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Stable expression of human cytochrome P4502E1 in HepG2 cells: Characterization of catalytic activities, cytotoxicity with acetaminophen and degradation by carbon tetrachloride

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Stable Expression of Human Cytochrome P4502E1 in HepG2 Cells: Characterization of Catalytic Activities, Cytotoxicity with Acetaminophen and Degradation by Carbon Tetrachloride

by

Yan Dai

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ABSTRACT

Stable Expression of Human Cytochrome P450 2E1 in HepG2 Cells: Characterization of Catalytic Activities, Cytotoxicity with Acetaminophen and Degradation by Carbon Tetrachloride

by

Yan Dai

Advisor: Professor Arthur I. Cederbaum

Chronic excessive alcohol consumption induces cytochrome P450-2E1 in the liver. Elevated expression of this form of P450 has been correlated with the degree of liver damage in alcoholic patients. Extensive studies have been carried out using animal models to investigate the involvement of oxygen radicals produced by P450-2E1 and the role of the P450 in the activation of a variety of compounds and hepatotoxins and the subsequent production of alcoholic liver damage. To study the function of the human isoform of P450-2E1, the following series of experiments were carried out: (**A**) A stable cell line that constitutively expresses the human P450-2E1 in HepG2 cells was established by a recombinant retroviral expression method. Western blot analysis indicated that the transduced clones produced a protein band with molecular weight of 54,000. Northern blot and Southern blot analyses confirmed the expression and that the CYP2E1 was transcribed from a single copy of CYP2E1 cDNA integrated into the chromosome. The expressed human 2E1 was functionally active and could metabolize *p*-nitrophenol, *N*-dimethylnitrosamine, aniline and ethanol in the microsomal fraction of the transduced cells. The MVh2E1-9 clone expressed the P450 at a level of about 15 pmol/mg microsomal protein. No such activities were found with the MV-5 clone in which HepG2 cells were infected with retrovirus lacking the CYP2E1 cDNA. (**B**) The microsomal fraction of this clone was able to produce superoxide radical as measured by ESR spectroscopy and generate increased levels of hydrogen peroxide as compared to the control clone, MV-5

cells. Microsomes from MVh2E1-9 cells could initiate lipid peroxidation with a low iron concentration while microsomes from MV-5 cells essentially showed no lipid peroxidation at such low concentrations of iron catalyst. This suggests that human P450-2E1 is especially reactive in production of reactive oxygen species and in catalysis of lipid peroxidation at physiologically relevant low iron concentration. (**C**) When the cells were treated with acetaminophen, a typical hepatotoxin, MVh2E1-9 cells showed a dose-dependent toxicity as measured by an LDH leakage method while no toxicity was observed with MV-5 cells. Cytotoxicity by acetaminophen required removal of cellular GSH with BSO. This toxicity could be blocked by 2E1 ligands such as 4-methylpyrazole and ethanol, by antioxidants such as *N*-acetylcysteine and by a spin trapping agent, PBN, but not by inhibitors of lipid peroxidation, suggesting the involvement of reactive species such as the benzoquinone imine which are produced during the P450-dependent metabolism of acetaminophen. Using ^{14}C-labeled acetaminophen, protein adducts were produced in the MVh2E1-9 cells. Moreover, this adduct formation was inhibited by the same agents which prevented the cytotoxicity induced by acetaminophen, suggesting a close correlation between adduct formation and cytotoxicity. Acetaminophen at a lower concentration inhibited cell proliferation of both MVh2E1-9 and MV-5; this anti-proliferative effect of acetaminophen was similar in the two cell lines and was independent of CYP2E1. Thus acetaminophen has a dual action in the MVh2E1-9 cells, a CYP2E1-independent effect which prevents cell proliferation and a CYP2E1-dependent action which results in cellular cytotoxicity. (**D**) When the cells were treated *in situ* with CCl_4, the CYP2E1 expressed in MVh2E1-9 cells was inactivated and then degraded. This CCl_4-dependent inactivation and degradation could be prevented by 2E1 ligands but not by antioxidants or inhibitors of lipid peroxidation. These actions of CCl_4 were enhanced in the presence of cycloheximide to prevent new enzyme synthesis. CCl_4 was able to form adducts with cellular macromolecules suggesting that the human P450-2E1 expressed in MVh2E1-9 cells metabolizes CCl_4, generating reactive metabolites which directly inactivate and labilize the

P450 for degradation by proteases, including lysosomal protease, present in the HepG2 cells.

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LIST OF ABBREVIATIONS

2E1	CYP2E1 or cytochrome P450-2E1 (EC 1.14.14.1)
PNP	*para*-nitrophenol
DMN	*N,N*-dimethylnitrosamine
DDC	diethyldithiocarbamate
4-MP	4-methylpyrazole
DAS	diallyl sulfide
GSH	reduced glutathione
$\cdot O_2^-$	superoxide anion radical
·OH	hydroxyl radical
SOD	superoxide dismutase
TBA	thiobarbituric acid
TCA	trichloroacetic acid
ESR	electron spin resonance
TEMPO	2,2,6,6-tetramethylpiperidinyloxy free radical
PBN	*N*-t-butyl-α-phenylnitrone
DMEM	Dulbecco's modified Eagle's medium
MEM	minimal essential medium
PBS	phosphate-buffered saline
DMSO	dimethylsulfoxide
APAP	acetaminophen or *N*-acetyl-*p*-aminophenol
NAPQI	*N*-acetyl-*p*-benzoquinone imine
BSO	*L*-buthionine sulfoximine
LDH	lactate dehydrogenase
BCNU	1,3-bis-(2-chloroethyl)-1-nitrosourea
TPMP	triphenylmethylphosphonium

INTRODUCTION

It is well known that excessive alcohol consumption can directly or indirectly cause profound toxic effects in virtually every part of the body, including the liver, cardiovascular system, immune system, endocrine system and central nervous system, and result in a series of alcohol related disorders including alcoholic liver disease such as cirrhosis and cancer, metabolic disorders such as hypoglycemia, ketosis, and gout, central nervous system degeneration, cardiovascular disorders, alterations in drug metabolism, chronic peripheral neuropathies, and alcoholic fetal syndrome (Goedde and Agarwal, 1989; Hall, 1985). The most important direct toxic effect of alcohol is on the liver. Chronic alcohol abuse is unquestionably among the most important causes of morbidity and mortality from liver disease (alcoholic hepatitis and cirrhosis) in the United States (Grant et al., 1988). Alcohol-induced liver disease usually involves three histologically distinct types of lesions: alcoholic steatosis (fatty liver), alcoholic hepatitis and fibrosis, and cirrhosis. In 1988 the highest mortality from cirrhosis occurred in nonwhite males (18.3 per 100,000), followed by white males (12.2), nonwhite females (8.6), and white females (5.1) (Grant et al., 1988). Factors contributing to the alcoholic liver damage include quantity and duration of drinking, association of prior liver diseases such as viral hepatitis, nutritional deficiencies, diets and gender difference. Diets that are high in fat, particularly unsaturated fatty acids, may increase the susceptibility of the liver to alcohol-induced damage by activating fat-storing liver cells known as Ito cells (Takahashi et al., 1989). Serious forms of alcoholic liver disease are more frequent in women than men probably because of higher peak blood alcohol levels due, in part, to a decreased gastric alcohol dehydrogenase (Frezza et al., 1990) and a slightly higher rate of hepatic metabolism of alcohol (Mishra et al., 1989) resulting in a higher rate of production of acetaldehyde, which may play a part in alcohol-induced liver injury.

Potential mechanisms of alcohol-induced liver damage include: 1) production of free radicals, 2) disturbances in antioxidant defense, 3) direct effect of acetaldehyde, 4) malproduction of cytokines, 5) altered immunologic reactions, 6) Neutrophil chemoattraction, etc. Alcohol can also cause liver injury through its effects on production of a variety of toxic substances or metabolites from other xenobiotics taken into the body (Lieber, 1988; Lieber, 1990) including DMN, acetaminophen and CCl_4. Alcohol may induce oxidative stress by production of oxygen radicals (Diluzio, 1964; Diluzio, 1968) and by lowering the antioxidant defense mechanism; these actions of alcohol are mediated presumably through the ethanol oxidizing enzyme system in the microsomes (Lieber and DeCarli, 1968; Lieber and DeCarli, 1970).

The liver microsomal multisubstrate mixed function monooxygenase (MFO) system handles the constant onslaught of foreign chemicals that are being ingested and absorbed everyday. The key enzymatic components of this system are the cytochrome P450 enzyme superfamily and its NADPH-dependent cytochrome P450 oxidoreductase (P450 reductase). Cytochrome P450-2E1 (CYP2E1[1]), the ethanol inducible form, has been receiving increasing attention because of its inducibility by acute and chronic alcoholic ingestion and its ability of metabolizing and activating numerous hepatotoxins in the liver (Koop, 1992). The induced CYP2E1 in man increases the potential risk to low level exposure to those toxins which require CYP2E1 for activation (Koop, 1992); these activated toxins will in turn cause injury which may lead to a variety of alcohol related diseases and cancer. These toxins include industrial solvents such as benzene and carbon tetrachloride, drugs such as acetaminophen and isoniazid, carcinogens and procarcinogens such as nitrosamine derivatives, as well as ethanol itself[2] (Koop, 1992). Acetaldehyde, the

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[1]CYP is the standard abbreviation for cytochrome P450. CYP2E1 or 2E1, will also be used through the text as the abbreviation for P450-2E1.

[2]Ethanol is metabolized predominantly by alcohol dehydrogenase, a NAD^+-dependent enzyme, and to a lesser extent, by hydrogen peroxide-dependent peroxidatic activity of catalase in the liver. CYP2E1-dependent ethanol oxidation constitutes the third pathway which may contribute to as much as 1/3 of total

oxidized form of ethanol, and metabolites of other toxins can directly damage the targeted tissues and cause acute toxic effects and symptoms especially when the secondary enzymes that degrade these metabolites are not induced or genetically varied (Goedde and Agarwal, 1989). Activation of carcinogens such as dimethylnitrosamine (Yang et al., 1985) by CYP2E1 has been shown *in vitro* to result in the formation of adducts with DNA causing genotoxicity (Nouso et al., 1992).

Besides the toxic effect produced from the metabolites from hepatotoxins oxidized by CYP2E1, this isoform may cause toxic effects on targeted tissues through production of oxygen radicals and initiation of lipid peroxidation. CYP2E1 has been shown *in vitro* to be a loosely coupled enzyme, which produces free radicals such as $\cdot O_2^-$ and other reactive oxygen species including H_2O_2 in higher amounts than any other P450 isoforms (Ingelman-Sundberg and Johansson, 1984). In the presence of iron, $\cdot O_2^-$ and H_2O_2 may serve as precursors of ·OH, the most reactive species on earth, via Fenton or Haber-Weiss types of reactions (Halliwell, 1978; McCord and Day, 1978). Alcohol has been proposed to cause tissue injury via lipid peroxidation presumably initiated by reactive oxygen radicals or an iron controlled mechanism (Diluzio, 1964; Diluzio, 1968). Ethanol may increase the production of oxidative stress include lowering of the protective antioxidant level including vitamin E and reduced glutathione level, damaging mitochondria and impairing respiration, conversion of xanthine dehydrogenase to xanthine oxidase, formation of acetaldehyde and hydroxyethyl radical which may also initiate lipid peroxidation. Therefore, CYP2E1 is a key enzyme that is induced by alcohol administration, activates various hepatotoxins and carcinogens in the liver, generates free radicals and other reactive oxygen species, and directly or indirectly causes damage to biological membrane via lipid peroxidation. Hence, the chain "alcohol-cytochrome P450 (2E1)-hepatotoxin-oxygen radical-tissue injury" is the theme of this thesis.

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metabolism when the blood ethanol concentration is high. This pathway is toxicologically the most important among the three.

A. Drug Metabolism and Cytochrome P450

A-1. Mixed Function Oxidation System in the Liver - Metabolism of various drugs and other xenobiotics can be carried out in organs such as the liver, lungs, kidneys and adrenal glands. However, the major site for such metabolism is the liver. Many lipid-soluble, weak organic acids or bases are not readily eliminated from the body and must be metabolized and/or conjugated to compounds that are more polar and less lipid-soluble in the liver before being excreted. Biochemical reactions involved in drug metabolism occur in two phases in the liver: phase 1 reactions (e.g., oxidation, reduction, hydrolysis) alter chemical reactivity and increase aqueous solubility; phase 2 reactions (e.g., conjugation) further increase the solubility to facilitate elimination.

Phase 1 reactions, which is the concern in this thesis, can happen in the microsomal and nonmicrosomal subcellular fractions. Microsomes, the subcellular components of the endoplasmic reticulum, are the major site for such metabolism and contain a set of membrane-associated enzymes called mixed-function oxygenases (MFO) because, as it is defined, one oxygen atom in molecular oxygen is incorporated into a substrate in the form of a hydroxyl (-OH) moiety, and the other is incorporated into water. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) is the preferred reducing agent. Several types of oxidative reactions are involved including hydroxylation, epoxidation, dealkylation, deamination, dehalogenation, and oxidative denitrosation (Mitchell and Horning, 1984), most of which are catalyzed by the combined actions of NADPH dependent cytochrome P450 reductase and cytochrome P450 (Ortiz de Montellano, 1986; Sato and Omura, 1978).

