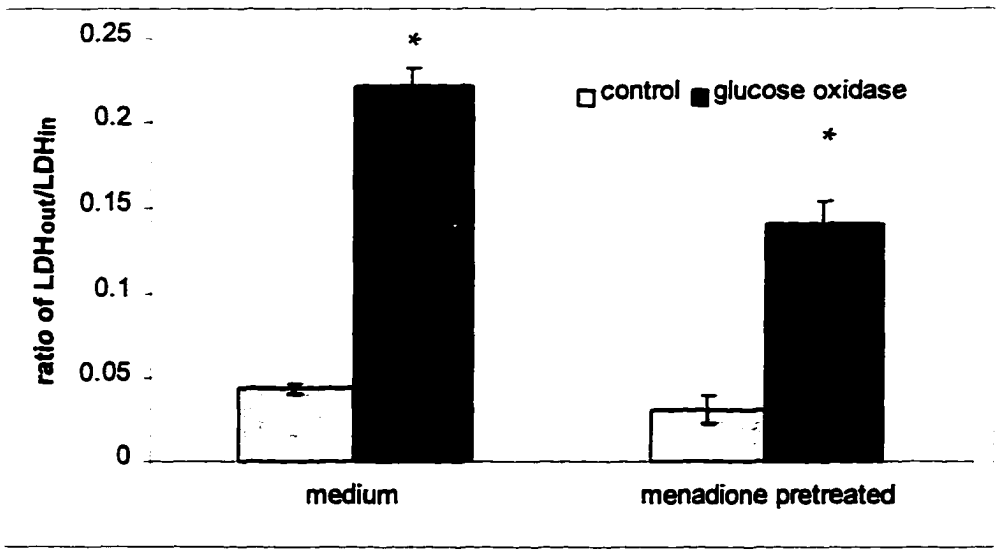


Fig. 8

Activation of NF-κB in Hep G2 cells

Nuclear extracts were prepared from Hep G2 cells treated with control medium (lanes 1, 4, 7), 50 ng/ml PMA (lanes 2, 5, 8), or 3 μM menadione (lanes 3, 6, 9) for 45 min and incubated with labeled oligonucleotide containing consensus sequence of NF-κB binding sites (lanes 1-3) for EMSA carried out as described under "Material and Methods". Twenty times more of unlabeled oligonucleotide containing NF-κB sequence (lanes 4-6) and unlabeled oligonucleotide containing mutant NF-κB sites (lanes 7-9) were present in the incubation period for competition.

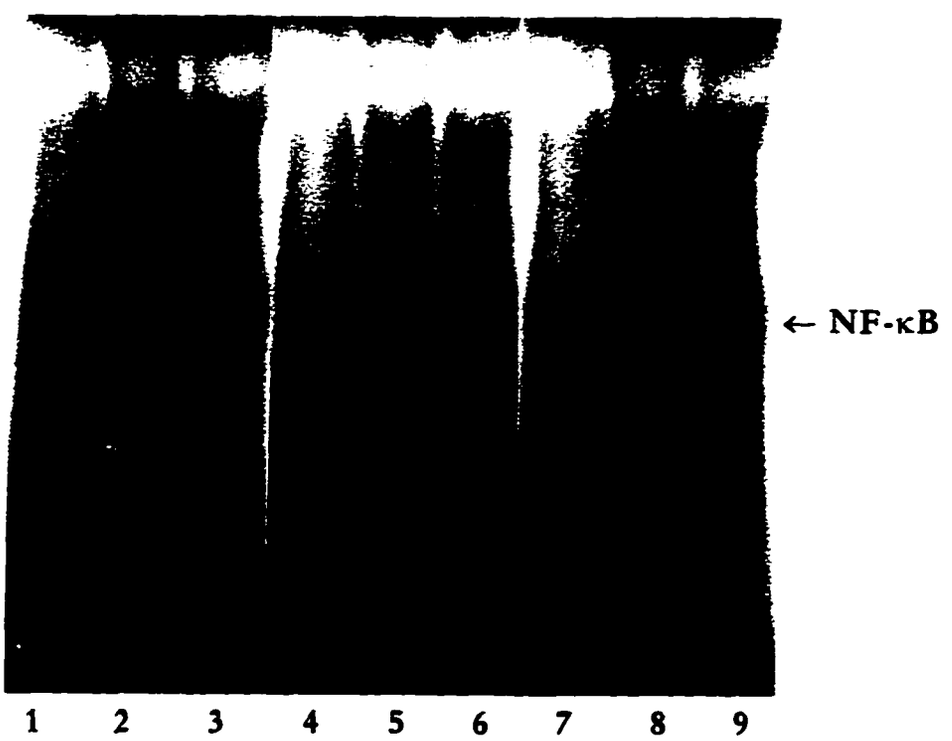


Fig. 9

Time course of NF-κB activation

EMSA was performed by utilizing nuclear extracts prepared from Hep G2 cells incubated with medium alone (lane 1), or 3 μM menadione for 5, 10, 15, 30, 45, 60, and 120 min respectively (lanes 2-8).

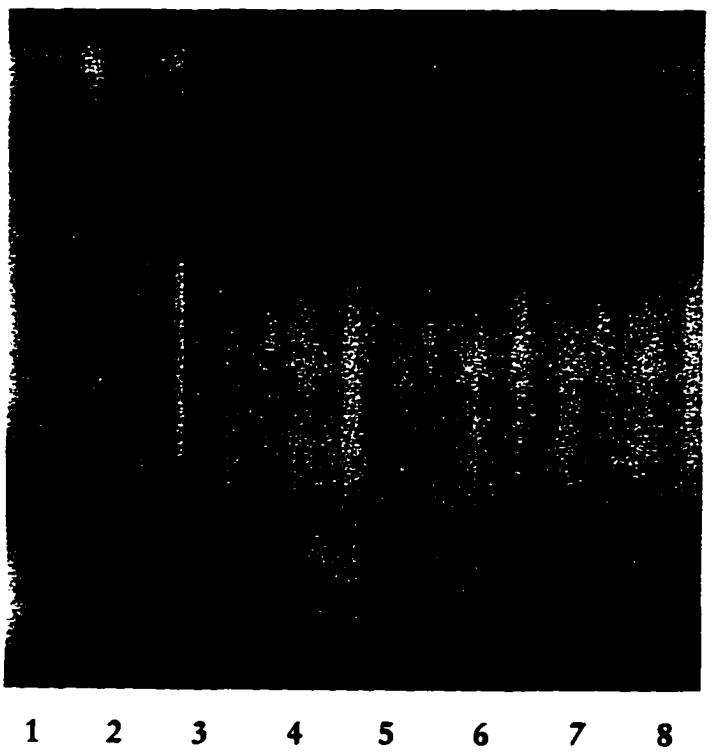


Fig. 10

Effect of anti-NF-κB antibodies on NF-κB binding to NF-κB consensus sequence oligonucleotide probe

EMSA was carried out as described under "Materials and Methods" with nuclear extracts prepared from the Hep G2 cells treated with control medium (lane 1) or 3 μM menadione for 45 min. Nuclear extract from the cells stimulated with menadione was incubated in the binding buffer in the absence of antibody (lane 2) or in the presence of 2 μg anti-p50 IgG (lane 3), 2 μg anti-p65 IgG (lane 4) or 2 μg preimmune IgG (lane 5) overnight at 4 °C. Labeled oligonucleotide containing the consensus sequence of NF-κB binding site was then added to each reaction mixture.

Fig. 11

Effect of salicylate, cycloheximide and antioxidants on NF-kB activation by menadione

EMSA was carried as described under "Materials and Methods" with nuclear extracts prepared from Hep G2 cells treated with 3 μM menadione in the absence of any additions (lane 1) or in the presence of 10 mM sodium salicylate (lane 2), 20 μg/ml cycloheximide (lane 3), 10 mM N-acetylcysteine (lane 4), 50 μM pyrrolidine dithiocarbamate (lane 5), 5 mM thiourea (lane 6), and 0.5 mM uric acid (lane 7) for 45 min.

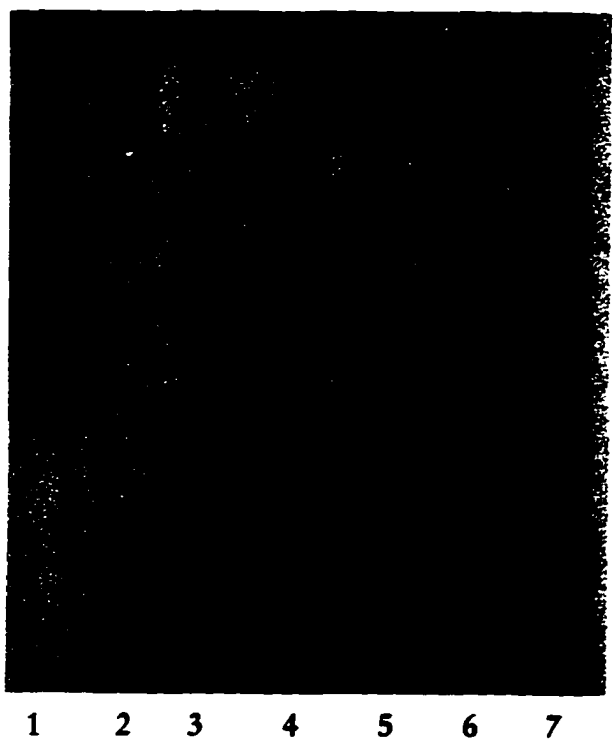


Fig. 12

Salicylate potentiation of menadione cytotoxicity to Hep G2 cells, dose dependence of salicylate concentration

Hep G2 cells were placed onto 24-well plates for assays, and viability was determined by the MTT assay and results expressed as the percent viability of cells exposed to menadione plus salicylate compared to cells exposed to salicylate alone. At indicated concentrations and for 6 h incubation, salicylate alone had no toxic effect. The cells were incubated with 10 μM menadione in the presence of different concentrations of sodium salicylate for 6 h.

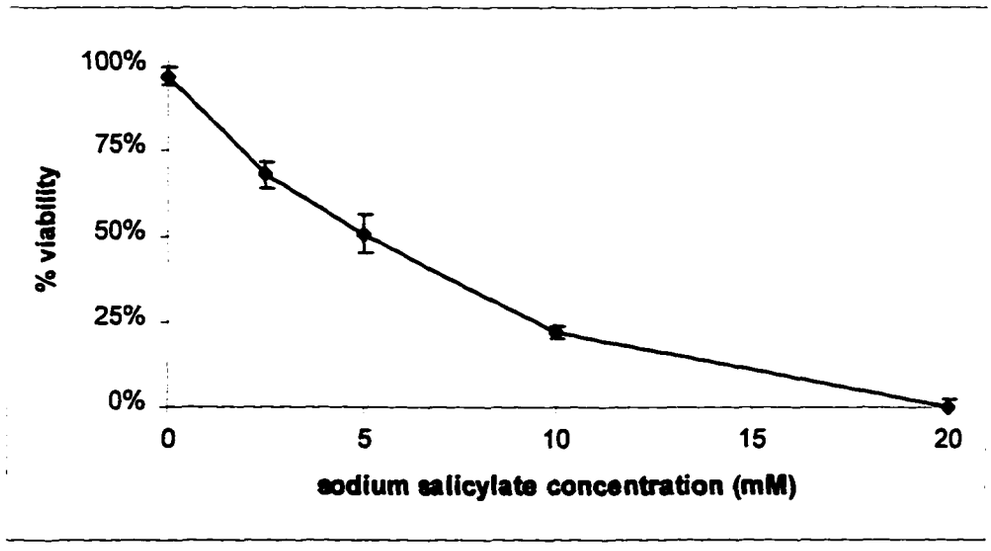


Fig. 13

Dose dependence curve of menadione cytotoxicity to Hep G2 cells potentiated by salicylate

Hep G2 cells were placed onto 24-well plates for assays, and viability was determined by the MTT assay and results expressed as the percent viability of cells exposed to menadione plus salicylate compared to cells exposed to salicylate alone. At indicated concentrations and for 18 h incubation, salicylate alone had no toxic effect. Dose dependence curves were made by incubation of Hep G2 cells with the combination of various concentrations of menadione and sodium salicylate for 18 h.

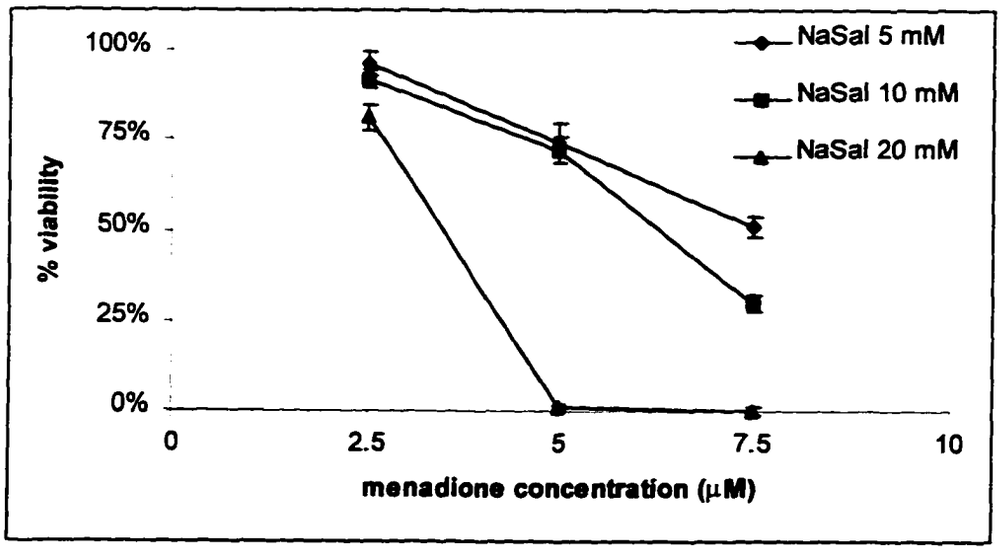


Fig. 14

Time course curve of menadione or H_2O_2 cytotoxicity to Hep G2 cells potentiated by salicylate

Hep G2 cells were placed onto 24-well plates for assays, and viability was determined by the MTT assay and results expressed as the percent viability of cells exposed to menadione plus salicylate compared to cells exposed to salicylate alone. At a concentration of 10 mM and for 18 h, salicylate alone had no significant toxic effect. Time course was carried out by incubating Hep G2 cells with 10 μM menadione or 50 μM H_2O_2 with or without 10 mM sodium salicylate, for the indicated time points.