A-2. Cytochrome P450 Superfamily - Cytochrome P450 was first identified as a CO-binding pigment in the liver expressing a characteristic absorbance peak at 450 nm by Klingenberg (Klingenberg, 1958) and Garfinkle (Garfinkle, 1958) in 1958. This pigment was further characterized as a P450 hemoprotein in the following years by Omura and Sato (Omura and Sato, 1961; Omura and Sato, 1964; Omura and Sato, 1964). As the role of P450 became more and more defined, many P450 isozymes have been isolated and purified (Guengerich, 1987; Lu and West, 1979). Molecular biology, especially the cloning technology, has accelerated the discovery and characterization of more enzymes belonging to this P450 superfamily in the past decade (Gonzalez, 1989). Up till now, there are about 150 isozymes identified, cloned, and sequenced which are classified into 27 families[3], ten of which exist in mammals, six in bacteria (Nebert et al., 1991). The ten mammalian P450 families, containing 18 subfamilies, can be functionally divided into two major classes: 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. The second class of P450s is the one involved in drug metabolism as the primary component of the MFO system in the liver (Gonzalez, 1992). Subfamily E of family 2, or P450-2E, is the focus of the thesis study.

A-3. Substrate Binding Spectra of P450 - CO binds to reduced P450, giving a characteristic absorbance peak at 450 nm. Substrates can change the optical properties of P450 to create so-called substrate binding spectra. The addition of various drugs to liver microsomes produced a blue or red shift in the Soret peak of microsomal P450 as a consequence of binding of the substrate to the oxidized form of P450 (Narashimhulu et al., 1965; Remmer et al., 1966). The substrate-induced difference spectra in the Soret region have been classified into three major types (Schenkman et al., 1967): (1) type I spectra,

[3]The classification of families and subfamilies is based on sequence homology. Any P450 member from one family should have greater than 40% identical amino acids to any other member within the family and less than 40% to any member from another family. Within the same family, proteins that have greater than 55% identical amino acids are classified into subfamilies

caused by substrates such as aminopyrine, phenobarbital, hexobarbital and testosterone, display a maximum absorbance at 385 - 390 nm and a minimum at ~ 420 nm; (2) type II spectra produced by nicotine, pyridine, and aniline have a maximum at ~ 430 nm and a minimum at 390 nm; (3) the third type, so-called modified type II spectra, which are induced by cortisol, corticosterone, cyanide, alcohol, etc., have a broad maximum absorbance ranging from 409 - 445 nm and a minimum at 365 - 410 nm (Vore et al., 1974). It has been suggested (Yoshida and Kumaoka, 1975) that type I changes occur when the substrate interacts with the protein moiety of a low-spin[4] P450, while type II changes reflect interaction of the amino group of the substrate with the heme iron of either high-spin or low-spin P450, and modified type II spectra result when the substrate hydroxyl group interacts with the heme of a high-spin P450. Conversion of P450 to P420 (i.e., Soret peak shifts from 450 to 420 nm) results in loss of substrate-induced spectral changes. All the information about substrate binding spectra and spectral changes are characteristic for the interaction between the heme group, substrates and the holo-enzyme. The magnitude of the CO-reduced P450 spectrum can be used to determine the expression level of P450 in a transfected cell line.

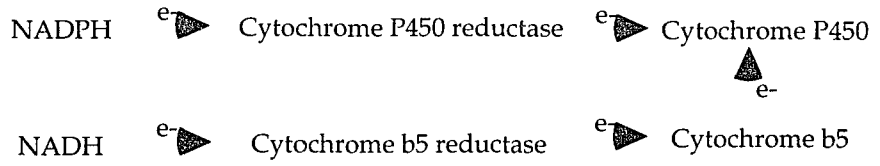
A-4. Catalytic Mechanism of P450 - Cytochrome P450 uses oxygen and NADPH via P450 reductase to catalyze the monooxygenation of a variety of substrates. The general hydroxylation reaction is

$$SH + NAD(P)H + O_2 = SOH + NAD(P)^+ + H_2O \qquad (1)$$

P450 accepts electrons from NADPH via P450 reductase (and an iron-sulfur protein for mitochondrial and bacterial species). However, the microsomal system, though preferring

[4]Spin state here refers to the energy state of ferric iron in the heme group which is affected by the distributaion of the five electrons in the five orbitals of the 3d subshell. If one electron is left unpaired it is low spin; if all five are unpaired it is high spin. The spin state is dependent on the energy of the system and the substrates (ligands) present, which is the cause the substrate binding spectra.

NADPH as reductant, can also accept electrons from NADH via cytochrome b_5 reductase, an FAD-containing flavoprotein, which reduces cytochrome b_5. Cytochrome b_5 normally transfers electrons to the cyanide-sensitive factor required for fatty acid desaturation, but can pass some of its electrons to P450 (Aoyama et al., 1990) as shown below.



A general mechanism for microsomal cytochrome P450 catalysis is shown in **Figure 1** (Coon et al., 1992; Guengerich, 1990). Though developed from the soluble bacterial $P450_{cam}$, it has been accepted, with some exceptions, by most of investigators as a model for mammalian P450. Substrate binds to the oxidized P450 with low K_m ranging from nanomolar to millimolar (step 1). This binding, with some exceptions (Guengerich, 1983), changes the spin state of the P450 from low to high (Waterman et al., 1973), which can be observed by substrate binding spectra (described above) The enzyme-substrate complex accepts the first electron from NADPH through P450 reductase (step 2). Molecular oxygen binds rapidly to ferrous P450 with a K_d of ~ 10^{-6} M and the unstable $[Fe^{2+}\text{-}O_2]$ complex can be observed by CO binding spectroscopy which measures the $[Fe^{2+}\text{-}CO]$ complex because CO has much higher affinity to heme iron than O_2. This competition between CO and O_2 forms the basis for spectral detection and quantitation of P450 in biological systems. The $[Fe^{2+}\text{-}O_2]$ complex can display a resonant structure in which an electron is transferred from the iron to oxygen producing a $[Fe^{3+}\text{-}\cdot O_2^-]$ complex. Decay of this complex produces the ferric P450-substrate intermediate, with release of superoxide radical, a reactive oxygen intermediate (Kuthan and Ullrich, 1982). The decay of oxygenated P450 appears to be a major source of superoxide radical ($\cdot O_2^-$), which can form hydroxyl radical ($\cdot OH$) in the presence of iron (discussed in details in Section C), in

biological systems (Kuthan and Ullrich, 1982) and is dependent on efficiency of input of the second electron and the form of P450. CYP2E1 has been shown in reconstitutive membrane systems to produce more hydroxyl radical than other P450 isozymes (Ingelman-Sundberg and Johansson, 1984). Enhanced superoxide radical release at this step may be a key mechanism for CYP2E1 causing toxic effects through oxygen radical production. The second electron (step 4) may come from the NADPH P450 reductase or from the reduced cytochrome b_5. Decay of peroxy P450 [Fe^{3+}-$O_2^{=}$] produces ferric P450 and hydrogen peroxide (H_2O_2). This H_2O_2 may be produced via this decay or via dismutation of $\cdot O_2^-$ (Reaction (5) in Section C). Heterolytic cleavage of the O-O bond, aided by the cysteinyl thiolate ligand (step 5), produces H_2O and the $[Fe\text{-}O]^{3+}$ complex (Dawson, 1988). This powerful oxidant abstracts a hydrogen from the substrate to yield a substrate radical (R·) and the bound equivalent of a "hydroxyl radical" (step 6). Rapid rebinding of oxygen to the substrate radical (radical recombination) yields the hydroxylated substrate (step 7) and regenerates ferric P450 (step 8).

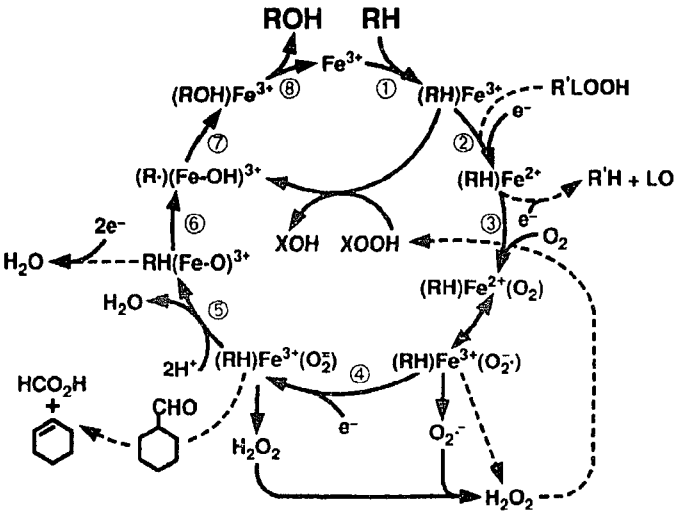


Figure 1. Overall scheme for catalytic mechanism of P450. Fe represents the heme iron atom in the active site, RH a substrate, and ROH the corresponding monooxygenation product. R'LOOH represents a lipid hydroperoxide and R'H and LO represent the corresponding reduction products (alkane and oxoacid, respectively). XOOH represents a peroxy compound that serves as an alternate oxygen donor to molecular oxygen (modified from (Coon et al., 1992)).

A-5. P450 Reductase - NADPH-dependent cytochrome P450 reductase is a FAD/FMN-containing flavoenzyme which functions, in the P450 system, as an electron transfer carrier from NADPH to P450. This enzyme, present in the microsomal membrane with a stoichiometry of 1:20 with respect to P450, can catalyze production of much more $\cdot O_2^-$ than P450 in the presence of iron, especially Fe-EDTA ($Fe^{2+} + O_2 = Fe^{3+} + \cdot O_2^-$, the reductase catalyzes regeneration of Fe^{2+}). This $\cdot O_2^-$ may lead to ·OH through an iron-catalyzed Haber-Weiss reaction. An enhanced activity of this reductase has also been reported after chronic ethanol intake (Ekström et al., 1986; Joly et al., 1973). However, the extent of this enhancement appears small compared to the changes in cytochrome P450 (Ekström et al., 1986). The ability of P450 reductase to generate oxygen radicals in the presence of iron complicates the measurement of oxygen radical production and lipid peroxidation from cytochrome P450 after alcohol administration by the conventional conditions. However, as will be shown in this thesis, we were able to distinguish the contribution of oxygen radical production from reductase or from P450 by careful adjustment of iron concentration.

B. Cytochrome P450-2E1

B-1. Functional Implication - Cytochrome P450-2E1 (CYP2E1), the ethanol inducible isozyme, was first purified from rabbits by Koop *et al.* (Koop et al., 1982) and later from rats (Palakodety et al., 1988; Patten et al., 1986; Ryan et al., 1985), and humans (Lasker et al., 1987; Wrighton et al., 1986). The cDNA for these enzymes including that of the mouse has also been cloned (Freeman et al., 1992; Khani et al., 1987; Song et al., 1986). CYP2E1 is the only enzyme in the CYP2E subfamily in almost all the species examined except rabbit, which possesses two enzymes, 2E1 and 2E2 (Khani et al., 1988). CYP2E1 is predominately expressed in the endoplasmic reticulum membrane of the liver and, to a lesser extent, in the kidneys, lungs, brain, GI tract and peripheral blood lymphocytes. In

the liver, it is distributed predominately in the pericentral (perivenous or centrilobular) region (Bühler et al., 1992; Ingelman-Sundberg et al., 1988; Tsutsumi et al., 1989). This distribution correlates with selective destruction of the centrilobular zone of the liver by toxins such carbon tetrachloride, acetaminophen or ethanol, which are oxidized by CYP2E1. CYP2E1 has been receiving increasing attention due to its capability of metabolizing and activating many toxicologically important substrates (Koop, 1992) (see **Table 1** for details). CYP2E1 has also been suggested to play a minor role in gluconeogenesis by converting acetone to acetol and methylglyoxal, which is then metabolized by glyoxylases I and II to produce D-lactate and finally pyruvate (Argeles, 1986; Caszza et al., 1984; Koop and Casazza, 1985). In the fasting human, 11% of the gluconeogenic demand could be satisfied by this acetone-derived regeneration pathway (Reichard et al., 1979). Substrates which are relatively specific for and commonly used for experimental detection of CYP2E1 include *p*-nitrophenol (Koop, 1986), aniline (Koop and Coon, 1986), as well as dimethylnitrosamine (Yang et al., 1985).