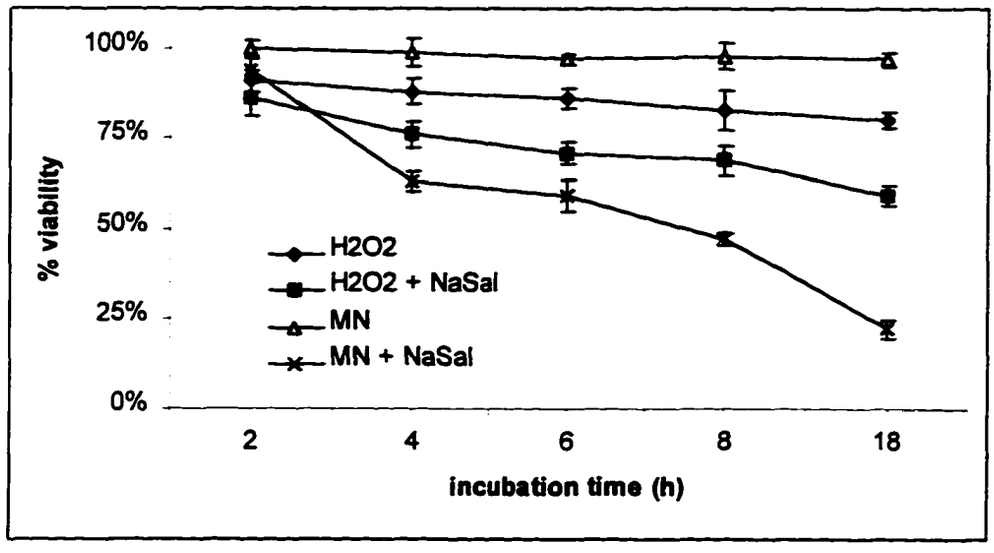


Fig. 15

Effects of long-term PMA treatment on the protection by menadione preincubation against menadione cytotoxicity

Hep G2 cells were treated with 100 ng/ml PMA or medium for 24 h, and then preincubated with normal medium or 3 μM menadione for 45 min, followed by incubation with 18 μM menadione and subsequent cytotoxicity assay using the MTT assay as described under "Material and Methods". Compared to control, * $p > 0.05$, ** $p < 0.001$.

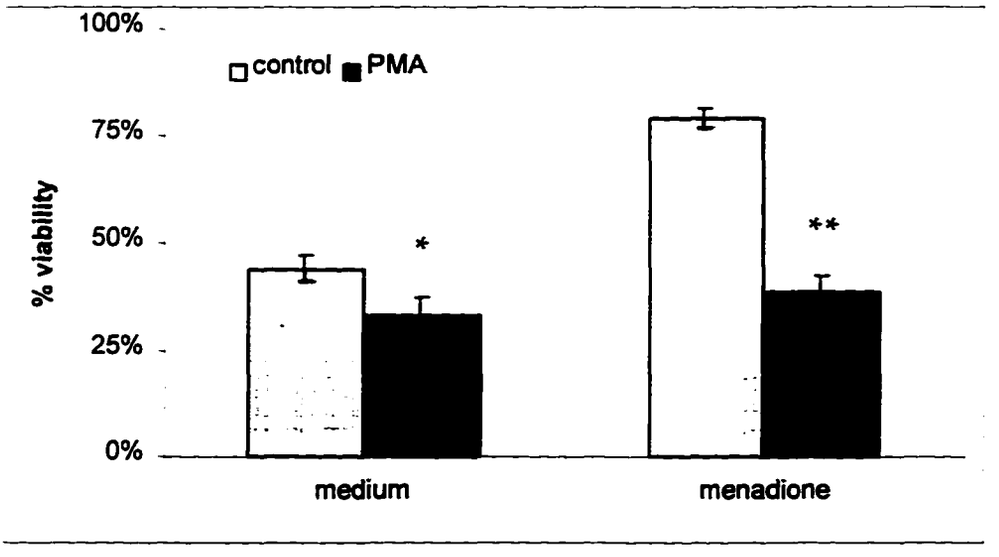


Fig. 16

Effect of long-term PMA treatment on menadione toxicity

After prolonged treatment with PMA or medium for 24 h, Hep G2 cells were exposed to a series of different menadione concentrations and followed by viability assay using the MTT assay as described under "Materials and Methods".

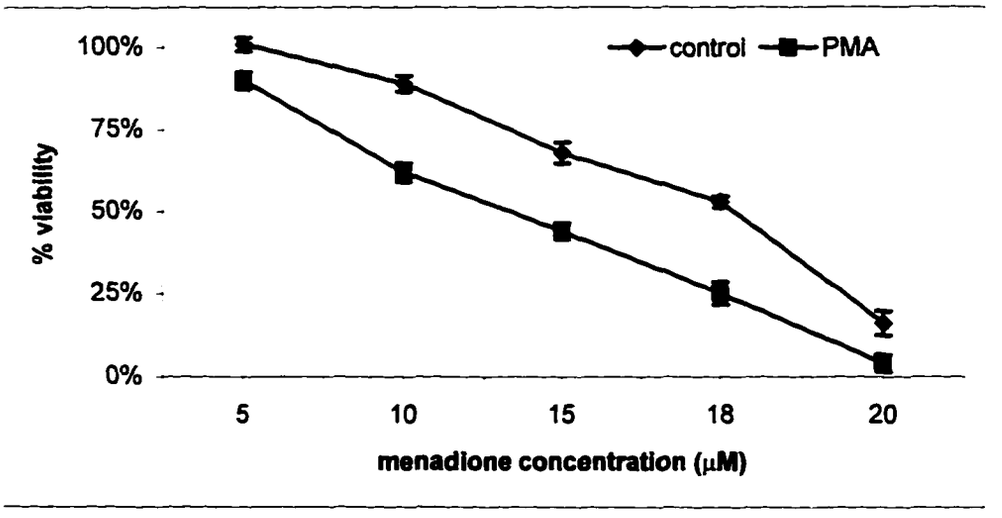


Fig. 17

Effect of short-term PMA treatment on menadione toxicity

Hep G2 cells were preincubated with medium, 3 μM menadione, or 25 ng/ml PMA for 25 min and than exposed to 18 μM menadione for cytotoxicity assay. The percent viability was determined using the MTT assay as described under "Materials and Methods". * $p < 0.001$, ** $p < 0.005$.

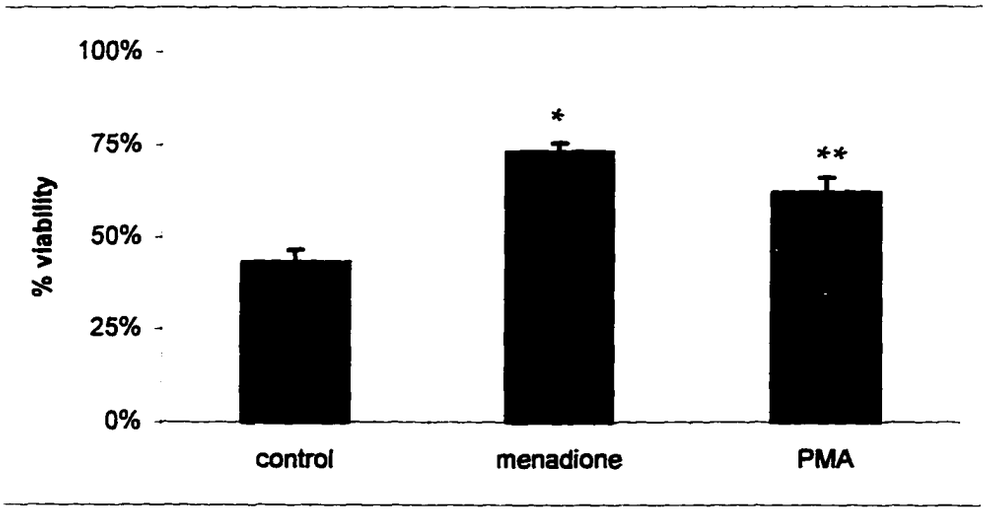


Fig. 18

Transient expression of IκBβ in Hep G2 cells

Transfection of the Hep G2 cells with control plasmid pCI-*neo* or pCI-IκBβ was carried out as described under "Materials and Methods". Western blot analysis was carried out on cell extracts using anti-IκBβ IgG (Santa Cruz Biotechnology, Santa Cruz, CA) as primary antibody and Goat anti-rabbit IgG-alkaline phosphatase as secondary antibody. From lane 1 to lane 4: molecular weight maker, Hep G2 cell, pCI transfectant, and pCI-IκBβ transfectant.

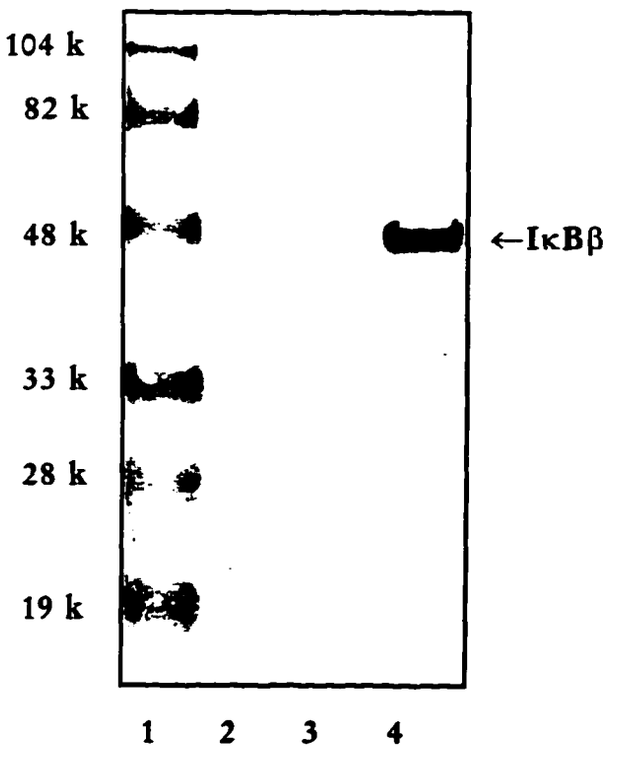


Fig. 19

Effect of overexpression of IκBβ on menadione cytotoxicity to Hep G2 cells

Transfection of the Hep G2 cells with control plasmid pCI-*neo* or pCI-IκBβ was carried out as described under "Materials and Methods". The cytotoxicity of the indicated concentrations of menadione in the control transfectant and the IκBβ-expressing Hep G2 cells was determined after an 18 h incubation period using the MTT assay as described under "Materials and Methods". Compared to control (pCI-neo), * $p < 0.001$, ** $p < 0.01$.

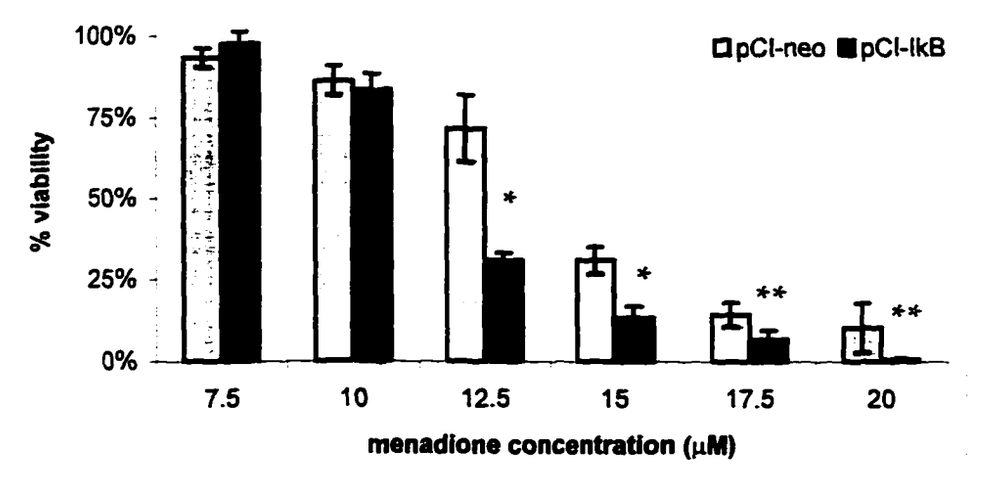


Fig. 20

Effect of BSO on menadione toxicity to Hep G2 cells

Hep G2 cells were incubated with or without 0.1 mM BSO for 2 h followed by incubation in the presence of a series of different menadione concentrations for 18 h. The cytotoxicity was determined as described under "Materials and Methods". * $p < 0.001$, ** $p < 0.01$.