Table 1. Substrates Metabolized by CYP2E1 (modified from (Koop, 1992))

Substrates	Product	Reference
Aromatic compounds		
Pyridine	Pyridine N-oxide	(Kim et al., 1988)
3-Hydroxypyridine	2,5-Dihydroxypyridine	(Kim and Novak, 1990)
p-Nitrophenol	4-Nitrocatechol	(Koop, 1986)
Benzene	Phenol	(Johansson and Ingelman-Sundberg, 1988)
Phenol	Hydroquinone, catechol	(Koop et al., 1989)
Acetaminophen	Glutathione conjugates	(Raucy et al., 1987)
Pyrazole	4-Hydroxypyrazole	(Clejan and Cederbaum, 1990)
Chlorzoxazone	6-Hydroxychlorzoxazone	(Peter et al., 1990)
Aniline	p-Aminophenol	(Koop and Coon, 1986)
Halogenated alkanes and alkenes/alkanes		
Chloroform	Glutathione conjugates	(Brady et al., 1989)
Pentane	product not measured	(Terelius and Ingelman-Sundberg, 1986)
Ethyl carbarmate	1,N-Ethenoadenosine	(Guengerich et al., 1991)
Trichloroethylene	Chloral	(Guengerich et al., 1991)
Enflurane	Fluoride	(Hoffman et al., 1989)
Halothane	Trifluoroacetic acid	(Gruenke et al., 1988)
1,1,1,2-Tetrafluorothane	Fluoride	(Olson et al., 1990)
Alcohols/ketones/nitriles		
Ethanol	Acetaldehyde	(Koop and Coon, 1986)
Propanol	Propionaldehyde	(Koop and Coon, 1986)
Isopropanol	Acetone	(Koop and Coon, 1986)
Glycerol	Formaldehyde	(Winters et al., 1988)
Acetone	Acetol	(Koop and Coon, 1986)
Acetol	Methylglyoxal	(Koop and Coon, 1986)
Acetonitrile	Cyanide	(Feierman and Cederbaum, 1989)
Nitrosamines/azocompounds		
N,N-Dimethylnitrosamine	Formaldehyde/nitrite	(Yang et al., 1990)
N,N-Diethylnitrosamine	Acetaldehyde	(Yang et al., 1990)
Methylazoxymethanol	Methanol/formic acid	(Sohn et al., 1991)
N-Nitrosopyrrolidine	Acetaldehyde	(McCoy and Koop, 1988)
Reductive substrates		
Carbon tetrachloride	Chloroform/lipid peroxidaton	(Guengerich et al., 1991)
t-Butylhydroperoxide	Methane/acetone	(Vaz et al., 1990)
Oxygen	Superoxide/peroxide/water	(Gorsky et al., 1984)

B-2. Regulation of CYP2E1 - CYP2E1 expression appears to be regulated at several levels (Koop and Tierney, 1990; Porter and Coon, 1991). Transcriptional regulation/activation of CYP2E1 occurs at birth which is apparently associated with demethylation at the 5' region of the gene (Jones et al., 1992; Umeno et al., 1988). Messenger RNA levels reach maximum 6 days after birth and are associated with increased 2E1 protein and catalytic activity. CYP2E1 mRNA levels and acetone hydroxylase activity in the liver of pregnant rat were lower than the control suggesting that some factor(s) present during pregnancy may limit the expression of CYP2E1 (Casazza et al., 1990). Post-transcriptional mRNA stabilization has been observed in the streptozotocin-induced diabetic animal model by nuclear run on assay (Song et al., 1987). This mRNA stabilization could be reversed by insulin administration. Increased translational efficiency was seen in pyridine treated rats (Kim and Novak, 1990) presumably through an increase in polysomal 2E1 mRNA species compared to mainly monomeric species found in saline controls.

Post-translational protein stabilization (up to 20-fold) has been shown in a variety of *in vivo* and *in vitro* experimental models by various 2E1 ligands such as acetone (Koop et al., 1985; Patten et al., 1986), pyrazole (Ingelman-Sundberg and Jörnvall, 1984), imidazole (Ingelman-Sundberg and Jörnvall, 1984; Koop and Coon, 1984), and ethanol (Ingelman-Sundberg and Jörnvall, 1984; Koop et al., 1982; Ryan et al., 1986). It is a primary regulatory mechanism of the CYP2E1 level in adult animals by ethanol administration and is a major factor for alcohol-induced toxicity via CYP2E1. This type of induction by protein stabilization was initially characterized by a biphasic (half lives of 7 and 37 hours) to monophasic (half live 37 hours) half life shift after acetone treatment (Song et al., 1989). Recent observations have suggested that under certain conditions, e.g., very high blood alcohol levels, ethanol induction of CYP2E1 is associated with elevated mRNA levels and increased transcription. This induction of CYP2E1 by ethanol

may involve both transcriptional and post-transcriptional mechanisms. The ligand-bound enzyme complexes appear to be protected from a rapid degradation by uncharacterized intracellular proteolytic pathways specific for CYP2E1 (Koop and Tierney, 1990). The rapid degradative pathway appears to be regulated by hormone-regulated phosphorylation of CYP2E1 (Eliasson et al., 1988; Johansson et al., 1991). Ingelman-Sundberg and his coworkers recently proposed a potential MgATP-dependent microsomal proteolytic pathway which rapidly degrades the CYP2E1 labilized by either phosphorylation or carbon tetrachloride treatment (Eliasson et al., 1990; Eliasson et al., 1992). Other investigators found that a ubiquitin-dependent degradation pathway could be involved in the rapid degradation of CYP2E1 labilized by CCl_4 (Koop and Tierney, 1990). Therefore, it is still unclear so far as to which proteolytic pathway(s) is involved in degrading CYP2E1.

B-3. Cytotoxicity of CYP2E1- As mentioned above, most of the drugs metabolized by cytochrome P450 become more water-soluble and are more readily excreted; however, metabolism does not always inactivate the drugs. Some drugs or other xenobiotics are activated by P450 to form more reactive or toxic metabolites. CYP2E1 can activate various substrates to form cytotoxic and genotoxic substances. These substrates include industrial solvents such as benzene (Johansson and Ingelman-Sundberg, 1988) and carbon tetrachloride (Johansson and Ingelman-Sundberg, 1985), drugs such as acetaminophen (Morgan et al., 1983; Raucy et al., 1987) and isoniazid, anesthetics such as halothane (Gruenke et al., 1988) and enflurane (Hoffman et al., 1989), carcinogens and procarcinogens such as dimethylnitrosamine (Yang et al., 1985), azoxymethane (Sohn et al., 1991) and nitrosopyrrolidine (McCoy and Koop, 1988), as well as alcohols (Koop, 1992) (**Table 1**).

The other mechanism by which CYP2E1 may cause toxic effects is production of reactive oxygen intermediates such as superoxide radical (from the decay of the resonance

form of [Fe^{2+}-O_2] after the third step in the P450 catalytic mechanism described above in Section 1), which may result in production of hydroxyl radical and initiation of lipid peroxidation and eventually toxicity. Indeed, chronic alcohol intake has been shown in rats to enhance the production of reactive oxygen species such as superoxide (Boveris et al., 1983; Ekström and Ingelman-Sundberg, 1989), hydrogen peroxide (Ekström and Ingelman-Sundberg, 1989; Lieber and DeCarli, 1970; Thurman, 1973), and hydroxyl radical (Dicker and Cederbaum, 1987; Klein et al., 1983) by isolated microsomes. However, it is unclear whether production of the reactive oxygen species in ethanol-induced microsomes can be directly attributed to CYP2E1. The concepts and chemistry of these reactive oxygen intermediates are summarized below.

C. Reactive Oxygen Species and Their Measurement

A free radical is a species which contains an unpaired electron in an outer orbital. Such radicals may be neutral, e.g., vitamin E, glutathione radical; positively charged, e.g., paraquat radical; or negatively charged, e.g., superoxide radical (Cederbaum, 1992). Free radicals are very reactive because of the tendency of the unpaired electron to pair with another electron. It is now clear that the toxicity of molecular oxygen is not due to oxygen *per se*, which by itself possesses weak reactivity, but is due to the production of reactive oxygen intermediates such as superoxide radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\cdot OH$) (Cederbaum, 1992).

C-1. Interrelationships between $\cdot O_2^-$, $\cdot OH$, and H_2O_2 - As described in the P450 catalysis mechanism, a loosely-coupled[5] enzyme such as CYP2E1 may generate superoxide radical ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2) from [Fe^{3+}-$\cdot O_2^-$] and [Fe^{3+}-$O_2^=$]

[5] P450 enzymes such as CYP2E1 are considered "loosely-coupled" because it consumes NADPH even in the absence of its substrates. The electrons from NADPH are used to reduce oxygen molecules producing free radicals. Other P450 enzymes such as bacterial camphor P450 do not consume NADPH in the absence of camphor, and therefore are tightly-coupled.

respectively. P450 reductase, in the presence of iron, can also catalyze the formation of $\cdot O_2^-$ by donating one electron to the oxygen molecule (described above). These $\cdot O_2^-$ radicals can produce hydroxyl radical ($\cdot OH$), the most reactive oxygen species in the world, by a Haber-Weiss type of reaction (Halliwell, 1978; McCord and Day, 1978):

$$\cdot O_2^- + H_2O_2 = \cdot OH + OH^- + O_2 \qquad (2)$$

Although thermodynamically feasible, this reaction is kinetically very slow and therefore has been replaced by the proposed metal-catalyzed Haber-Weiss reaction (reaction 3 and 4). Reaction 4 is also called the Fenton reaction.

$$Fe^{3+}(Cu^{2+}) + \cdot O_2^- = Fe^{2+}(Cu^{+}) + O_2 \qquad (3)$$

$$Fe^{2+}(Cu^{+}) + H_2O_2 = Fe^{3+}(Cu^{2+}) + \cdot OH + OH^- \qquad (4)$$

In both reactions, iron acts as a redox cycling catalyst. Two $\cdot O_2^-$ dismute spontaneously or as catalyzed by superoxide dismutase (SOD), to produce H_2O_2 (reaction 5), which can be further degraded into H_2O via catalatic or peroxidatic modes of catalase (reaction 6a and 6b), or via the GSH peroxidase system (reaction 7). GSH can be regenerated from GSSG by GSH reductase.

$$\cdot O_2^- + \cdot O_2^- + 2H^+ = O_2 + H_2O_2 \qquad (5)$$

$$H_2O_2 + H_2O_2 = 2H_2O + O_2 \qquad (6a)$$

$$H_2O_2 + AH_2 = 2H_2O + A \qquad (6b)$$

$$H_2O_2 + 2GSH = 2H_2O + GSSG \qquad (7)$$

C-2. Electron Spin Resonance Spectroscopy - Measurement of free radical production has been carried out extensively using spectrophotometry, HPLC and gas chromatography and using chemical compounds which react with the radicals as the probe. The ESR technique, however, has been claimed to be more sensitive and specific and is the only method to detect certain radicals such as the hydroxyethyl radical. Therefore, ESR was chosen as the method to be used in this study to measure production of $\cdot O_2^-$ and ·OH from the HepG2 clones. The basic theory about ESR is briefly summarized below.

An electron possesses a spin magnetic moment that can assume two orientations in space and thus should interact with a magnetic field. In most chemical compounds, electrons are paired so that the net magnetic moment is zero and there is no condition of resonance for absorbance or radiation. However, there do exist substances that possess an unpaired electron such as certain ions of transition elements, excited photochemical intermediates, molecules like O_2 that have a triplet ground state, as well as free radicals. Such substances are called paramagnetic and many can be detected by electron spin resonance (ESR) or electron paramagnetic resonance (EPR) spectroscopy. The paramagnetic moment of the unpaired electron in these substances can be split into two nonequal energy sublevels under an applied magnetic field. Resonance occurs when the magnetic frequency ν corresponds to the energy of separation of the two electron sublevels ΔE, $\Delta E = g\beta H = h\nu$ where h is the Plank constant, β is the Bohr magneton which equals $eh/4\pi m$, H is the strength of magnetic field and g is a factor which identifies the radical (Atherton, 1979; Wertz and Bolton, 1974).

A normal ESR spectrum consists of a cluster of lines, called hyperfine structure (see **Figure 9** for example), which are a result of the interaction of the magnetic moment of the unpaired electron and the magnetic fields of nearby spinning nuclei. Splitting pattern and the distance between individual lines are characteristic of each radical. The number of

lines per cluster is determined by the number of possible orientations of the magnetic moment of the particular nucleus with which the electron interacts. In general, if the spin quantum number of the nucleus is n, the ESR spectrum has a cluster of 2n + 1 lines of equal intensity. The spacing between adjacent lines of a cluster is constant for a particular cluster but differs for each molecular type. If there are n equivalent nuclei that the unpaired electron interacts with, the spectrum consists of n + 1 lines, whose intensity distribution is given by the coefficients of the binomial distribution (Atherton, 1979; Wertz and Bolton, 1974).

In biological systems, highly reactive species such as $\cdot O_2^-$ and ·OH are very difficult to detect directly by ESR because of their very low steady-state concentration. However, such reactive radicals can be detected by employing the spin-trapping technique (Manzen and Haire, 1990; Motley and Mason, 1989). The principle of spin-trapping is that the unstable reactive free radical reacts with the spin-trap, e.g., DMPO, PBN and 4-POBN for detection of oxygen radicals, to produce a product which is relatively more stable, therefore has a longer lifetime, and can accumulate to a measurable concentration (Finkelstein et al., 1980; Yamazaki et al., 1990). ESR and spin trapping techniques are the basis for detection of superoxide and hydroxyl radical production in this study.

C-3. Lipid Peroxidation - One important mechanism by which reactive oxygen intermediates damage cells appears to be via peroxidation of membrane lipids (Diluzio, 1964; Diluzio, 1968). The basis for lipid peroxidation is that the allylic divinyl methane groups of polyunsaturated fatty acids are very sensitive to hydrogen abstraction by ·OH and other oxidants to yield the lipid radical, L·. Propagation of L· via interaction with oxygen and with other lipids readily occurs with the subsequent breakdown of membrane lipids before termination (Farber et al., 1990; Horton and Fairhurst, 1987; Tribble et al., 1987). Metals such as iron often play a central role in the lipid peroxidation process (Aust

et al., 1985; Ryan and Aust, 1992). Antioxidants such as vitamin E, which is present in most biological membranes, can also react with lipid radicals to cause chain termination (Cederbaum, 1992).

Initiation	$LH + \cdot X$ (a radical or oxidant) $= L\cdot + H_2O$	(8)
	$L\cdot + O_2 = LOO\cdot$	(9)
Propagation	$LOO\cdot + LH = LOOH + L\cdot$	(10)
	$LOOH + Fe^{2+} = LO\cdot + Fe^{3+} + OH^-$	(11)
	$LOOH + Fe^{3+} = LOO\cdot + Fe^{2+} + H^+$	(12)
Termination	$LOO\cdot + L\cdot = LOOL$	(13)
	$L\cdot + \text{Vit E} = LH + \text{Vit E}\cdot$	(14)

Although extensive investigation has been done in the past twenty years on lipid peroxidation produced by ethanol in *in vivo* models (Diluzio, 1964; Diluzio, 1968) and various *in vitro* and reconstitutive experiments with ethanol-treated microsomes, NADPH P450 reductase, P450 and reductase plus P450 (Cederbaum, 1987; Cederbaum, 1989), the ability of ethanol to cause lipid peroxidation is still controversial (Dianzani, 1985) probably because of numerous variables existed in the experimental system and the actual parameters being measured (Cederbaum, 1989). Therefore, it is important to re-evaluate this theory in another system containing only CYP2E1, which is the only known ethanol-inducible enzyme in microsomes. This system was created by employing molecular approaches. Lipid peroxidation was measured by assaying for TBA-reactive substances such as malondialdehyde, which is a major breakdown product of lipid hydroperoxides (Buege and Aust, 1978).

D. Expression of Cytochrome P450

Most of the investigations of human CYP2E1 were performed with isolated liver microsomes, which contain various other, characterized or uncharacterized, forms of P450, or with the purified enzyme, which lacks the integrity of the natural membranous environment for the P450 because mammalian cytochrome P450 is associated with membranes of microsomes or mitochondria, where it can interact with P450 reductase. The efficiency of this interaction may be very important because in naturally existing loosely coupled enzymes such as CYP2E1, free radicals can be generated presumably due to slow transfer of the second electron from the reductase or cytochrome b_5 which may result from inefficient coupling within the complex. In addition, in reconstituted systems, the most effective ratios of reductase and P450 are very different than the ratios existing in the endoplasmic reticulum, generally 1 reductase per 10 or 20 P450s (Miwa and Lu, 1978). To determine the true biochemical properties of the enzyme it would be advantageous to develop a pure enzyme system free of other forms of P450. This could be accomplished by the *in vitro* expression technology. A number of systems have been used to express mammalian cytochrome P450 in bacteria, yeast, insect cells, and mammalian cells via transfection or transformation of plasmid DNA or infection of recombinant viruses including vaccinia virus, and baculovirus (Waterman, 1991). Stable expression of P450 has also been accomplished using Chinese hamster cells (Doehmer and Oesch, 1991) and a human B lymphoblastoid cell line (Crespi, 1991).

In the present study, the retrovirus mediated stable transduction (Battula, 1989; Nouso et al., 1992) was used to express human CYP2E1 in HepG2, a human hepatoma derived cell line, because of the precise transmission of the recombinant viral DNA sequences into the target cells (Battula, 1989). The reason we chose the HepG2 cell line is that it is a human liver-derived cell line containing the P450 reductase and cytochrome b_5.

HepG2 does not produce significant (spectrally detectable) amounts of P450 (Patten et al., 1992; Waxman et al., 1991). However, there have been some reports showing that HepG2 cells have certain catalytic activities presumably from endogenous cytochrome P450 isoforms. These activities include catalyzing metabolism of benzopyrene (Diamond et al., 1980), ethoxyresorufin, pentoxyresorufin and benzoxyresorufin (Grant et al., 1988) and ethoxycoumarin (Sassa et al., 1987) to polar products, and conversion of 2-aminoanthrecene, benzidine, acetylbenzidine and aflatoxin B_1 to mutagenic derivatives (Zhou et al., 1986). These activities from HepG2 cells were very low as compared to corresponding activities from human liver samples. For example, arylhydrocarbon hydroxylase and 7-ethoxycoumarin de-ethylase activities from HepG2 cells were a few pmol/hr/mg microsomal proteins (Sassa et al., 1987). There was also a report showing expression of P450-3A in HepG2 cells (Schuetz et al., 1993). However, there have not been any reports showing the constitutive expression of CYP2E1 in HepG2 cells. Therefore, HepG2 was chosen as the target cell line to express the human CYP2E1. In this study, microsomes of successfully transduced clones were used to characterize CYP2E1 biochemical properties, including oxygen radical production and lipid peroxidation. Intact cells from the clone were also used to test other properties associated with CYP2E1 as well as cytotoxicity *in situ* caused by hepatotoxins. The transient expression systems such as SV40-COS cell (Clark and Waterman, 1991) or vaccinia virus (Gonzalez et al., 1991), although of value, are not as appropriate for aspects of this study which requires a long-term production of the enzyme in a stable environment without interference which might be caused by the process of expulsion of the plasmid or cell death.

E. Hepatotoxicity of Acetaminophen

Acetaminophen (APAP) is a widely used over-the-counter drug with analgesic and antipyretic activity. Although considered safe at recommended therapeutic doses, APAP,

when administered in excess, can cause severe centrilobular hepatic necrosis (Black, 1984; Hinson, 1980; Prescott, 1983). At a normal dosage level, the majority of APAP is metabolized via glucuronidation and sulfation in the liver and excreted out of the body (Jollow et al., 1973; Mitchell et al., 1973). A small amount of APAP is metabolized through a third metabolic pathway, in which APAP is bioactivated by hepatic cytochrome P450 to form a reactive intermediate, *N*-acetyl-*p*-benzoquinone imine (NAPQI), which is believed to play an important role in APAP-induced toxicity (Dahlin et al., 1984; Potter et al., 1973; Raucy et al., 1989). At typical doses of APAP, only a trace amount of NAPQI is formed; this reactive metabolite can be conjugated with and reduced by intracellular GSH and therefore displays no significant toxicity. However, at high dosage levels, or under conditions of enhanced activation by cytochrome P450, intracellular GSH is rapidly depleted (Albano et al., 1985; Moore et al., 1985) and can no longer compensate for the massive production of NAPQI, which subsequently can initiate lipid peroxidation (Albano et al., 1983; Younes et al., 1986), form adducts with cellular proteins (Pumford et al., 1989), damage nuclear DNA (Hongslo et al., 1988), and eventually alter intracellular homeostasis; these effects result in severe necrosis of the liver (Landon et al., 1986; Moore et al., 1985; Ray et al., 1993).

Several P450 isozymes including CYP1A2 (Raucy et al., 1989), CYP3A4 (Thummel et al., 1993), and CYP2E1 (Raucy et al., 1989) have been shown by experiments involving antibody inhibition and reconstitutive systems with purified enzymes to be responsible for the bioactivation of APAP. These three isozymes were also confirmed in a transient transfection study using a vaccinia virus expression system to have high APAP metabolic activity compared to eight other human P450 isozymes which were expressed (Patten et al., 1993). In animal model studies, administration of ethanol, a CYP2E1 inducer, increased binding and metabolism of APAP in the rat (Prasad et al., 1990). Similarly, isoniazid, another CYP2E1 inducer, potentiated APAP hepatotoxicity

(Burk et al., 1990). Perfused livers from ethanol-treated rats show enhanced vulnerability toward APAP and extensive GSH depletion in the perivenous region where CYP2E1 is induced (Anundi et al., 1993). Hepatic necrosis has been observed in chronic alcoholics after therapeutic doses of APAP administration (Pezzano et al., 1988). APAP toxicity in the kidneys of male mice has also recently been correlated with CYP2E1 (Hu et al., 1993). Therefore, CYP2E1 appears to be a key component which activates APAP and hence mediates APAP toxicity *in vivo* and *in vitro*.

To investigate further the potential cytotoxic effect of APAP via bioactivation solely by human CYP2E1 under controlled conditions, the MVh2E1-9 cell line, which was established in this study to express human CYP2E1 in HepG2 cells, was used. The control MV-5 cell line, which was infected with retrovirus lacking the 2E1 cDNA and did not display detectable CYP2E1 on immunoblots or catalytic activity with typical CYP2E1 substrates, was used as a control cell line identical to MVh2E1-9 except for the expression of CYP2E1. The interaction of APAP with these two cell lines was therefore compared to assess the contribution by CYP2E1 to changes in cellular viability and cell proliferation induced by APAP. The mechanism of APAP toxicity was evaluated by studying the effect of CYP2E1 ligands and inhibitors, antioxidants, and by measuring protein adduct formation.

F. Degradation of Cytochromes P450

The cytochromes P450 are a superfamily of hundreds of hemoproteins that serve as the terminal oxidases in the metabolism of a variety of endogenous substrates and xenobiotics (Gonzalez, 1989). The various forms of P450 are different in their distinct and yet overlapping substrate specificities and their characteristic regulation of expression at different levels (Gonzalez, 1989; Porter and Coon, 1991). Cytochrome P450 2E1, the

alcohol-inducible isozyme, has been shown to be able to metabolize and activate many toxicologically important compounds such as acetaminophen, DMN and CCl_4 (Koop, 1992). As a poorly-coupled enzyme, CYP2E1 is able to generate reactive oxygen intermediates and initiate lipid peroxidation which may directly contribute to alcoholic hepatotoxicity (Albano et al., 1988; Albano et al., 1991; Ekström et al., 1986; Gorsky et al., 1984).

Regulation of CYP2E1 expression has been extensively studied and shown to occur at various levels including gene transcription, post-transcriptional mRNA stabilization, translational, and post-translational enzyme stabilization (Gonzalez, 1989; Porter and Coon, 1991). The post-translational regulation of CYP2E1 is reflected by the balance of the enzyme stabilization and degradation. Although most CYP2E1 substrates induce its enzyme activity and protein by stabilizing the holoenzyme, some of these substrates labilize and enhance enzyme degradation. For example, treatment of adult rats *in vivo* or cultured hepatocytes *in situ* with acetone or ethanol increased 2E1 half-life (Song et al., 1989), whereas similar treatment of mice with CCl_4 and 3-aminotriazole resulted in rapid loss of PNP hydroxylase activity and CYP2E1 protein (Tierney et al., 1992). While CCl_4 can cause rapid loss of enzyme activity for several P450s including 1A, 2C and 2E1, it only induces a significant loss of protein for 2E1 (Sohn et al., 1991). The mechanism by which CCl_4 labilizes CYP2E1 is not clear and may involve metabolites produced via bioactivation of CCl_4 or CCl_4-induced lipid peroxidation or nonspecific CCl_4-induced cytotoxicity.

In this study, we will use our MVh2E1-9 cell line, which constitutively expresses human CYP2E1 in HepG2 cells, to initiate studies on the CCl_4-induced inactivation and degradation of CYP2E1. This cell line expresses CYP2E1 at a level of about 15 pmol per mg microsomal protein, and microsomes isolated from these cells are catalytically active in oxidizing PNP, DMN, aniline and ethanol. The control MV-5 cell line, which was infected

with retrovirus lacking the 2E1 cDNA, did not display detectable CYP2E1 on immunoblots or catalytic activity with the above substrates. We found that CCl_4 could inhibit microsomal PNP oxidation and induce 2E1 degradation; the latter could be completely or partially prevented by 2E1 ligands such as 4-MP, DMN and ethanol. Adduct formation from ^{14}C-labeled CCl_4 was measured to evaluate reactivity of the CCl_4 metabolites. These results suggest that the human CYP2E1, like its counterpart in rat, may be labilized by CCl_4 metabolic intermediates produced via 2E1 and subjected to a proteolytic system present in the HepG2 cells.