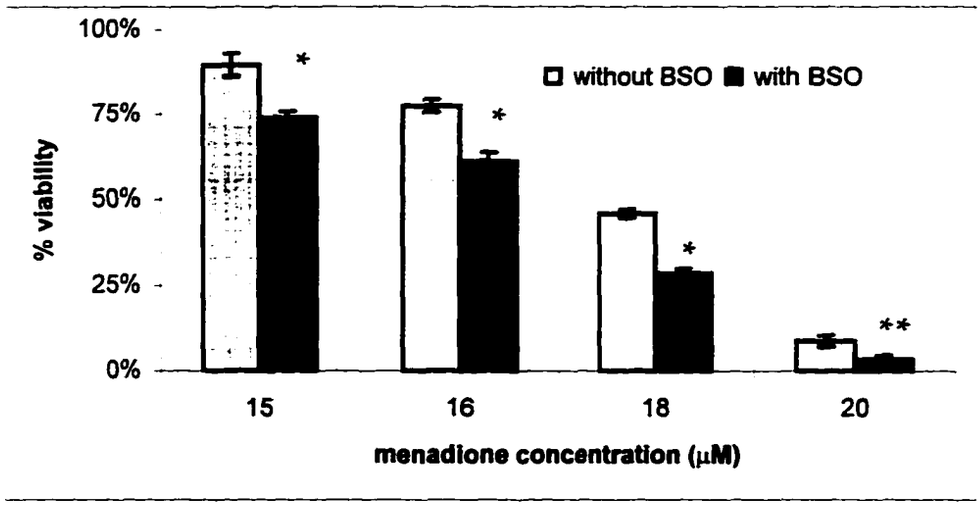


Fig. 21

Intracellular GSH levels after menadione preincubation

Hep G2 cells were preincubated with control medium, 3 μM menadione, 10 mM sodium salicylate, or 3 μM menadione + 10 mM sodium salicylate for 45 min (salicylate was added 15 min before menadione), and then were rinsed twice with medium followed by further incubation of 2, 4, or 8 h with medium. Cells were harvested at the indicated time for GSH assay as described under "Materials and Methods". Compared to the zero time, * $p<0.001$, ** $p>0.05$.

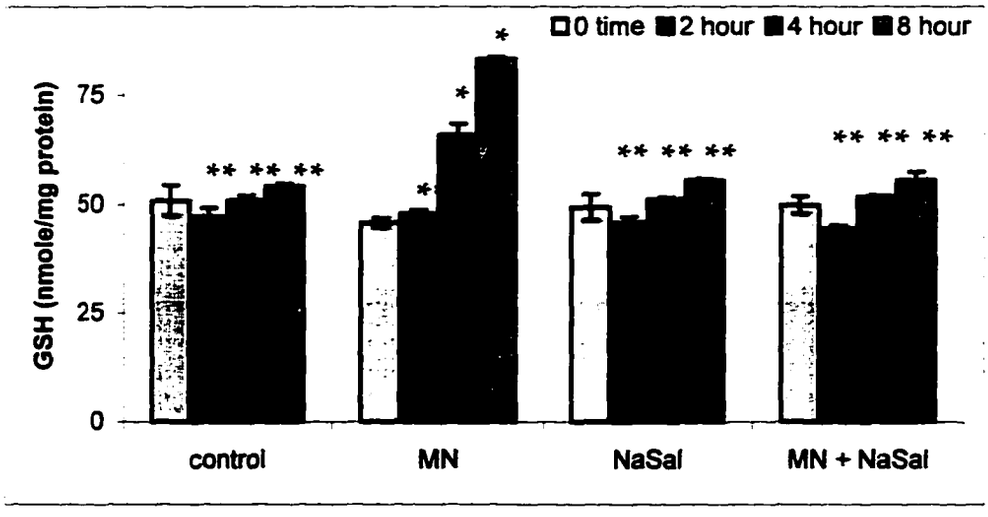


Fig. 22

Arachidonic acid induced cytotoxicity as measured by the LDH release assay

E9 and MV5 cells were cultured in control medium, medium containing 0.03 mM oleic acid, or medium containing 0.03 mM arachidonic acid for 24 h. The medium was then replaced with normal MEM medium for an additional 24 h of incubation. At the end of this treatment, the supernatants were collected to measure the LDH activity (designated as LDH_{out}) and cells were harvested by scraping for measurement of LDH activity (designated as LDH_{in}). The cytotoxicity index was expressed as the ratio of LDH_{out}/LDH_{in}. * $p<0.001$, ** $p>0.1$.

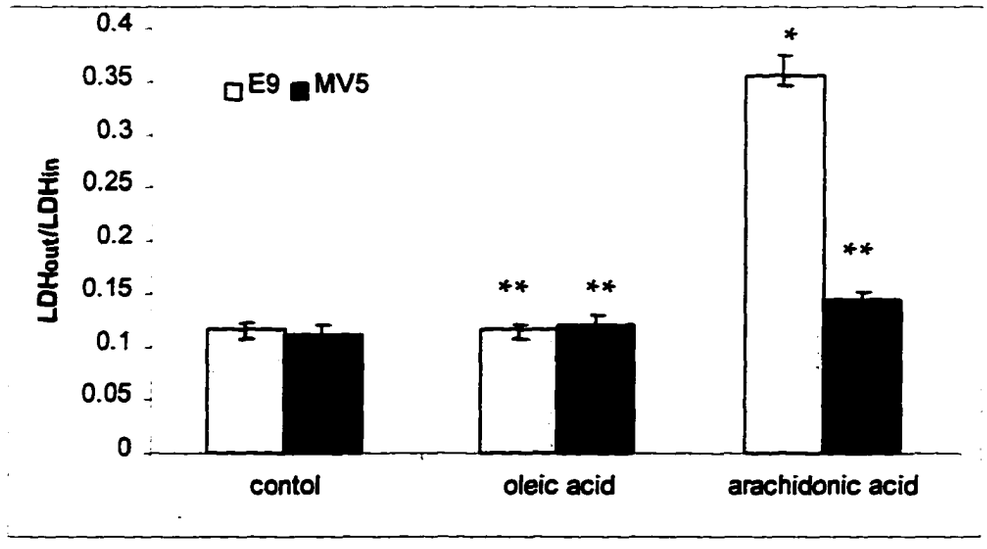


Fig. 23

Morphology of E9 and MV5 cells in the absence and presence of arachidonic acid

E9and MV5 cells were incubated for 24 h with or without 0.03 mM arachidonic acid. The medium was removed, the cells were rinsed with MEM and incubated for an additional 24 h with normal medium, and visualized under the light microscope (magnification, 10X20).

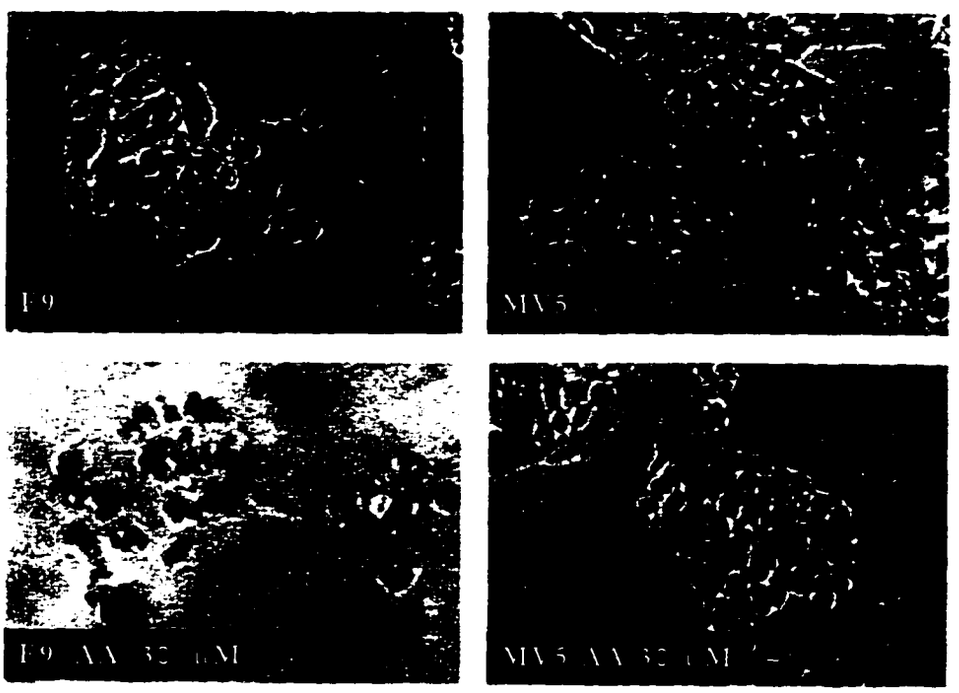


Fig. 24

Dose-dependence curve for arachidonic acid cytotoxicity

E9 and MV5 cells were cultured in control medium, or medium containing arachidonic acid at a series of concentrations ranging from 0.01 to 0.05 mM for 24 h in the absence or presence of 0.1 mM BSO. The medium was then replaced with normal medium for an additional 24 h of incubation, followed by viability measurement using the MTT assay as described under "Materials and Methods".

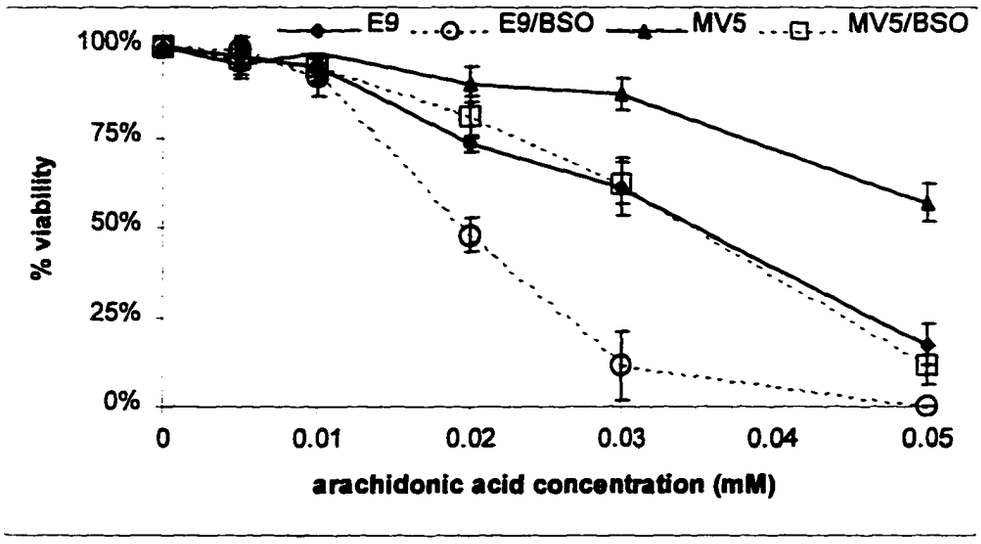


Fig. 25

Time course curve for arachidonic acid cytotoxicity

E9 and MV5 cells were cultured in control medium, or medium containing 0.03 mM arachidonic acid for 24 h, and then were transferred into normal medium for the additional designated periods of time. At the end of treatment, cell viability was determined by the MTT assay as described under "Materials and Methods". * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

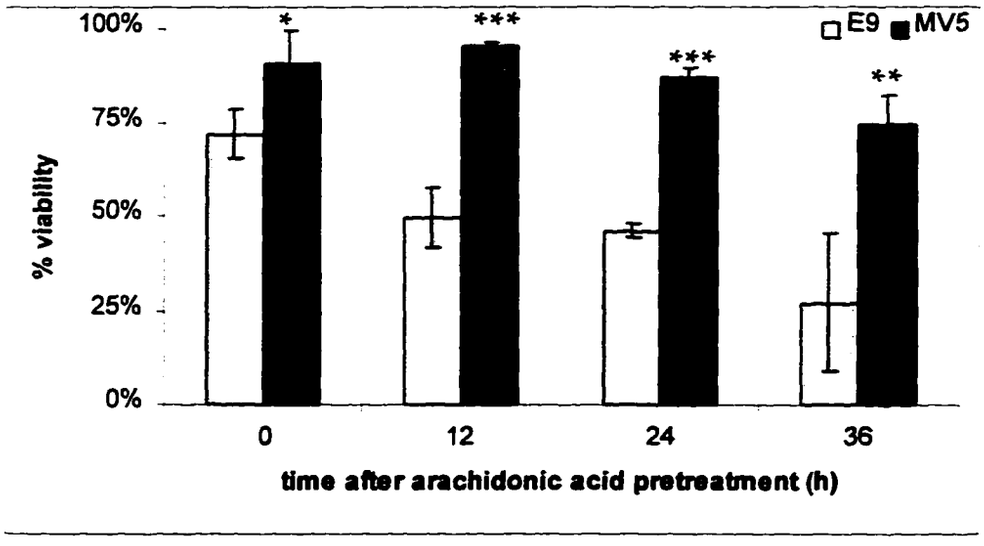


Fig. 26

Lipid peroxidation in E9 and MV5 cells after arachidonic acid pretreatment: intracellular

After 24 h of arachidonic acid (0.01-0.03 mM) pretreatment followed by an additional 24 h of culture in normal MEM, E9 and MV5 cells were harvested and assayed for lipid peroxidation. The lipid peroxidation level was monitored by the production of MDA plus 4-HNE using the LPO-586 kit as described under "Materials and Methods". * $p < 0.001$, ** $p < 0.05$.