MATERIALS AND METHODS

A. Materials and General Methods

A-1. Bacterial Strains and Growth Conditions - Several bacterial strains were used in this study. XL1-Blue (Stratagene), *E. coli* strain (recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac, [F' proAB, lacI^{q}ZΔM15, Tn10(tet^r)]) is commonly used for transformation and propagation of plasmid constructs. It carries the F' episome encoding for pili that allows infection by filamentous M13 phage or the pBluescript II phagemid vectors. The F' episome contains the lacI^{q}ZΔM15 mutation which provides α-complementation of the β-galactosidase gene, which allows blue/white color selection of recombinant colonies or plaques on plates supplemented with X-gal and IPTG (Bullock et al., 1987). XL1-Blue was grown on LB plates or LB liquid medium with or without appropriate amount of antibiotics such as 60 μg/ml of ampicillin.

A-2. Cell Lines and Culture Conditions - NIH 3T3-derived amphotropic retrovirus packaging cell line, PA317 (Miller and Buttimore, 1986) was provided by Dr. H. Stulhmann (Mount Sinai School of Medicine, New York, NY). This cell line, containing helper virus DNA that has several alterations in addition to deletion of the packaging signal, features high titer of virus production but extremely low titer of production helper virus after introduction of retrovirus vector. HepG2, a human liver carcinoma cell line (Aden et al., 1979), was provided by Dr. G. Acs's laboratory (Mount Sinai School of Medicine, New York, NY). PA317 and HepG2 were grown in Dulbecco's modified Eagle's medium (DMEM) and minimum essential medium (MEM) respectively supplemented with 1% PSN antibiotics mixture (Gibco/BRL) and 10% fetal calf serum (Sigma). For selection of G418 resistance, 0.5 mg/ml and 0.4 mg/ml of the active compound of G418 (Gibco/BRL) were

used on PA317 and HepG2 respectively. Half concentration of G418 was used to maintain the selected pools and clones.

A-3. Subcloning I: Digestion, DNA Fragment Purification and Ligation - All plasmids were digested with the appropriate amount of restriction endonuclease and buffer purchased from either New England Biolabs or Promega at suggested temperature and for a sufficient period of time. DNA insert in agarose gels was purified by the method of Vogelstein *et al.* (Vogelstein and Gillespie, 1979) with GeneClean II kit (BIO 101) and, for TBE containing gel, the TBE modifier solution. Briefly, agarose gel slab containing the DNA is added into 3 volumes of NaI solution, melted at 50°C for about 5 minutes. Completely resuspended glass milk (5 μl for 5 μg or less DNA) was added into the mixture. The DNA-bound glass milk was washed three times with the washing solution provided with the kit before elution with sterile H_2O at 50°C. Vectors linearized with one restriction enzyme were extracted with phenol-chloroform and directly precipitated with 2.5 volumes of ethanol plus 0.1 volume of 3 M sodium acetate, pH 5.2, at -20°C for overnight or -70°C for at least 1 hour without further gel purification. Ligation was carried out with an insert to vector molar ratio of 3:1 with Klenow enzyme (New England Biolabs) in the provided buffer at 12 - 16°C for 4 hours to overnight.

A-4. Subcloning II: Transformation - Competent bacteria were made according to the method of Hanahan (Hanahan, 1983). Briefly, bacteria grown in SOB medium to the early log phase (i.e., reaching $O.D._{600}$ of 0.4) were harvested and centrifuged at 3,000 rpm in a Sorvall RCS centrifuge with the SS34 rotor at 4°C. Bacterial pellets were treated with 1/3 volume of FSB, left on ice for 1 hour, and centrifuged as before at 4°C. The final pellets were resuspended in 1/12 volume of FSB and immediately kept at -70°C. The quality of competent bacteria would directly effect transformation efficiency and therefore the procedures should be carefully followed. Ampicillin agar plates were prepared in advance

by adding ampicillin stock (50 mg/ml) to cooled (around 55 - 60°C) autoclaved LB-agar solution to make a final concentration of 60 μg/ml, followed by immediately pouring into petri dishes (about 25 ml per plate). For transformation, an appropriate amount of ligation reaction was mixed with 50 - 100 μl of competent bacteria which had been treated with β-mercaptoethanol (0.2% final concentration) for 30 minutes, heat-shocked at 42°C for 1 minute, and shaken at 37°C for 1 hour with an additional 0.9 ml of SOC medium. The mixture was then briefly centrifuged to condense the bacteria into about 0.1 ml, which was then spread onto ampicillin agar plates. Overnight incubation at 37°C should result in ampicillin resistant colonies. If color selection for pBLUEscript plasmid was desired, prior treatment of the ampicillin agar plates with 4 μl 20 mg/ml X-gal and 40 μl 20 mg/ml IPTG was required before transformation. White (not blue) colonies should be the ones with the plasmid containing insert.

A-5. Plasmid DNA Preparations - Two methods for plasmid preparations were used in this study for different purposes. Mini-prep was a modified quick alkaline lysis method. Briefly, 3 ml of an overnight-cultured bacteria pellet was resuspended in 100 μl Solution I (0.9% glucose, 25 mM Tris, and 10 mM EDTA, pH 8.0) supplemented with 10 μl 50 mg/ml lysosyme stock for 10 minutes on ice. Then 200 μl of Solution II (0.2 N NaOH and 1% SDS) were added to the mixture to break the bacterial cell wall and release the protein and DNA. Finally, 200 μl of Solution III (3 M potassium acetate) was added and incubated on ice with the mixture for about 15 minutes, which was then centrifuged to remove most of the macromolecules except the plasmid DNA, RNA and some proteins. Treatment with RNase A at 37°C for 1 hour and proteinase K at 37°C for 1 hour followed by further phenol-chloroform extractions and ethanol precipitation degrades and removes most of the contaminants (RNA and proteins).

Large-prep was the alkaline lysis/CsCl density gradient centrifugation method of plasmid DNA preparation that results in a large amount of high quality supercoiled plasmid DNA, which can be used for transfection in tissue culture. The initial steps were similar as described for the mini-prep. Briefly, for 500 ml culture, 20 ml of solution I and 40 ml of solution II and III were needed. At Solution III step, the crude prep was loaded into ultracentrifuge tubes with 1 mg/ml CsCl solution and 20 μg/ml ethidium bromide. Tubes were sealed and centrifuged at 38,000 rpm in a Ti-60 rotor at 20°C for 48 hours. Plasmid bands were collected, extracted with isopropanol to remove ethidium bromide, and precipitated with 2 volumes of ethanol at - 20°C overnight. The concentration of DNA was determined by O.D. at 260 nm and 1 O.D. unit equals 50 mg/ml DNA. The quality of the preparation can be checked by the ratio of $O.D._{260}/O.D._{280}$ (1.8 is considered good) for protein contamination and by gel electrophoresis for supercoilicity. The quality of plasmid DNA for transfection is extremely important as it directly determines the transfection efficiency.

B. Stable expression of human cytochrome P450-2E1 in HepG2 cells

Stable expression of human CYP2E1 in HepG2 cells consists of several steps (**Figure 2**) which can be divided in three blocks: 1) construction of the retrovirus shuttle vector consisting of human CYP2E1 cDNA; 2) transfection and selection of packaging cell lines/pools which produce the recombinant retrovirus containing 2E1 cDNA; and 3) infection and isolation of HepG2 clones that express CYP2E1 protein.

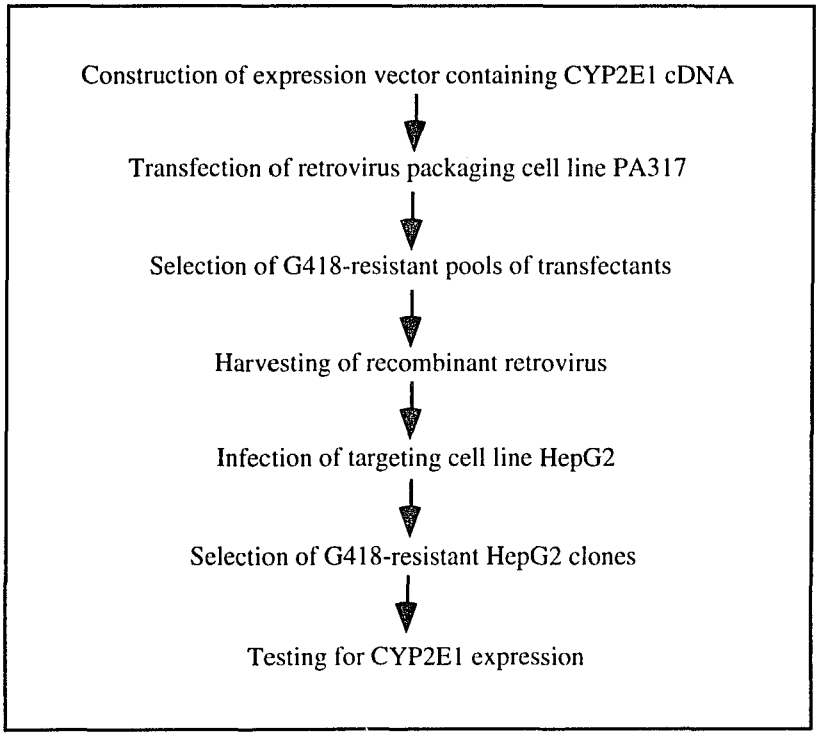


Figure 2. Strategy for construction of HepG2 cell lines expressing human cytochrome P450-2E1, including construction of the expression vector, the transfection step and the infection step.

B-1. Construction of the Expression Vector - Plasmid p91023(B)-IIE1 (Umeno et al., 1988), containing a 1.65 kB full-length human cytochrome P450IIE1 (CYP2E1) complementary DNA, was kindly provided by Dr. F. J. Gonzalez (National Cancer Institute, Bethesda, MD). The retrovirus shuttle vector, pMV7 (Housey et al., 1988; Kieschmeier et al., 1988), was kindly provided by Dr. R. S. Krauss (Mount Sinai School of Medicine, New York, NY). This Moloney murine leukemia virus (MoMuLV) based plasmid contains a RNA packaging signal, a modified herpes simplex virus thymidine kinase (TK) promoter driven bacterial Neo^r gene for selecting stable expression clones and two, *Eco*RI and *Hin*dIII, cloning sites down-stream to the 5' LTR promoter. The full-length CYP2E1 cDNA insert was excised by digesting p91023(B)-IIE1 with *Eco*RI and subcloned into the *Eco*RI site of pMV7. The subclone containing the cDNA insert in the sense orientation, assured by restriction mapping with *Bam*HI and *Kpn*I, was designated as pMV7-2E1 (**Figure 3**).

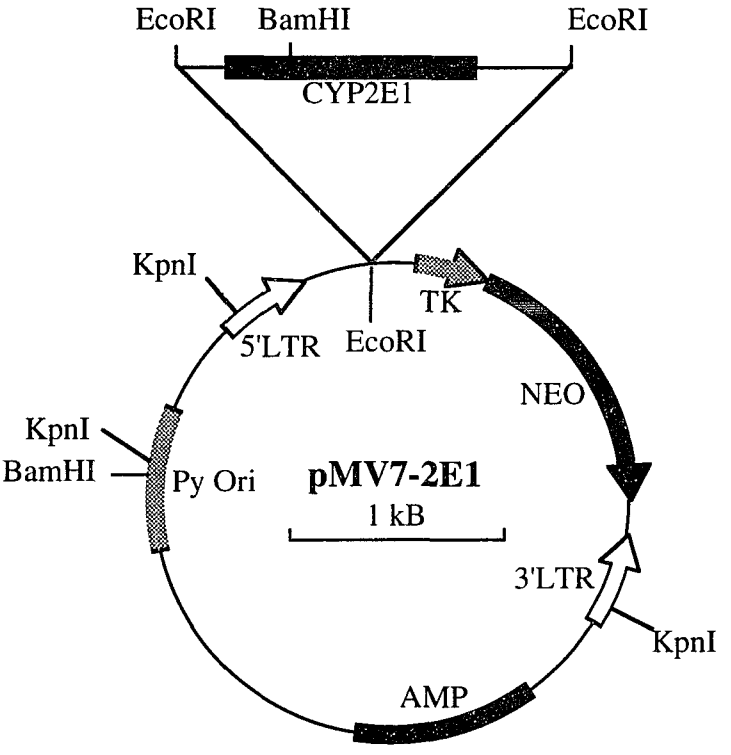


Figure 3. Partial restriction map and construction strategy of pMV7-2E1 plasmid. The MoMuLV long terminal repeats (LTR) are shown as empty boxed arrows. The modified herpes simplex virus thymidine kinase (TK) promoter and direction are shown as shaded arrow. The bacterial neomycin phosphotransferase (NEO) gene, ampicillin resistance (AMP) gene, and polyoma virus origin of replication (Py Ori) are also shown as indicated. Full-length human CYP2E1 cDNA was inserted into *Eco*RI site of pMV7 expression vector.