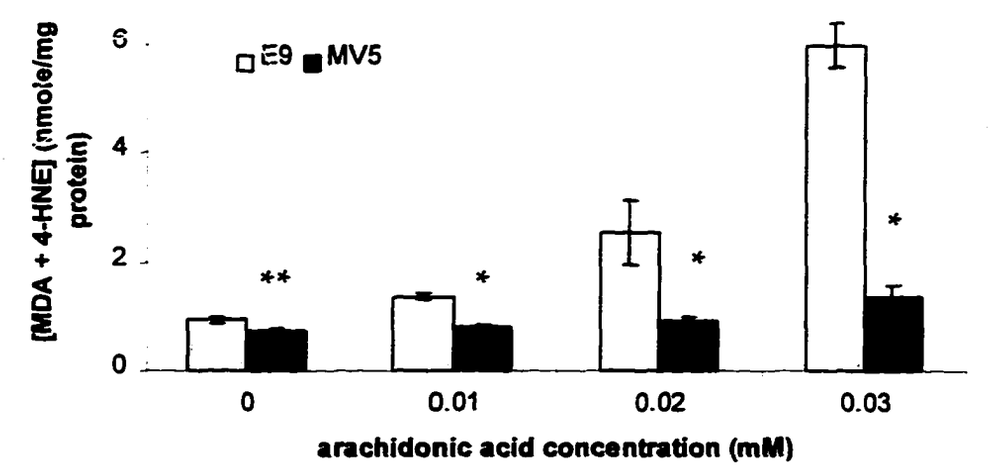


Fig. 27

Lipid peroxidation in E9 and MV5 cells after arachidonic acid pretreatment: supernatants

The culture medium was collected at the end of the arachidonic acid treatment for 24 h, and at the end of the additional 24-h culture, and the combined total was used to assay for lipid peroxidation. The lipid peroxidation level was monitored by the production of MDA plus 4-HNE using the LPO-586 kit as described under "Materials and Methods". * $p>0.05$, ** $p<0.001$.

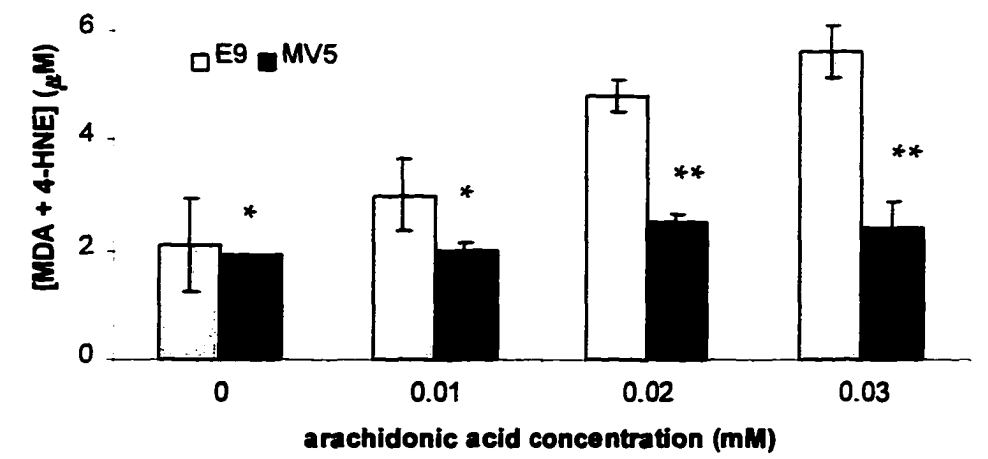


Fig. 28

Intracellular GSH levels after arachidonic treatment

E9 cells were cultured in medium containing 30 μM arachidonic acid for the indicated times, and then harvested for measurement of intracellular GSH levels as described under "Materials and Methods". The cells collected before arachidonic acid treatment were considered the time zero control. * $p<0.01$.

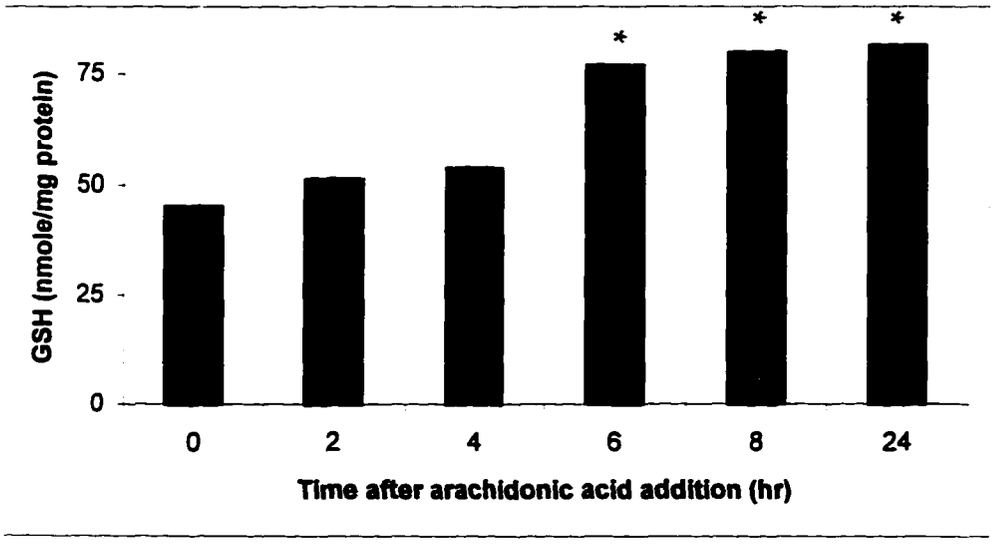


Fig. 29

Effect of sodium salicylate and antioxidants on the elevated intracellular GSH level induced by arachidonic acid in E9 cells

E9 cells were cultured in medium containing 30 μM arachidonic acid with or without 10 mM sodium salicylate, 0.5 mM sodium ascorbate, or 20 μM trolox for 8 h, and then harvested for measurement of intracellular GSH levels as described under "Materials and Methods". Parallel cultures of E9 cells in normal medium, or medium with 10 mM sodium salicylate, 0.5 mM sodium ascorbate, or 20 μM trolox in the absence of arachidonic acid were assayed as controls. * $p<0.01$, ** $p>0.05$.

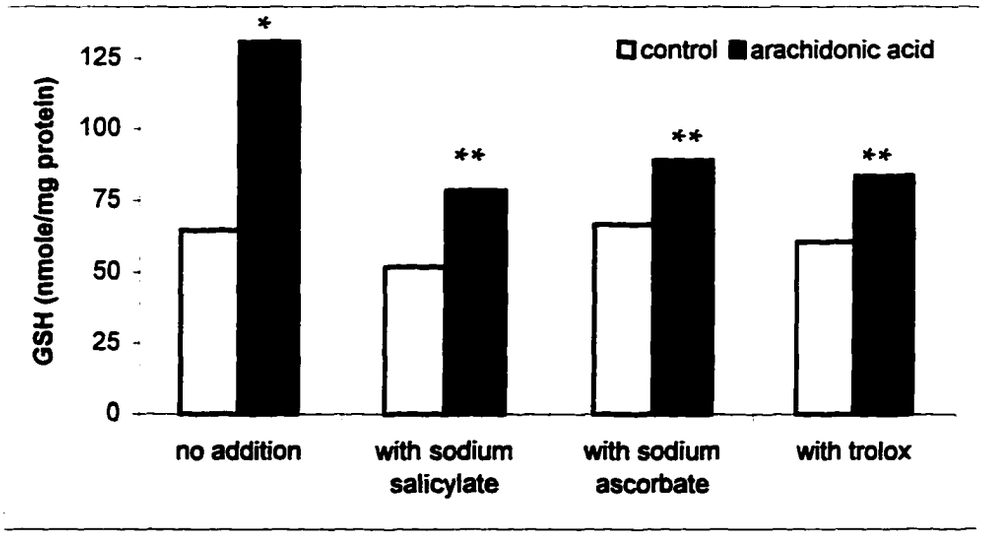


Fig. 30

Effect of salicylate on arachidonic acid cytotoxicity in E9 cells

E9 cells were preloaded with 30 μM arachidonic acid in the absence or presence of 10 mM sodium salicylate or 10 mM sodium salicylate plus 20 μM trolox for 24 h. The medium was then replaced with normal medium, or medium with 10 mM sodium salicylate, or medium with 10 mM salicylate and 20 μM trolox, and incubated for an additional 24 h. The cells were subjected to the MTT assay as described under "Materials and Methods". * $p < 0.05$, ** $p > 0.05$.

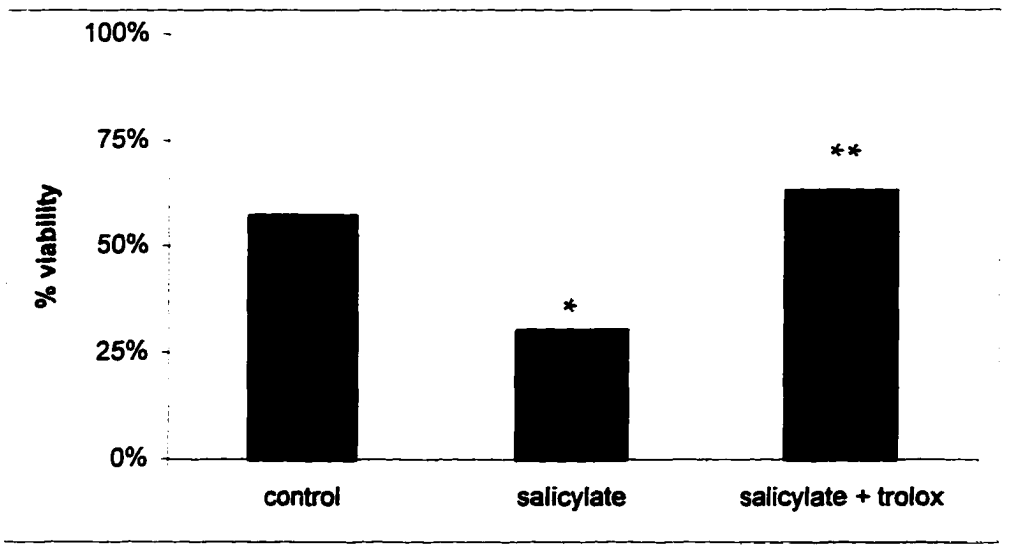


Fig. 31

Western blot analysis of the content of CYP2E1 and bcl-2 after transfection

E9 cells were transfected with plasmids pCI-2E1 (Top lane 5), pCI-as-2E1 (Top lane 1), pCI-bcl-2 (Top lane 4 and Bottom lane 2), pCI-as-bcl-2 (Top lane 2 and Bottom lane 1), or pCI-*neo* (Top lane 3 and Bottom lane 3) by utilizing LipofectAMINE reagent as described under "Materials and Methods". 50 μg of cell lysate was loaded into each lane for SDS-PAGE, followed by Western blot analysis with polyclonal rabbit anti-human CYP2E1 (upper panel) or monoclonal anti-human Bcl-2 antibodies (lower panel).

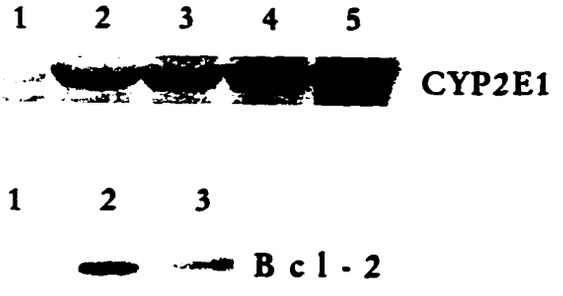


Fig. 32

Role of CYP2E1 in arachidonic acid-induced cytotoxicity

E9 cells were transiently transfected with plasmids pCI-*neo*, pCI-2E1, or pCI-as-2E1 and then subjected to arachidonic acid treatment for 24 h. After removal of medium containing PUFA followed by an additional 24 h of culture in normal medium, cell viability was determined by the MTT assay described under "Materials and Methods".

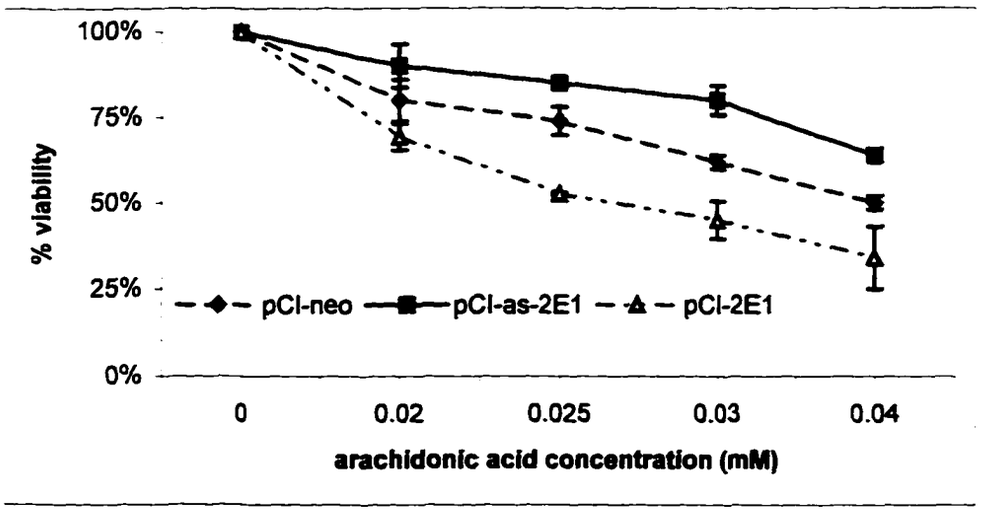


Fig. 33

Effect of 4-MP on CYP2E1-induced arachidonic acid toxicity

E9 cells were pretreated with 0.02, 0.03 or 0.04 mM arachidonic acid for 24 h, in the absence or presence of 2 mM 4-MP or absence or presence of 0.1 mM BSO. After removal of the arachidonic acid medium, fresh control medium (with the same amount of 4-MP or BSO respectively) was added and cell viability was determined after an additional 24 h incubation period by the MTT assay described under "Materials and Methods". * Compared to the control (without 4-MP), $p>0.05$; **compared to control (without 4-MP but with BSO) $p<0.05$.

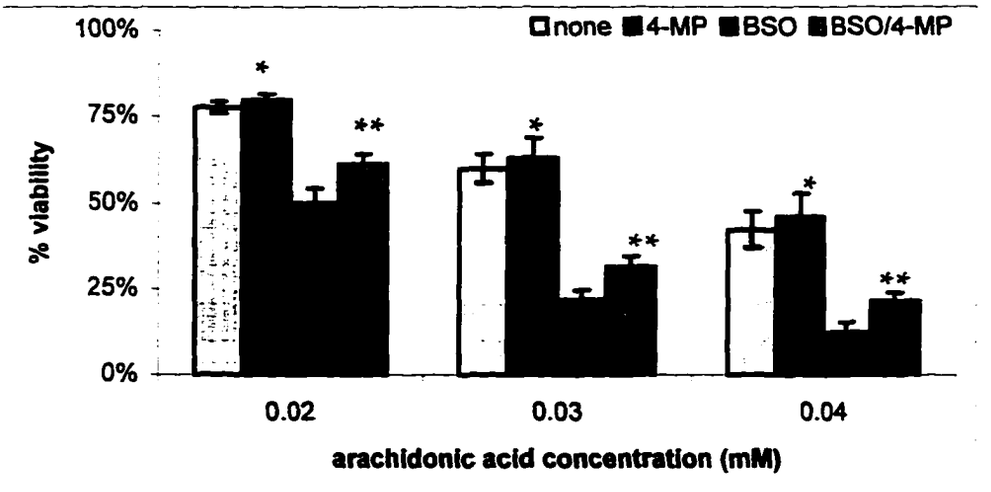


Fig. 34

Histograms of *in situ* DNA nick end labeling of E9 and MV5 cells

Cells were preincubated in MEM medium 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.

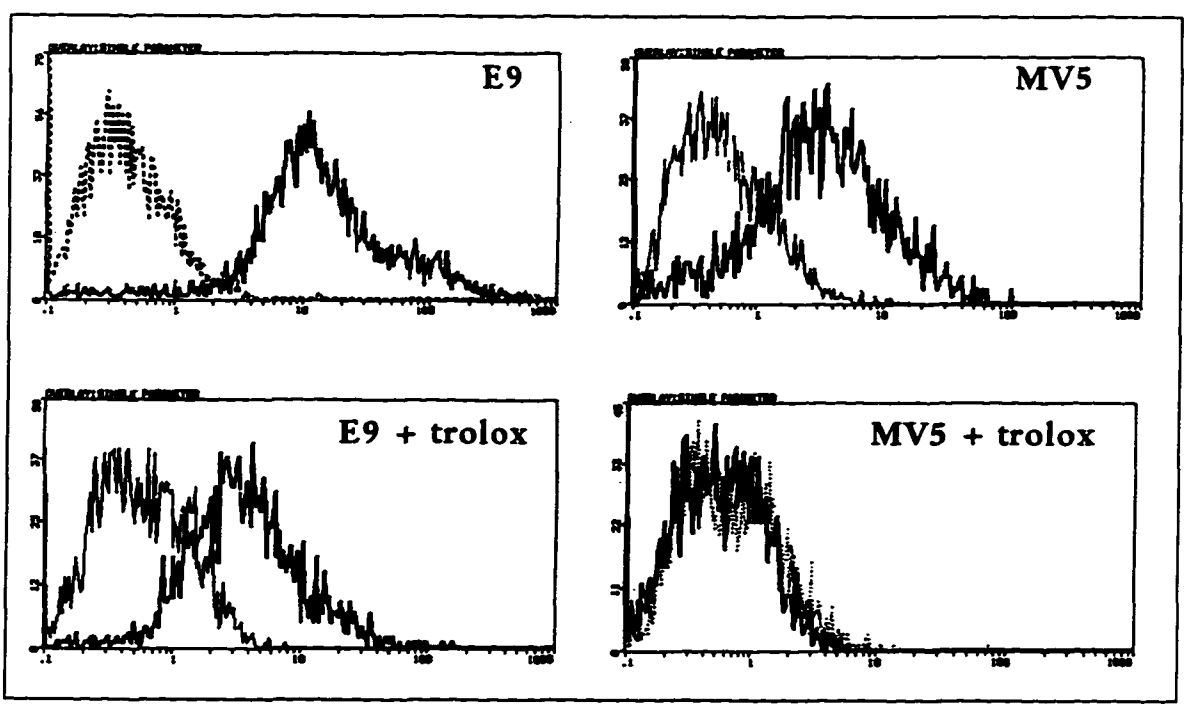


Fig. 35

Dose dependent curve of *in situ* DNA nick end labeling of E9 and MV5 cells

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. 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.

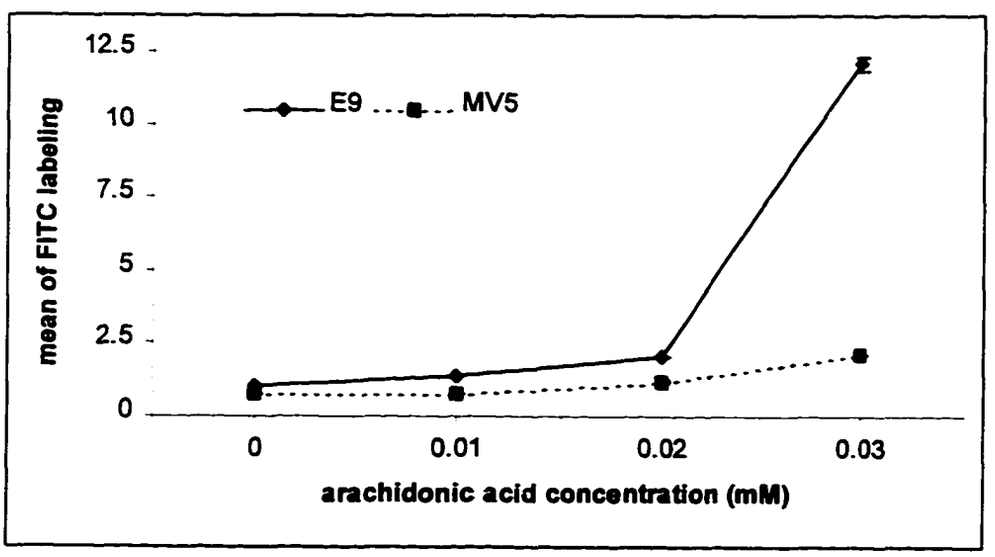


Fig. 36

Effect of trolox on *in situ* DNA nick end labeling of E9 and MV5

The effect of 0.03 mM arachidonic acid in the absence or presence of 0.1 mM trolox on *in situ* DNA nick end labeling was determined. Trolox was present throughout the 24-h and the 8-h incubation periods. 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. Compared to the control (without trolox), * $p>0.05$, ** $p<0.001$.

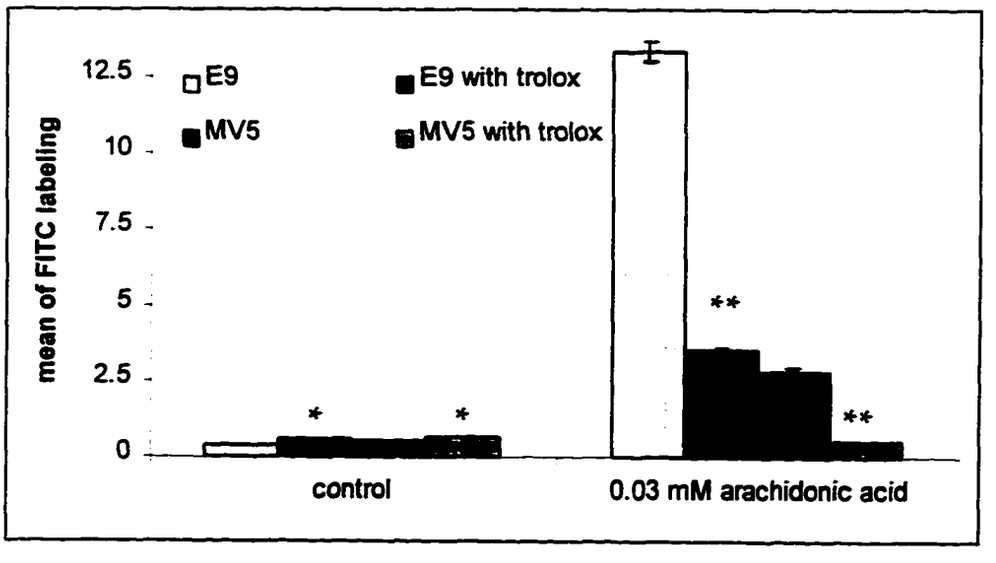


Fig. 37

Apoptosis induced by arachidonic acid in E9 cells

E9 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 were 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: lane 1, 100 bp ladder standard; lanes 2, 6, 10, and 14, no arachidonic acid added, and incubation for 6, 12, 24, or 24 plus additional 8 h respectively; lanes 3, 7, 11, and 15, 0.02 mM arachidonic acid and incubation for 6, 12, 24, and 24 plus 8 h, respectively; lanes 4, 8, 12, and 16, 0.03 mM arachidonic acid and incubation for 6, 12, 24, and 24 plus 8 h, respectively; lanes 5, 9, 13, and 17, 0.04 mM arachidonic acid and incubation for 6, 12, 24, and 24 plus 8 h, respectively. Cells were harvested by scraping, followed by DNA separation and DNA ladders were displayed by agarose gel electrophoresis as described under "Materials and Methods".

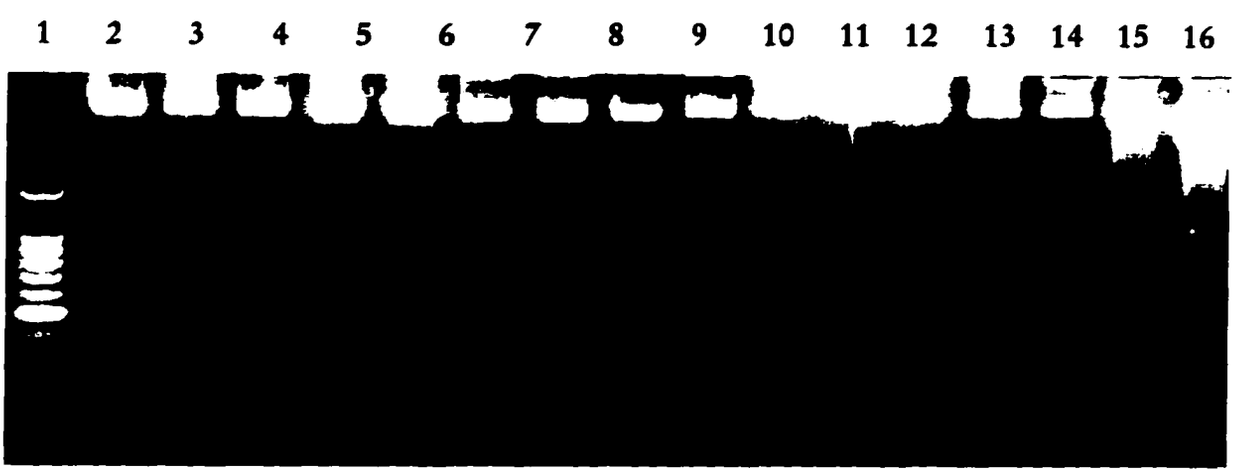


Fig. 38

Effect of trolox on apoptosis induced by arachidonic acid in E9 cells

E9 and MV5 cells were preincubated with (lanes 6 to 9) or without (lanes 2 to 5) 0.03 mM arachidonic acid for 24 h followed by additional 8 h of incubation in medium without arachidonic acid. Two groups of Hep G2-MV2E1-9 cells were given 0.04 or 0.1 mM trolox together with 0.03 mM arachidonic acid. Lane 1, 100 base-pairs DNA ladder standard; lane 2, MV5 cells without arachidonic acid; lane 3, E9 cells without arachidonic acid; lane 4, Hep G2-MV2E1-9 cells without arachidonic acid but with 0.04 mM trolox; lane 5, E9 cells without arachidonic acid but with 0.1 mM trolox; lane 6, MV5 cells with arachidonic acid; lane 7, E9 cells with arachidonic acid; lane 8, E9 cells with arachidonic acid plus 0.04 mM trolox; lane 9, E9 cells with arachidonic acid plus 0.1mM trolox. Cells were harvested by scraping, followed by DNA separation and DNA ladders were displayed by agarose gel electrophoresis as described under "Materials and Methods".

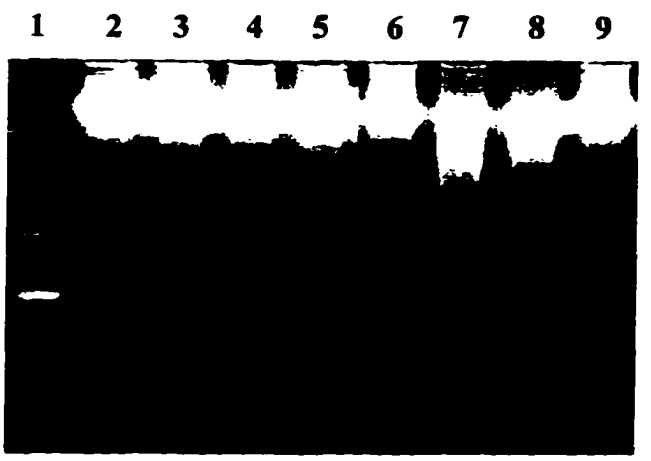


Fig. 39

The effect of *bcl-2* on arachidonic acid cytotoxicity

E9 cells were transiently transfected with plasmids pCI-*neo*, pCI-bcl-2, or pCI-as-bcl-2 and then subjected to arachidonic acid treatment for 24 h. After an additional 24 h of culture in normal medium without arachidonic acid supplement, cell viability was evaluated by the MTT assay as described under "Materials and Methods".