B-2. Transfection of the Packaging Cell Line - Plasmid pMV7-2E1, the subclone with the insert in the correct orientation assured by restriction mapping, and pMV7 were used to transfect the PA317 packaging cell line to generate stable transfected pools producing MV2E1 virus with CYP2E1 insert and the control MV virus without CYP2E1 cDNA, respectively. Transfection of PA317 was carried out by the calcium phosphate method (Ausubel et al., 1990) with minor modifications. Briefly, 20 μg of either plasmid DNA precipitated with 125 mM calcium phosphate in 19 mM HEPES buffer (pH 7.08) was applied onto about 30% confluent PA317 cells in 90 mm dishes for 5 hours. Transfected cells were grown to confluence and then split with 1:10 ratio into selecting DMEM medium containing 0.5 mg/ml G418. Resistant colonies were formed in about 10 days. For preparing the recombinant virus, pooled clones from both transfections were grown to subconfluence before changing to normal medium without G418 for two days. Virus stock was made by harvesting and sterilizing the medium through a 0.4 mm microfilter and kept at -70°C.

B-3. Infection of HepG2 Cells - Virus infection (Ausubel et al., 1990) of HepG2 cells was carried out by applying 3ml of undiluted MV2E1 virus stock (1:10 diluted for MV control virus stock) onto 30% confluent cells in 90 mm dishes for 3 hours with 8 mg/ml polybrene (Sigma). Infected cells were grown to confluence before splitting at a 1:10 ratio into selecting MEM medium containing 0.4 mg/ml G418. Resistant colonies were formed in about 2 weeks. Clones were isolated by ring isolation, grown to large scale, and maintained in MEM containing 0.2 mg/ml G418.

B-4. Selection of Virus Transduced Clones and Western Blot Analysis - To select the clones that express the most CYP2E1 protein, Western blot analysis (Laemmli, 1970; Towbin et al., 1979) was carried out with microsomes prepared from the CYP2E1-transduced and control clones (for preparation of microsomes see below) using a 10%

running and 4% stacking polyacrylamide gel. Electrophoresis and electrotransfer procedures were done with the BioRad mini-PROTEAN II system. Nitrocellulose membrane (Schleicher & Schuell) with transferred protein was incubated with anti-human CYP2E1 polyclonal antibody provided by Dr. J. M. Lasker (Mount Sinai Medical Center, New York, NY), and alkaline phosphatase conjugated goat anti-rabbit antibody as second antibody, and developed by the NBT-BCIP mixture (Promega). In between each incubation, extensive (about 5 times) washes with 10 mM TBS, pH 8.0 with 0.05% Tween were performed to lower the nonspecific recognition.

B-5. Southern Blot analysis of Integrated Copy of Viral DNA - High molecular weight genomic DNA was extracted from HepG2 clones by a standard DNA extraction method (Sambrook et al., 1989). The genomic DNA was completely digested with *Kpn*I, which cuts once within each LTR of the integrated copy of viral DNA, and *Bam*HI, which cuts once in the CYP2E1 cDNA but not anywhere else in the integrating region of the vector (**Figure 3**). Digested DNA was run on 0.6% agarose gel and transferred onto a BAS-NC supported nitrocellulose membrane (Schleicher & Schuell), which was pre-hybridized with buffer containing 50% formamide at 42°C for at least 2 hours, and then hybridized in buffer containing 50% formamide with either human CYP2E1 cDNA or bacterial Neo gene probe with 10^6 cpm/ml at 42°C for overnight. All DNA probes were labeled with α-^{32}P-dCTP (NEN) with Klenow enzyme (New England Biolabs) by the random primer labeling method (Feinberg and Vogelstein, 1983) and purified through a G-50 quick spin column (Boehringer Mannheim) to remove the unincorporated radioactive isotopes and random primers from the reaction buffer. Southern blot analysis would provide information as to whether the CYP2E1 cDNA was successfully transduced into the HepG2 cells, whether there were potential alterations such as deletion in the integrated copy of CYP2E1 (with *Kpn*I digestion), and the copy number (with *Bam*HI digestion).

B-6. Northern Blot Analysis of RNA Transcripts - PolyA RNA was isolated with a mRNA mini purification kit (Pharmacia) from the CYP2E1 transduced clones and control clones according to the method of Chirgwin *et al.* (Chirgwin et al., 1979). Two μg of polyA RNA was electrophoresed on a 1% denaturing agarose gel containing 3% (v/v) formaldehyde. RNA was transferred to nitrocellulose membranes and hybridized to either ^{32}P-labeled human CYP2E1 cDNA or bacterial Neo gene probe (described above). Northern blot analysis would provide information as to whether HepG2 itself had CYP2E1 expression from its endogenous copy of the gene and whether the transduced clones express the correct size of the CYP2E1 RNA.

B-7. Microsome Preparation - Stable-transduced HepG2 clones were grown in the maintaining medium in the presence of 2 mM 4-methylpyrazole (Larson et al., 1991) till confluence. Cells were washed once with phosphate buffered saline, pH 7.4 and harvested by scraping and subsequent sonication for 45 seconds. The sonicated samples were centrifuged at 7,000 g (10,000 rpm in Ti-50 rotor) for 10 minutes to remove unbroken cells and large subcellular particles. The supernatants were transferred to new tubes and centrifuged at 100,000 g (37,000 rpm in Ti-50 rotor) for 60 minutes to bring down the microsomal fraction. The microsomal pellets were rinsed and resuspended in 125 mM KCl and 10 mM potassium phosphate, pH 7.4, with a Dounce homogenizer. The final microsomes were stored in the same buffer containing 20% glycerol and kept at -70^{o}C. Protein concentration was determined by the method of Lowry *et al.* (Lowry et al., 1951). with known concentration of BSA as standard.

B-8. Quantitation of Expressed CYP2E1 - Microsomes from the transduced clones as well as the control clones were measured for total P450 content (presumably from the transduced 2E1) by CO binding spectrum using the method of Omura and Sato (Omura and Sato, 1964a; Omura and Sato, 1964b) and by quantitative Western blot analysis. For CO

binding spectrum, 2.5 mg/ml microsomal protein in 0.2 ml 100 mM potassium phosphate buffer, pH 7.4, containing 0.5% sodium cholate plus 20% glycerol in sample cuvette were very gently bubbled with CO for about 30 seconds. A few mg of fresh sodium dithionite (Sigma) were added to both sample and reference cuvette, and the spectrum from 500 to 400 nm was recorded on a Perkin-Elmer 554 dual beam spectrophotometer. The sample and reference cuvettes were corrected for baseline with both cuvettes before bubbling with CO to compensate for any differences between them. The difference in O.D. from 490 to 450 was used to calculate the concentration of P450. The extinction coefficient is 91 $mM^{-1}cm^{-1}$ (Omura and Sato, 1964a; Omura and Sato, 1964b).

Since the P450 expression level may be too low to accurately assess by spectrophotometry, quantitative Western blot analysis was used in combination with P450 spectrum to measure the yield of expressed CYP2E1. Different amounts of purified human CYP2E1 protein standard (from Dr. J. M. Lasker, Mount Sinai Medical Center) was loaded on the same gel with sample microsomes. Western blot technique was carried out according to the method described above. Immunoreactive proteins were quantified by scanning the blots with a LKB UltroScan XL densitometer.

B-9. Assays of Enzyme Activities - Enzymatic activity of the transduced clones was determined in the microsomal fraction by metabolism of *p*-nitrophenol (PNP) to *p*-nitrocatechol, aniline to *p*-aminophenol, *N*-dimethylnitrosamine (DMN) to formaldehyde, and ethanol to acetaldehyde. Reactions were carried out with 0.2 mM PNP, 1 mM aniline, 4 mM DMN, or 100 mM ethanol in Eppendorf tubes in 0.1 M potassium phosphate buffer, pH 7.4 (50 mM Tris buffer, pH 7.4, for ethanol oxidation), initiated with 1 mM NADPH, reacted at 37°C for 60 minutes except where stated, and stopped by 0.3 volume of 20% trichloroacetic acid (1 N HCl for the ethanol reaction). The samples for PNP, aniline, or DMN were centrifuged and aliquots of the supernatant are assayed for a) *p*-nitrocatechol by

adding 0.1 ml of 10 N NaOH per ml supernatant and immediately determining the absorbance at 546 nm; b) assayed for *p*-aminophenol by an incubation at room temperature with 0.2 ml of 2.5 M sodium carbonate per ml of supernatant and 0.1 ml of a 5% phenol in 2.5 M NaOH solution in the dark for 30 minutes, followed by immediately determining the absorbance at 630 nm; c) assayed for formaldehyde by an incubation with an equal volume of Nash reagent (Nash, 1953) at 60°C for 10 minutes and determining the absorbance at 412 nm. Acetaldehyde produced from ethanol oxidation was measured by gas chromatography on a Hewlett Packard 5840A gas chromatograph using a carbowax 20M column. Operating conditions were: column, 50°C; inlet 100°C; detector 150°C; nitrogen flow, 35 ml/min. Concentrations of *p*-nitrocatechol, *p*-aminophenol, and formaldehyde are calculated using extinction coefficients of 9.5, 15, and 8 $mM^{-1}cm^{-1}$ respectively (Mieyal and Blumer, 1976; Reinke and Mooyer, 1985; Werringloer, 1978).

Inhibition by CYP2E1 antibodies was carried out by pre-incubation of the antibody with the complete system for 5 minutes at 37°C before adding NADPH (Clejan and Cederbaum, 1992). Other CYP2E1 specific inhibitors such as diethyldithiocarbamate (DDC) at 0.1 mM, a relative specific inhibitor for CYP2E1; 4-methylpyrazole (4-MP) at 1 mM, a good ligand of 2E1; ethanol at 100 mM, a good substrate for 2E1; and miconazole at 0.2 mM, a general P450 inhibitor were used to test the specificity of the PNP reaction for the human CYP2E1.

B-10. In Situ PNP Metabolism - Transduced HepG2 clones were grown in the complete MEM medium without phenol red (Gibco/BRL) till confluence before additions of sterile PNP to a final concentration of 0.4 mM into the culture dishes for 24 hours. *p*-Nitrocatechol produced from PNP secreted to the medium was assayed according to the *in vitro* method with microsomes described above.

C. Characterization of generation of reactive oxygen species and initiation of lipid peroxidation in the human CYP2E1 transduced HepG2 clones

C-1. ESR Assay of Superoxide Radical Production [6]- Superoxide generation was determined by assaying the generation of stable nitroxide radicals formed in the reaction of hydroxylamines with $\cdot O_2^-$ (Rashba et al., 1990). The hydroxylamine, 4-oxo-2,2,6,6-tetramethylpiperidine-1-hydroxyl (4-oxo-TEMPOH) was synthesized and purified (Krinitskaya and Volodarskii, 1982) and dissolved in triply distilled, chelex-treated water. 4-oxo-TEMPOH reacts with $\cdot O_2^-$ to form 4-oxo-TEMPO radical, which is very stable and easily measured by ESR, giving a characteristic three line spectra. Microsomes from MVh2E1-9 and MV-5 clones (usually 1 mg per ml) were incubated in 100 mM potassium phosphate buffer, which had been passed through Chelex-100 resin to remove metal impurities, pH 7.4, plus 10 mM of hydroxylamine at room temperature. Reactions were initiated by the addition of NADPH to a final concentration of 1.2 mM. The samples were immediately transferred to a WG-812Q flat quartz cuvette (Wilmad) and spectra were recorded on a Bruker ESP 300 spectrometer equipped with a 4102ST probe. Kinetics of the reaction were monitored as a function of time by measuring the increase of the intensity of the second component of the three line nitroxide spectrum. A standard spectrum was recorded with a stable nitroxide radical, 4-oxo-TEMPO (Aldrich). Instrument settings for the spectra were as follows: sweep width 70.0 G, sweep time 20.97 sec, modulation amplitude 0.5 G, microwave power 11 mW. For the calculations of the rate of superoxide radical production, only the SOD-inhibited part of the nitroxide radical accumulation was taken into consideration since SOD should compete with the nitroxide for $\cdot O_2^-$.

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[6] ESR assays of superoxide radical production and hydroxyl radical production were carried out in collaboration with Dr. Julia Rashba-Step, who was a postdoctoral fellow in the lab.

C-2. ESR Assay of Hydroxyl Radical Production[5] - Production of ·OH was determined by assaying the formation of the POBN-HER adduct as described (Gonthier et al., 1991; Pou et al., 1992). In this system, ·OH produced from the clones reacts with ethanol to produce hydroxyethyl radical (HER), which forms a stable POBN-HER radical with POBN spin trap which can be detected and measured by ESR. Reactions were carried out in 0.1 M potassium phosphate buffer, pH 7.4, 0.05 mM iron chelate, 30 mM POBN (Sigma), 100 mM ethanol, 0.05 mM sodium azide, about 2 mg of microsomal protein in a final volume of 0.5 ml. Azide added in the system was used to inhibit potential contaminating catalase which removes H_2O_2 (reaction 6a and 6b in Introduction), which is required for production of ·OH in the system. Reactions were initiated by the addition of NADPH to a final concentration of 1.2 mM. Kinetics of the reactions were followed by measuring the intensity of the maximum of the 3rd low field line of the ESR spectra of the 6-line POBN-HER adduct. Instrument settings were the same as above and the spectra were recorded about 20 seconds after initiating the reaction by NADPH. The phosphate buffer and water used to prepare solutions were passed through Chelex-100 resin.

C-3. Assay for Production of Hydrogen Peroxide - The production of hydrogen peroxide was determined by measuring the formation of formaldehyde from the oxidation of methanol by the catalase compound I complex (Hildebrandt et al., 1978). Reactions were carried out in 200 mM KCl, 50 mM Tris, pH 7.4, 100 mM methanol, 300 units of catalase (or 1 mM sodium azide), and about 2.5 mg microsomal protein of the clone in a final volume of 100 µl. Reactions were initiated by the addition of NADPH (1.2 mM final concentration) and terminated by the addition of 30 µl of 20% TCA. The generation of formaldehyde was assayed by the Nash reaction (Nash, 1953) and measured at 412 nm. Catalase used in the reaction was to complete the oxidation of methanol to formaldehyde by its peroxidatic mode of action (reaction 6b in Introduction). Azide used in the control group is an inhibitor of catalase which may be present as a contaminant in isolated microsomes.

Rates of H_2O_2 production were calculated from the net difference in formaldehyde production between samples incubated in the presence of azide but without catalase (to evaluate formaldehyde arising from ·OH dependent or cytochrome P-450 dependent oxidation of methanol) from the samples incubated in the absence of azide but presence of catalase. Extinction coefficient is 8 $mM^{-1}cm^{-1}$ (Werringloer, 1978).

C-4. Measurement of Lipid Peroxidation - Lipid peroxidation was assayed by measuring production of TBA-reactive metabolites such as malondialdehyde, which are breakdown products of lipid peroxides (Buege and Aust, 1978). Experiments were performed in Eppendorf tubes containing 50 mM Tris-HCl, pH 7.4, 5 μM Fe-ATP, for most experiments, 1 mM NADPH, and about 2.5 mg/ml microsomes in a final volume of 0.1 ml. Reactions were initiated by adding NADPH and terminated after incubation at 37°C for 30 minutes by addition of 0.3 volumes of 30% ice cold TCA. After centrifugation, aliquots of the supernatants were mixed with equal volume of TBA (7.3 mg/ml) and heated at 100°C for 10 minutes. After cooling on ice for 2 minutes, absorbance was determined at 535 nm (Niehaus and Jamuelson, 1968) and the concentration of TBA-reactive product was calculated using an extinction coefficient of 156 $mM^{-1}cm^{-1}$ (Wills, 1969).

D. Cytotoxicity of Acetaminophen in Human P450 2E1-transduced HepG2 Cells

D-1. LDH Leakage Assay - Cells were subcultured at about 20% confluence in 35 mm 6-well culture plates and grown for 2 days before treatment with APAP and other chemicals. In most cases, cytotoxicity was assayed three days after the treatment by a LDH leakage method. Briefly, culture medium was first saved; then the cells were trypsinized, pelletted, resuspended in 1 ml PBS, and sonicated for 10 seconds to prepare the total cell extract. LDH activity in both culture medium and the total cell extract was determined using

the LDH-20 Diagnostic kit. LDH leakage was calculated by dividing the LDH activity in the culture medium by the LDH activity in the total cell extract and is expressed as this ratio.

D-2. Measurement of Radioactive APAP Adduct Formation - Cells were subcultured into 35 mm 6 well plates and grown to about 80% confluence. Cells were treated with 2 μCi/ml ^{14}C-labeled APAP in the presence or absence of BSO and/or other chemicals, and grown for an additional 24 hours. Cells were then harvested and sonicated in 1 ml PBS. Total cellular proteins were precipitated with 0.3 ml 20% TCA and radioactive protein adduct was measured according to Streeter et al. (Streeter et al., 1984). Briefly, precipitates were washed once in an equal volume with 80% methanol, three times with ethanol-diethyl ether (3:1) mixture, and once again with 80% methanol. The final pellets were dissolved in 0.2 ml of 1 M sodium hydroxide. A one-half aliquot was neutralized with 0.1 volume of 10 M HCl, thoroughly mixed with 4 ml of Soluscint liquid scintillation cocktail and counted in a Beckman liquid scintillation counter. The other half was saved for protein estimation.

For cell fractionation experiments, MV-5 and MVh2E1-9 cells were grown to ~80% confluence before being treated with 2 μCi/ml ^{14}C-labeled APAP in the presence of 100 μM BSO for 24 hours. Cells were then harvested and sonicated in PBS buffer for 45 seconds. The total cell extracts were centrifuged at 600 g for 10 minutes to yield the nuclear pellet and the post-nuclear supernatant. The supernatant was centrifuged at 8,000 g for 10 minutes to provide the mitochondrial pellet, and the post-mitochondrial supernatant was centrifuged at 100,000 g for 1 hour to give the microsomal pellet and the cytosolic fraction. 0.25 mg protein of each fraction was precipitated with TCA, washed as described above and counted in a Beckman liquid scintillation counter.

E. CCl_4-induced Degradation of Human Cytochrome P450 2E1 in the Transduced HepG2 Cells

E-1. Assay for 2E1 Inactivation and Degradation in situ - MVh2E1-9 and MV-5 cells were grown in 100 mm dishes to about 80% confluence. Cells were treated with 2 mM CCl_4 (or 10 mM APAP) in the absence or presence of 0.1 μg/ml PMA and, where indicated, other chemicals, and grown for an additional 24 hours (or 48 hours for APAP treatment). Cells were then harvested and sonicated in 10 ml PBS. Microsomes were prepared by centrifugation of the sonicated cell extracts at 100,000 g for 60 minutes and resuspended in PBS. Protein content was determined by the method of Lowry et al. (Lowry et al., 1951). Inactivation of 2E1 was determined by assaying for microsomal PNP oxidation while degradation of 2E1 was evaluated by assaying the content of 2E1 by Western blot analysis.

E-2. Assay for CYP2E1 Degradation in MVh2E1-9 Microsomes - MVh2E1-9 cells were grown to confluence and treated with 0.1 μg/ml PMA for 24 hours. Microsomes were then prepared and a concentration of 2.5 mg/ml was incubated with the indicated additions in 10 mM phosphate buffer (pH 7.5) in a final volume of 100 μl. Reactions were initiated with 1 mM NADPH, allowed to continue for 4 hours, and stopped by an addition of PAGE gel loading buffer and heat inactivation. One fifth of the reaction was loaded to each lane for Western blot analysis of the 2E1 content.

E-3. Measurement of Radioactive CCl_4 Adduct Formation - Cells were subcultured into 35 mm 6 well plates and grown to about 80% confluence. Cells were treated with 1 mM ^{14}C-labeled CCl_4 in the presence of BSO and/or other chemicals, and grown for an additional 24 hours. The BSO was added to lower GSH levels and thereby enhance protein adduct formation. Cells were then harvested and sonicated in 1 ml PBS. Total cellular

proteins were precipitated with 0.3 ml of 20% trichloroacetic acid and radioactive protein adducts were measured according to Streeter et al. (Streeter et al., 1984). Briefly, the TCA precipitates were washed once in a equal volume with 80% methanol, washed three times with an ethanol-diethyl ether (3:1) mixture, and once again with 80% methanol. The final pellets were dissolved in 0.2 ml of 1 M sodium hydroxide. A one-half aliquot was neutralized with 0.1 volume of 10 M HCl, thoroughly mixed with 4 ml of Soluscint liquid scintillation cocktail and counted in a Beckman liquid scintillation counter. The other half was saved for protein estimation.

RESULTS

A. Stable Expression of Human Cytochrome P450-2E1 in HepG2 Cells and Characterization of Catalytic Activities.

A-1. Transduction of 2E1 into HepG2 Cells - After infection of HepG2 cells with the recombinant retrovirus MV2E1 and the subsequent G418 selection, 38 clones were isolated and grown separately. Western blot analysis was carried out to measure the expression level of CYP2E1 protein in the microsomal fraction of these clones. 20 of these clones expressed varying amounts of CYP2E1 protein and 4 of them (MVh2E1-9, 15, 19, and 37) appeared to express the most. All four clones produced a protein band at 54 kD (lanes 5 to 8) that comigrated with the CYP2E1 band from human liver microsomes (lane 2) (**Figure 4**). All control MV clones infected with MV virus lacking the 2E1 insert (two are shown in **Figure 4**) as well as uninfected HepG2 cells did not produce this band. The varying intensity of the CYP2E1 band in different clones in the Western blot suggested differential expression of the protein in different clones. Clone MVh2E1-9, which seems to express the most, was chosen for further characterization.

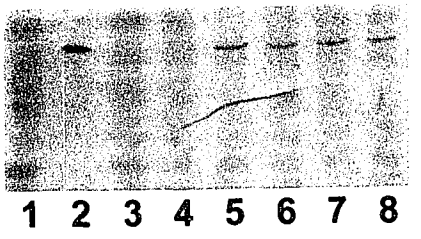


Figure 4. Western blot analysis using anti-human P450-2E1 IgG for detection of P450-2E1 expression in the transduced HepG2 clones. Forty micrograms (4μg for human liver microsomes) of microsomal protein was loaded onto each lane. Lane 1, HepG2 cells; lane 2, human liver microsomes; lanes 3 and 4, MV-3 and -5 control clones; lanes 5-8, MVh2E1-9, -15, -19, and -37, respectively.

A-2. Analysis of the Integrated CYP2E1 DNA in the Transduced Clones - To demonstrate that the CYP2E1 protein detected above was truly from the transduced CYP2E1 DNA, Southern blot analysis was carried out. DNA isolated from different clones and HepG2 cells was digested with *Kpn*I, which cuts once within each LTR (Kieschmeier et al., 1988) (**Figure 3**) and hybridized with the CYP2E1 cDNA probe. The result showed one band at about 4.8 kb (**Figure 5B**), which was present in all four MV2E1 clones (lanes 4 to 7) but absent in the control MV clones (lanes 2 and 3) or HepG2 (lane 1) cells indicating that an unaltered DNA copy was integrated into the genome of each MV2E1 clone. Southern blot analysis of the same set of DNA digested with *Bam*HI, which cuts once in the CYP2E1 cDNA (Umeno et al., 1988) but not anywhere else in the integrating (LTR to LTR) region of the vector (Kieschmeier et al., 1988) (**Figure 3**), showed no more than 2 exogenous bands in the MV2E1 clones (lanes 4 to 7 in **Figure 5A**) indicating only one copy of the CYP2E1 DNA was introduced into the genome in all four MV2E1 clones. All other bands present in all lanes in **Figure 5** result from the endogenous copy of CYP2E1 gene and are consistent with the restriction map of CYP2E1 genomic sequence (Umeno et al., 1988). Therefore, the varying amount of CYP2E1 protein detected in the Western blot in the four different clones appears to be due to the insertional effect of the integration site rather than to a different copy number.

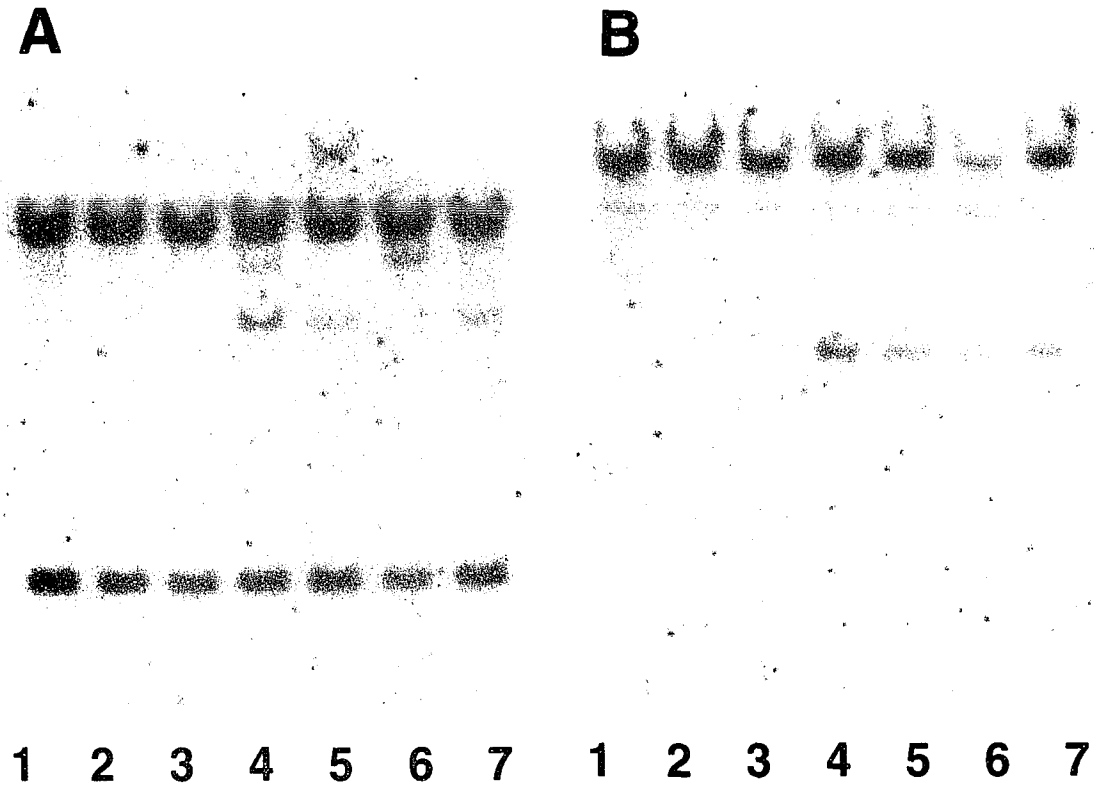


Figure 5. Southern blot analyses of CYP2E1 transduced clones. Ten micrograms of genomic DNA which was extracted from the transduced clones and HepG2 cells was digested with BamHI (A) or KpnI (B) and loaded onto each lane. The integrated viral DNA was identified by hybridization with a ^{32}P-labeled human CYP2E1 cDNA probe. For both (A) and (B): lane 1, HepG2 cells; lanes 2 and 3, MV-3 and -5 control clones; lanes 4-7, MVh2E1-9, -15, -19, and -37, respectively.