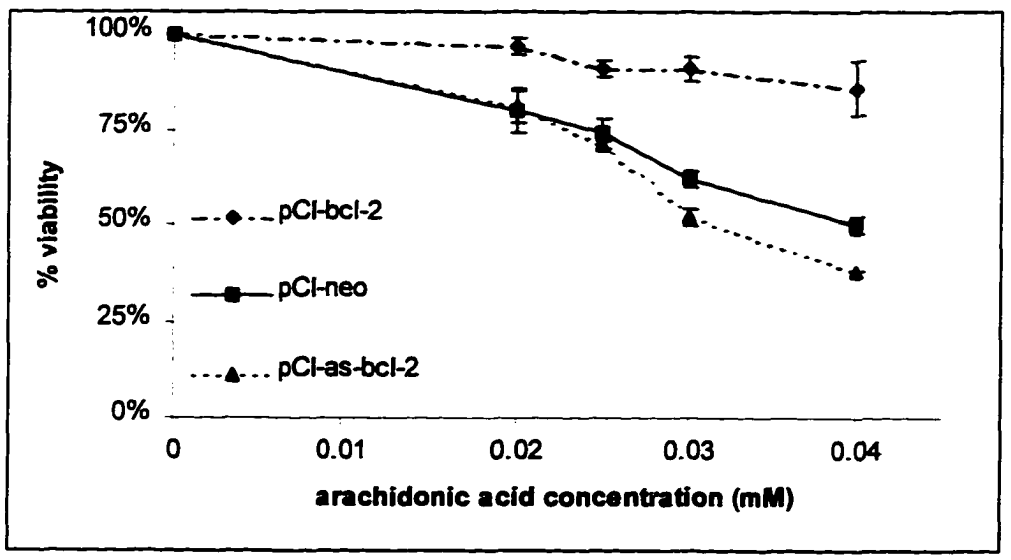


Fig. 40

Western blot analysis of CYP2E1 in transduced Hep G2 cell subclones

CYP2E1 expression levels in parental Hep G2 cells (lane 5), Hep G2 subclones, C34 (lane 1), C37 (lane 2), E43 (lane 3), E47 (lane 4), as well as Hep G2-MV2E1-9 cells (lane 6) were determined by Western blot analysis with polyclonal rabbit anti-human CYP2E1 antibody as described under "Materials and Methods".

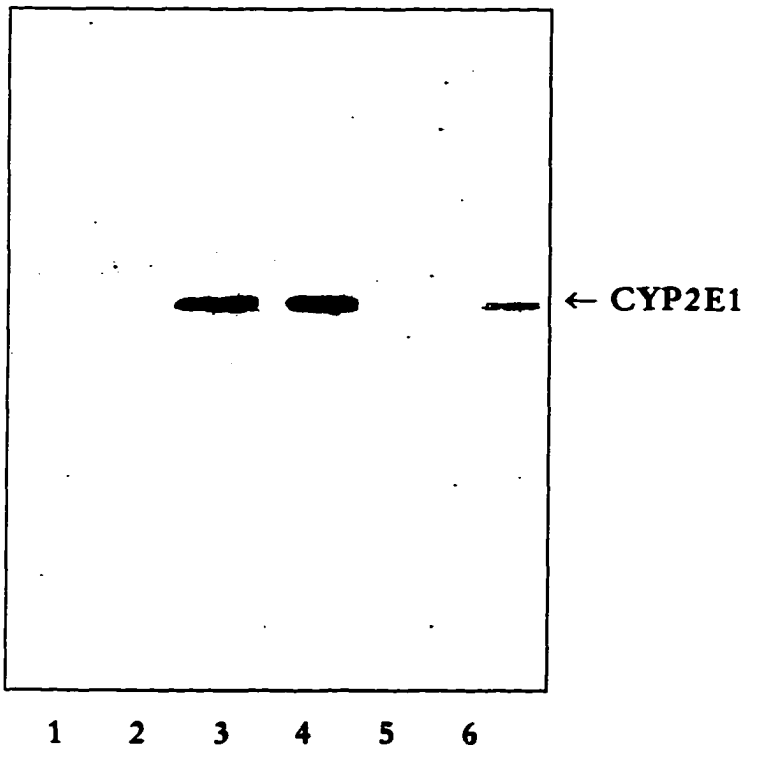


Fig. 41

Western blot analysis of CYP2E1 in E47 transfected with antisense plasmid

CYP2E1 expression levels in E47 cells four days after transfection with pCI-neo (lane 1) and pCI-as-2E1 plasmids (lane 3) were determined by Western blot analysis with polyclonal rabbit anti-human CYP2E1 antibody as described under "Materials and Methods". The none detectable level of CYP2E1 in cultures of C34 cells (lane 2) is shown for comparative purposes.

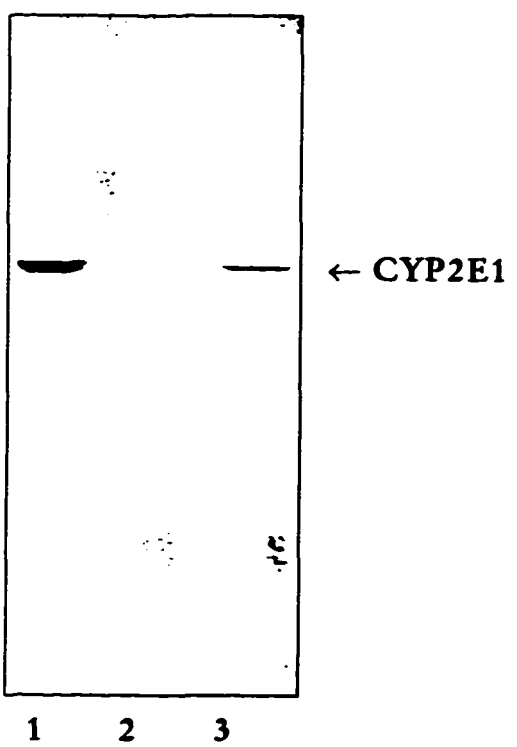


Fig. 42

Effect of 4-MP and Me_2SO on CYP2E1 expression in E47 cells

CYP2E1 expression levels in E47 cells two days after culture in MEM (control, lane 1), or medium containing 2 mM 4-MP (lane 2) or 25 mM Me_2SO (lane 3) were determined by Western blot analysis with polyclonal rabbit anti-human CYP2E1 antibody as described under "Materials and Methods".

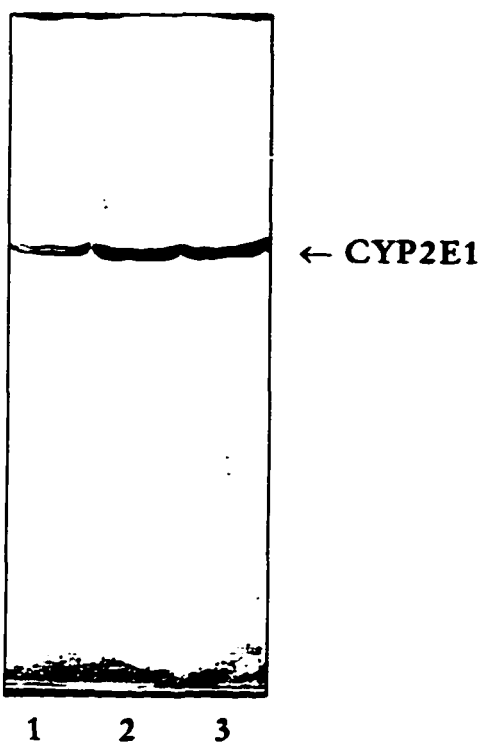


Fig. 43

Differential growth rates of Hep G2 cell subclones

1 X 10^5 cells were cultured in MEM. After indicated days of culture, cells were trypsinized and counted with a hemocytometer.

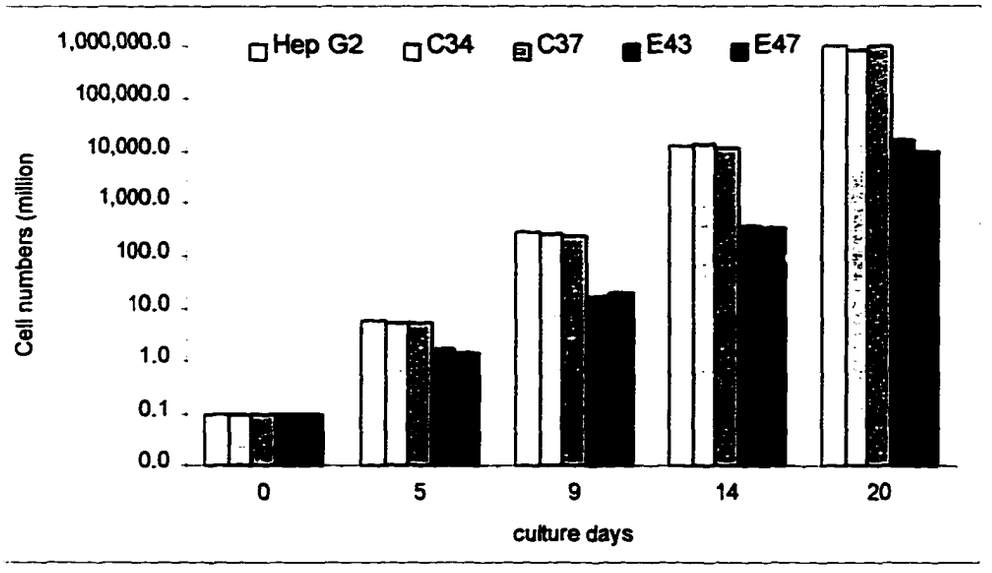


Fig. 44

Morphology of C34 and E47 cells in the absence or presence of BSO

C34 and E47 cells were cultured in MEM in the absence or presence of 0.1 mM BSO for four days and visualized under the light microscope (magnification, 10 X 20).

Fig. 45

Cytotoxicity of C34 and E47 cells in the absence or presence of BSO

C34 and E47 cells were cultured in MEM with or without 0.1 mM BSO for two or three days. Supernatants were collected, and the cells were harvested by scraping for measurement of LDH as described under "Materials and Methods". The cytotoxicity and the membrane integrity were determined by the ratio of LDH_{out}/LDH_{in}. Compared to the control (C34), * $p>0.05$, ** $p<0.001$.

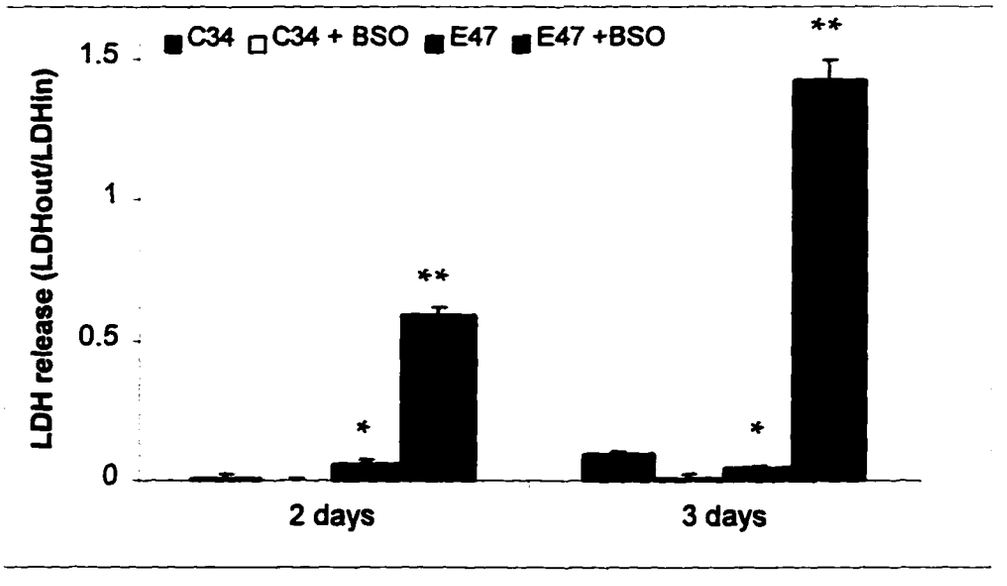


Fig. 46

Intracellular GSH levels of C34 and E47 cells after BSO treatment

Cells were cultured in the presence of 0.1mM BSO, and harvested by scraping 8 h or 24 h after BSO addition. Cells cultured in the absence of 0.1 mM BSO were collected as 0 time. The intracellular GSH level was measured as described under "Materials and Methods". Compared to the control (without BSO), * $p<0.01$, ** $p<0.001$, *** $p>0.05$.

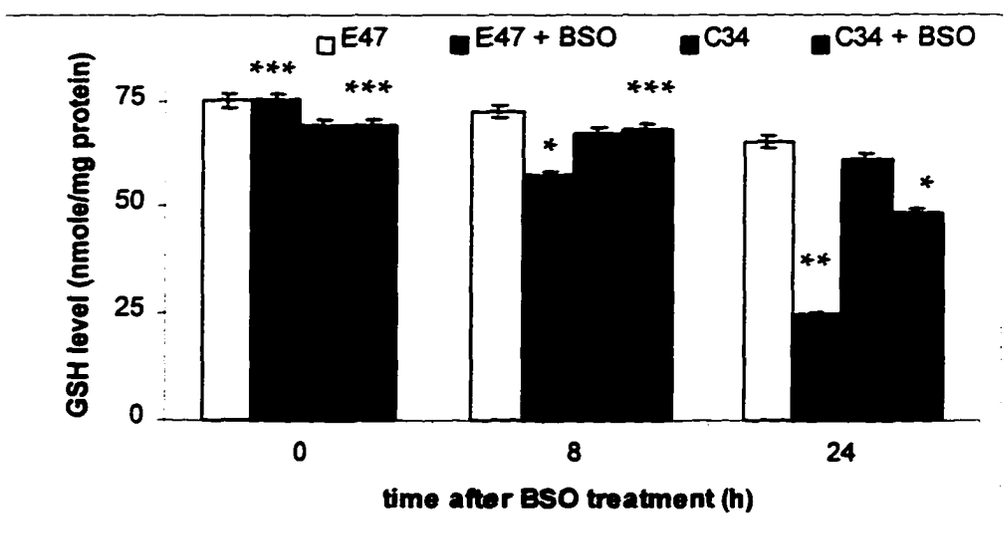


Fig. 47

Cytotoxicity in Hep G2 cells overexpressing CYP2E1 after BSO treatment

Hep G2 cells, or Hep G2 subclones were cultured in MEM in the absence or presence of 0.1 mM BSO for two days. The viability was measured using the MTT assay as described under "Materials and Methods". The net absorbance from the wells of cells cultured with control medium was taken as the 100% viability value. The percent viability of the BSO-treated cells was calculated by the formula: $(A_{570}-A_{630})_{BSO}/(A_{570}-A_{630})_{control}$ X 100. Compared to Hep G2 cell, * $p>0.05$, ** $p<0.001$.

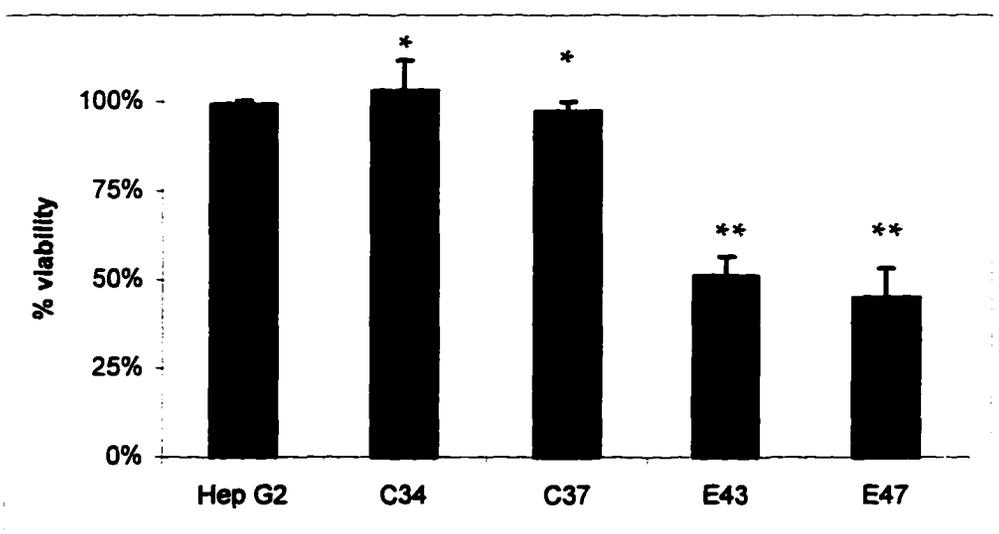


Fig. 48

Time course curve of cell viability after BSO treatment

C34 and E47 cells were cultured in MEM with or without 0.1 mM BSO for one to five days. Cell viability was measured by the MTT assay as described under "Materials and Methods". Compared to the control (without BSO), * $p>0.05$, ** $p<0.01$, *** $p<0.001$.

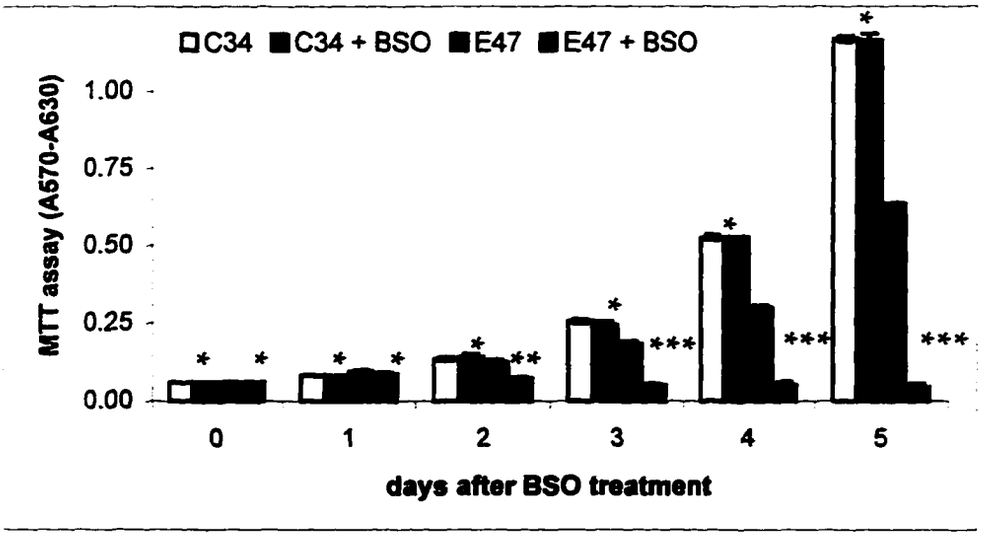


Fig. 49

Cytotoxicity in Hep G2 cells transiently expressing CYP2E1 after BSO treatment

Hep G2 cells were transiently transfected with vectors pCI-*neo* or pCI-2E1. Four days after transfection, the cells were subjected to BSO treatment for 2 days. Cell viability was measured by the MTT assay as described under "Materials and Methods". * $p < 0.001$.

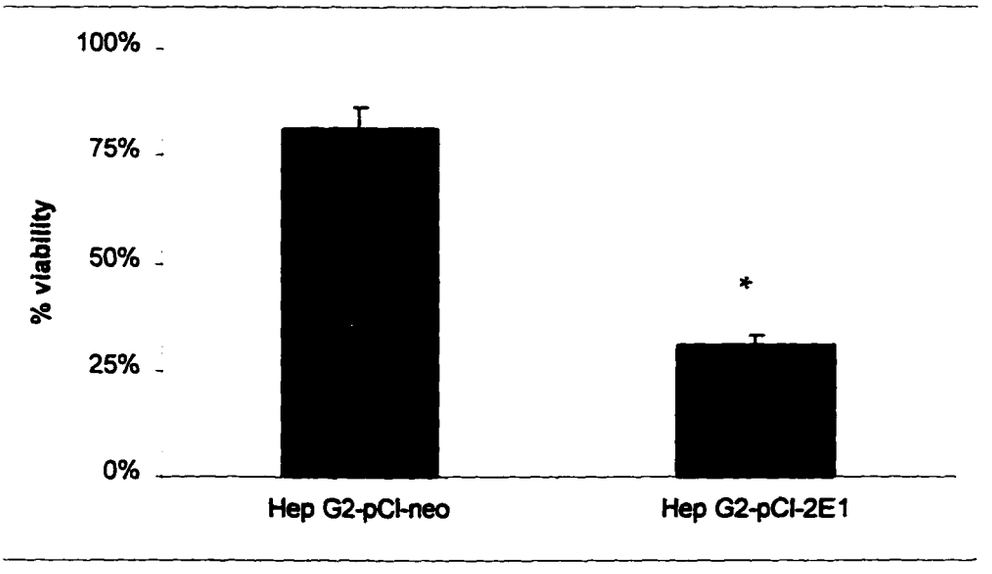


Fig. 50

Apoptosis produced in E47 cells after BSO treatment and the effect of vitamin E

C34 (lanes 1-6) and E47 (lanes 7-12) cells were treated with 0.1 mM BSO for 1 (lanes 2, 5, 8, and 11) or 2 (lanes 3, 6, 9, and 12) days with (lanes 4-6, and 10-12) or without vitamin E (lanes 1-3, and 7-9). Cells cultured in MEM in the absence of BSO were collected as controls (lanes 1, 4, 7, and 10). Cells were harvested by scraping, followed by DNA separation, and DNA ladders were visualized by agarose gel electrophoresis as described under "Materials and Methods".

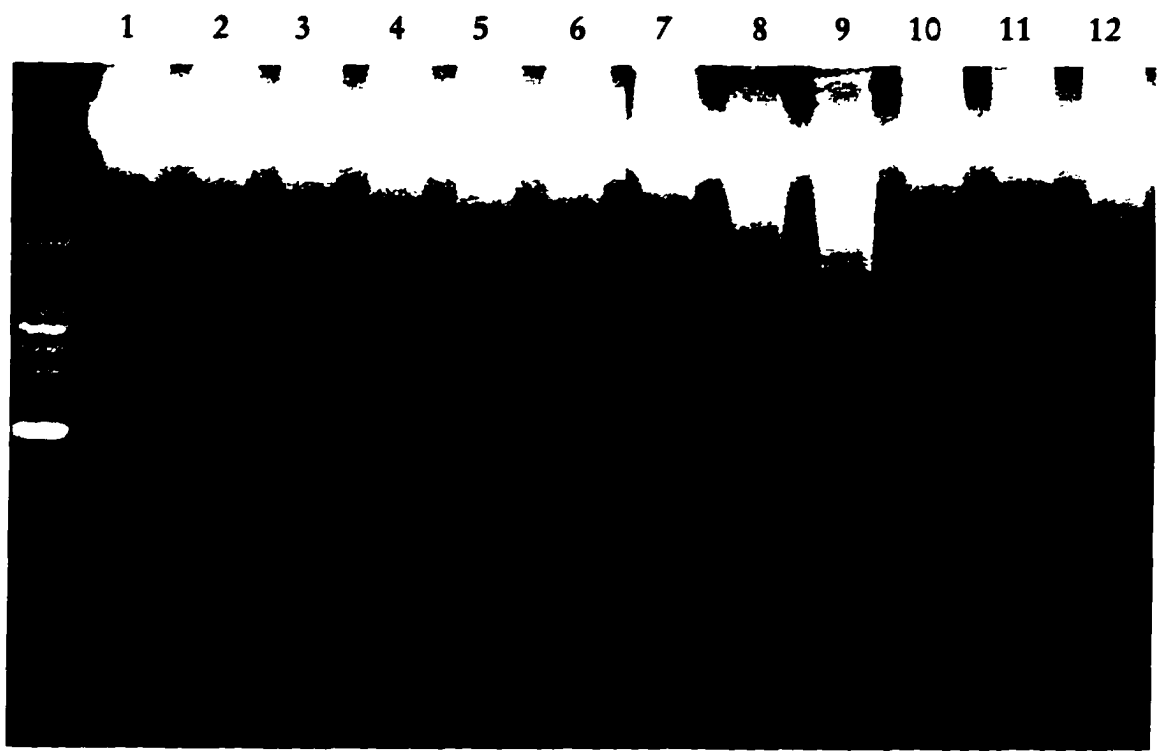


Fig. 51

Effect of antisense CYP2E1 vector on E47 growth rate

The same numbers of E47 cells were transiently transfected with pCI-*neo* or pCI-as-2E1 plasmid. Seven days after transfection, the cell numbers were counted. $p<0.001$.

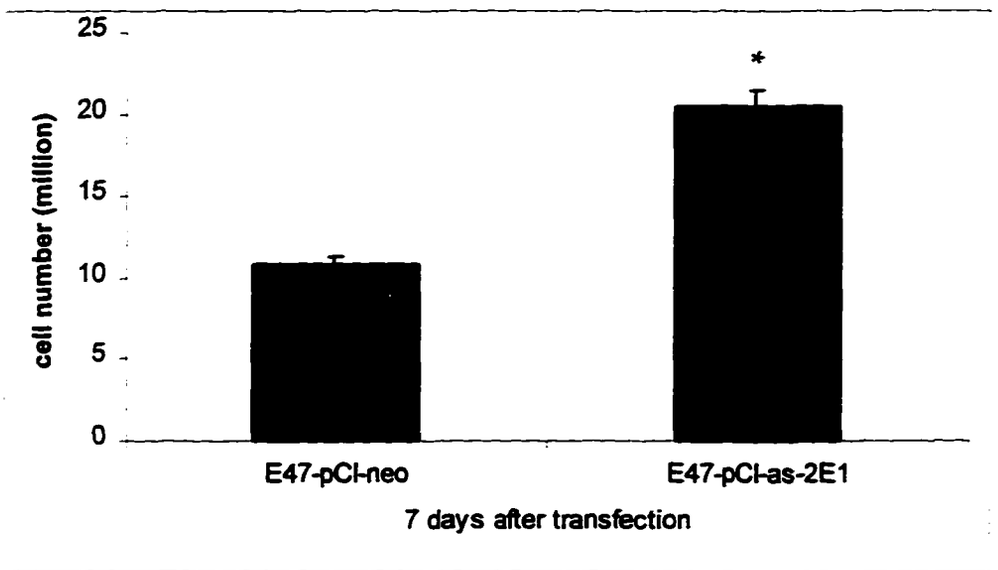


Fig. 52

Effect of antisense CYP2E1 vector on E47 viability in the presence of BSO

The same numbers of E47 cells were transiently transfected with pCI-*neo* or pCI-as-2E1 plasmid. Four days after transfection, pCI-*neo* or pCI-as-2E1 transfectants were treated with BSO for two days, followed by assaying for viability using the MTT reaction as described under "Materials and Methods". $p<0.001$.

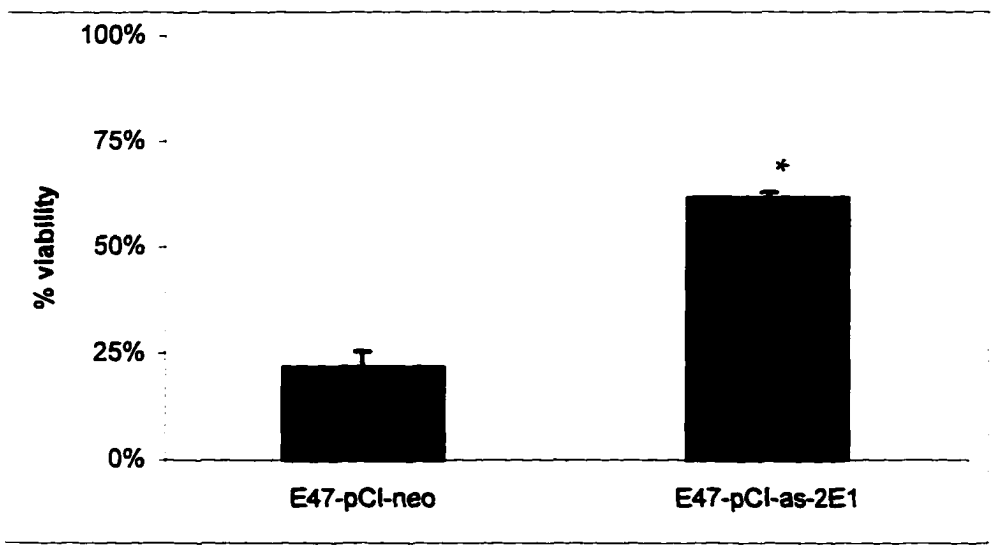


Fig. 53

Effect of 4-MP or Me_2SO on E47 viability in the presence of BSO

C34 and E47 cells were cultured in MEM with or without 4-MP or Me_2SO for two days and then subjected to 0.1 mM BSO treatment for an additional two days. Cell viability was determined by the MTT assay as described under "Materials and Methods". Compared to the no addition controls, * $p > 0.1$, ** $p < 0.05$, *** $p < 0.001$.

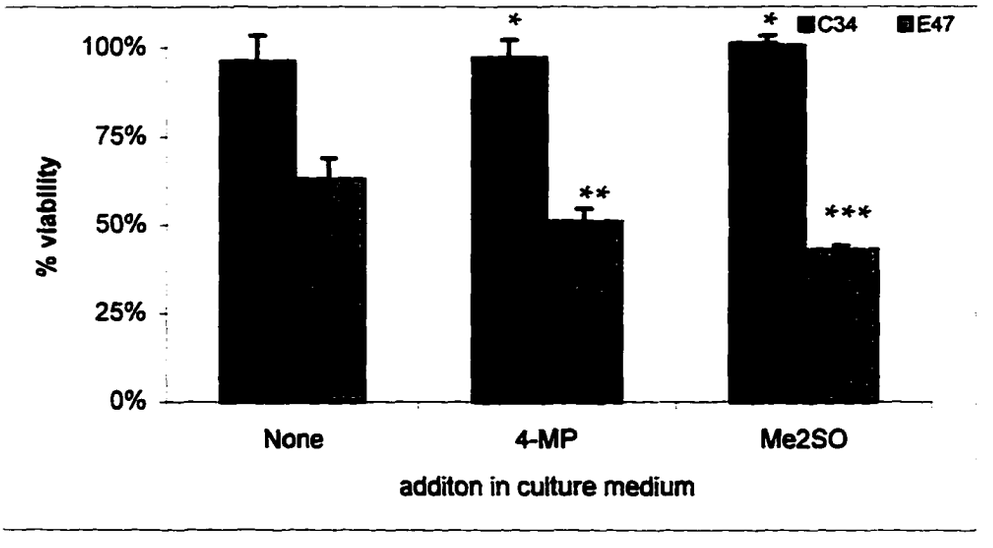


Fig. 54

Effect of antioxidants on CYP2E1 cytotoxicity

E47 cells were treated with BSO for two or four days in the absence or presence of the antioxidants, 5 μM vitamin E, or 0.2 mM vitamin C, or 20 μM trolox, followed by assaying for viability using the MTT reaction as described under "Materials and Methods". * $p < 0.001$.

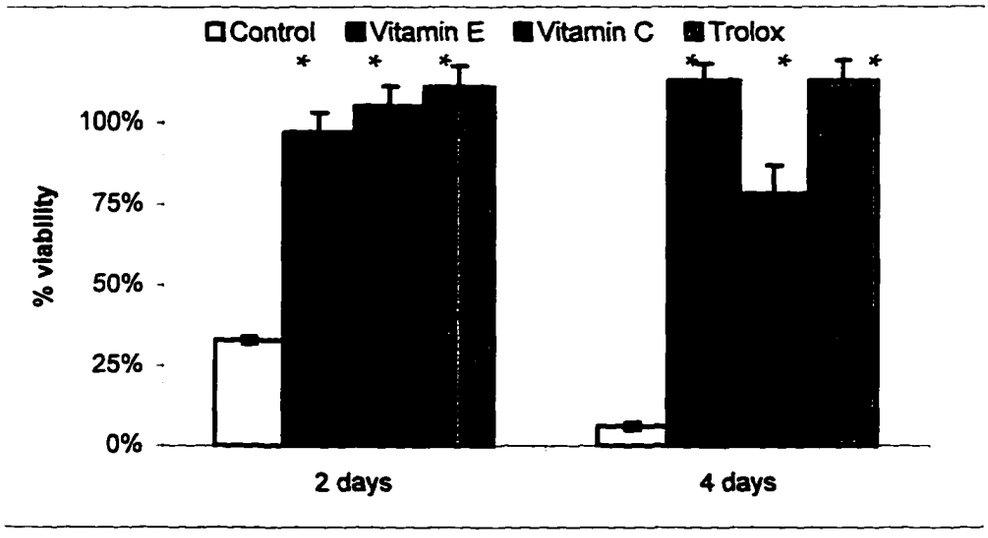


Fig. 55

Western blot analysis of Bcl-2 expression in Hep G2 subclones.

Bcl-2 expression levels in Hep G2 subclones, A14 (lane 1), A15 (lane 2), B27 (lane 3), B28 (lane 4), and C34 (lane 5), as well as parental Hep G2 cells (lane 6) were assessed by Western blot analysis with mouse anti-human Bcl-2 monoclonal antibody. A14 and A15 cells are Hep G2 subclones transfected with a vector containing anti-sense *bcl-2* cDNA, whereas B27 and B28 cells are Hep G2 subclones transfected with a vector containing a full length human *bcl-2* cDNA in the sense orientation.

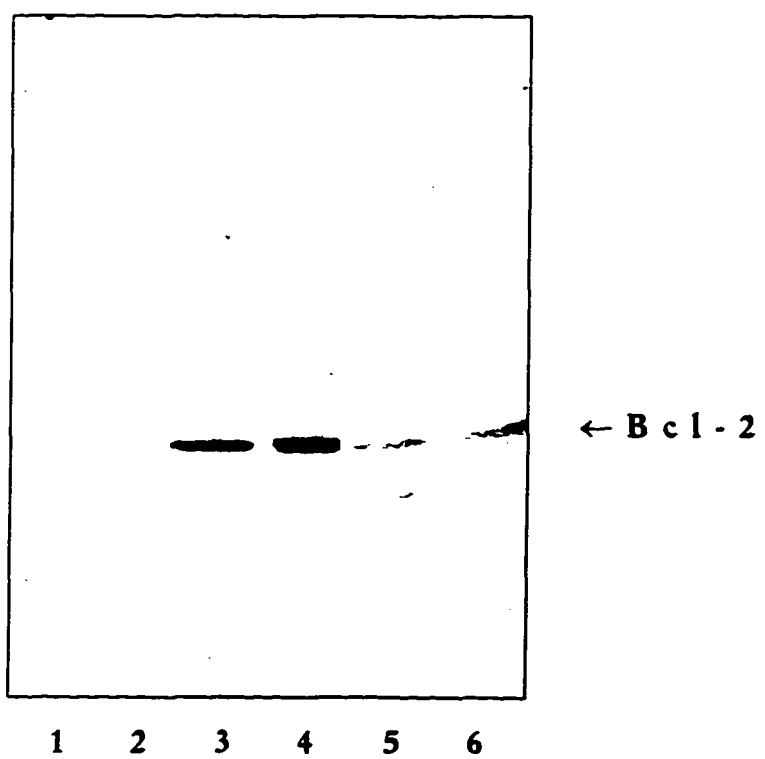


Fig. 56

CYP2E1 and Bcl-2 expression in CYP2E1 transfected B28, C34, and A14 cells

B28 (lane 1), C34 (lane 2), and A14 (lane 3) cells were transiently transfected with the same amount of pCI-2E1 vector. Four days after transfection, CYP2E1 and Bcl-2 expression levels were assessed by Western blot analysis with polyclonal rabbit anti-human CYP2E1 IgG (upper panel) or mouse anti-Bcl-2 monoclonal antibody (lower panel) as described under "Materials and Methods".

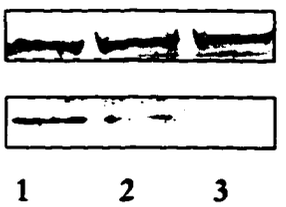


Fig. 57

Effect of *bcl-2* on CYP2E1-induced cytotoxicity

A14, B28, and C34 cells were transiently transfected with the same amount of pCI-2E1 plasmid. Four days after transfection, the transfectants were treated with BSO for two days, followed by determination of viability using the MTT assay as described under "Materials and Methods". Compared to control (C34-CYP2E1), * $p<0.05$, ** $p>0.05$.

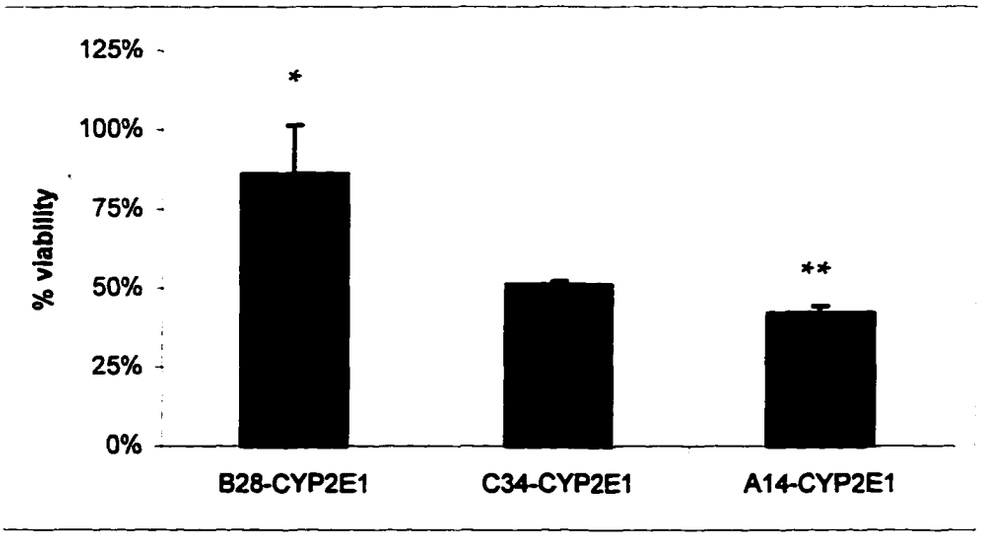
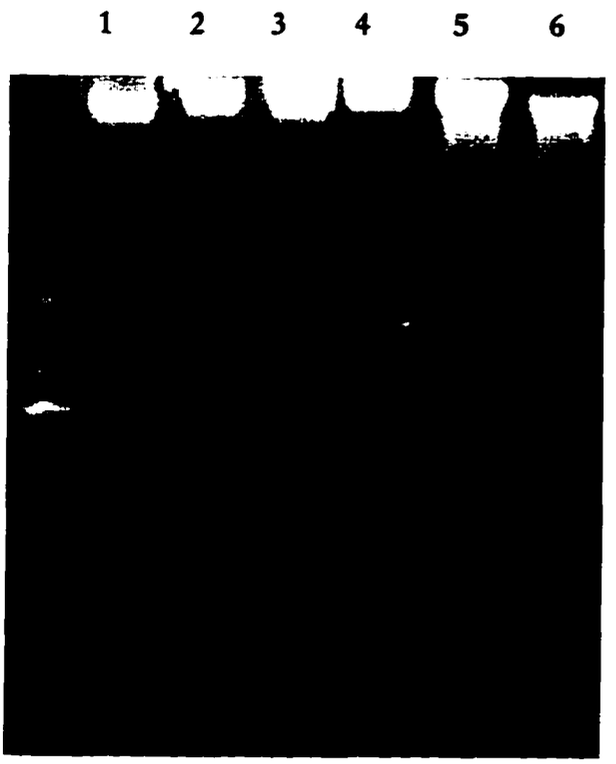


Fig. 58

Effect of *bcl-2* on CYP2E1-induced apoptosis

B28 (lanes 1 and 4), C34 (lanes 2 and 5) and A14 (lanes 3 and 6) cells were transiently transfected with the same amount of pCI-2E1 plasmid. Four days after transfection, the transfectants were treated with BSO (lanes 4-6) for two days, followed by agarose gel electrophoresis as described under "Materials and Methods".



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