A-3. Analysis of RNA Transcripts by Northern Blot - It has been shown by several groups that HepG2 cells do not normally express spectrally-detectable cytochrome P450-2E1 (Patten et al., 1992; Waxman et al., 1991). To test whether this is true for CYP2E1 at the transcriptional level in comparison to the transduced clones, Northern blot analysis was carried out using transduced MV2E1 clones, control MV clones as well as HepG2 cells. Only the transduced MV2E1 clones showed varying amount of RNA detected by the CYP2E1 cDNA probe (lanes 4 to 7, **Figure 6B**). Neither control MV clones (lanes 2 and 3) without 2E1 insert nor HepG2 cells (lane 1) showed detectable CYP2E1 RNA (**Figure 6B**). Different RNA species on these blots may be due to splicing events, particularly from the cryptic termination site in the vector (Kieschmeier et al., 1988) and polyadenylation sites at the 3' end of the CYP2E1 cDNA insert (Song et al., 1986). Northern blot analysis with the same set of RNA but hybridized with the neo^r gene probe detected bands in both transduced MV2E1 clones and control MV clones but not in HepG2 cells (**Figure 6A**). TK promoter generates the second RNA species detected by neo^r probe. These results demonstrated that the successfully transduced clones including control ones without 2E1 insert expressed the corresponding RNAs, full-length as well as spliced forms, from the recombinant virus and that only CYP2E1-transduced clones showed CYP2E1 RNA. None of these clones nor HepG2 cells expressed detectable CYP2E1 RNA from the endogenous copy of the CYP2E1 gene, which should be around 1.7 kb.

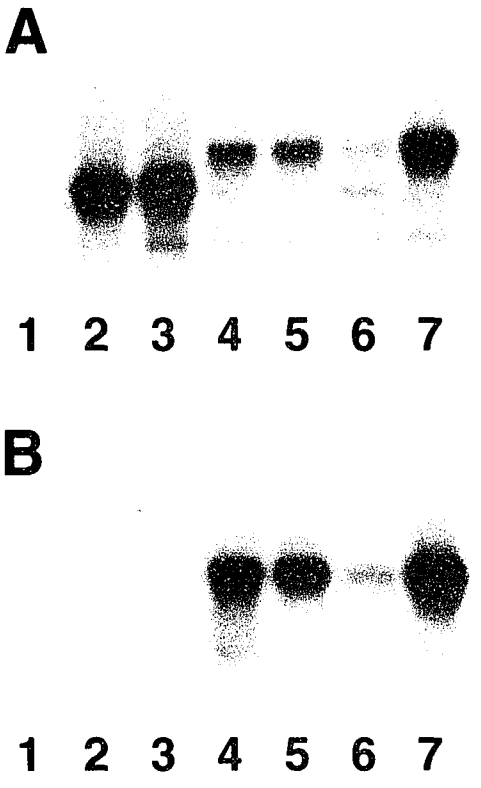


Figure 6. Northern blot analyses of CYP2E1 transduced clones. Two micrograms of poly(A) RNA isolated from the transduced clones and HepG2 cells was run on a 1% denaturing agarose gel. Viral RNA transcripts were identified by hybridization with either a ^{32}P-labeled human CYP2E1 cDNA probe (B) or bacterial NEO DNA probe (A). Lane 1, HepG2 cells; lanes 2 and 3 control clones MV-3 and-5, respectively; lanes 4-7 clones MVh2E1-9, -15, -19, and -37, respectively.

A-4. Quantitation of P450 in MVh2E1-9 Clone - CO reduced difference spectroscopy was used in an attempt to measure the P450 level in the transduced clones. However, as found by others (Clark and Waterman, 1991; Minowa et al., 1990), due to the high turbidity and low expression level, the spectral quantitation was found to be inaccurate and poorly resolved. Therefore, P450-2E1 was quantitated by Western blot analysis after preparing a standard curve with purified human P450-2E1 (kindly provided by Dr. J. M. Lasker, Mount Sinai Medical Center, New York, NY) (**Figure 7**). The final P450-2E1 content in MVh2E1-9 clone was estimated to be 10 pmol/mg of microsomal protein. This level of CYP2E1 protein is considerably less than that in human liver microsomes.

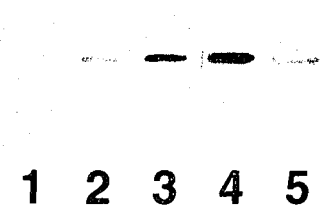


Figure 7. Quantitation of CYP2E1 expressed in MV2E1-9 clone. Quantitative Western blot. Lanes 1 - 4 were loaded with 0.25, 0.5, 1 and 2 pmol of human CYP2E1 protein respectively. Lanes 5 was loaded with 50 μg of microsomal protein from MV2E1-9 clone.

A-5. Enzyme Activity of the Transduced Clones - A complete *in vitro* system containing microsomes from the MVh2E1-9 clone, PNP and NADPH catalyzed PNP hydroxylation in a linear reaction for at least 30 minutes (**Figure 8A**). This reaction was dependent on the amount of microsomal protein and substrate concentration (**Figure 8B and C**). A Lineweaver-Burk plot of the substrate dependence was linear (**Figure 8D**) with an apparent K_m of about 110 μM and apparent V_{max} of about 0.1 nmol/min/mg microsomal protein. These values are in agreement with published data with CYP2E1 from other animal models. This would be equivalent to a V_{max} of about 10 nmol/min/nmol P450-2E1.

Oxidation of PNP by the human CYP2E1 was weakly inhibited by the anti-rat 2E1 antibody but was very sensitive to inhibition by anti-human 2E1 antibody (**Table 2**). Pre-immune IgG had no effect. The PNP activity was specifically inhibited by anti-human 2E1 indicating that the PNP catalytic activity was due to the expressed CYP2E1. The PNP activity was also inhibited by 0.1 mM diethyldithiocarbamate (DDC), a relative specific inhibitor for 2E1; by 4-methylpyrazole (4-MP), a good ligand of 2E1 (Feierman and Cederbaum, 1986); by ethanol, a good substrate for 2E1 (Morgan et al., 1982); and, to a lesser extent, by miconazole, a general inhibitor for P450 (**Table 3**). Besides PNP, microsomes from the MVh2E1-9 clone were also capable of oxidizing DMN to formaldehyde, aniline to *p*-aminophenol, and ethanol to acetaldehyde (**Table 4**). These are effective substrates for P450-2E1. Oxidation of DMN by MVh2E1-9 (**Table 4**) and by human liver microsomes (0.25 nmol/min/mg microsomal protein, **Table 8**) was about three-fold lower than rates of PNP oxidation by the clone (**Table 4**) and human liver microsomes (0.87 nmol/min/mg microsomal proteins, **Table 8**). These results differ from the comparable PNP and DMN oxidizing activities found with liver microsomes from pyrazole-induced rats (data not shown) suggesting that the human 2E1 may be a better catalyst of PNP oxidation relative to DMN oxidation as compared to the rat 2E1.

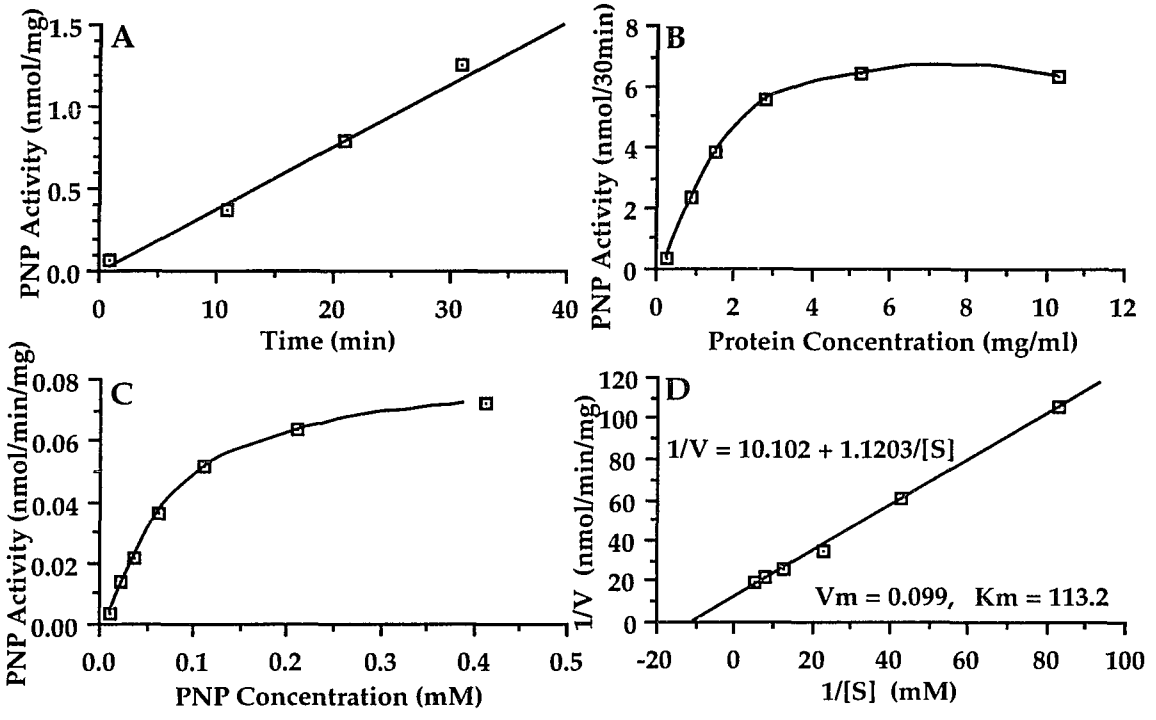


Figure 8. *In vitro* metabolism of *p*-nitrophenol by microsomes from the CYP2E1-transduced clone MV2E1-9 (see Materials and Methods for *in vitro* PNP assay). (A) Time course of PNP metabolism. 5 mg/ml microsomal protein concentration was used in 0, 10, 20, and 30 minute reactions. (B) Protein concentration dependence. Reaction time was 30 minutes. (C) Substrate concentration dependence. Various amounts of PNP were used in reactions with 2.5 mg/ml microsomal protein concentration and for 45 minute reactions. (D) Lineweaver-Burk plot for substrate concentration dependent reactions (C).

Table 2. Effect of Antibodies on Microsomal PNP Hydroxylase Activity of MVh2E1-9 Cells

Reaction	Concentration	Enzyme Activity in nmol/min/mg (Activity%)		
	mg/nmolP450	Rabbit Preimmune	Anti-rat 2E1	Anti-human 2E1
Control	-	0.059 (100%)	0.059 (100%)	0.048 (100%)
Antibody	1	0.060 (101%)	0.048 (81%)	0.021 (43%)
	2	0.059 (100%)	0.041 (70%)	0.013 (26%)
	4	0.058 (99%)	0.039 (67%)	0.008 (16%)
	8	0.059 (100%)	0.036 (62%)	0.004 (9%)

The oxidation of PNP by microsomes from clone MVh2E1-9 was determined as described under Materials and Methods in the presence of the indicated amounts of antibody (preimmune, anti-rat P450-2E1 IgG, or anti-human P450-2E1 IgG) Number in parentheses refer to percent of control activity.

Table 3. Effect of Chemical Inhibitors on Microsomal PNP Hydroxylase Activity of MVh2E1-9 Cells

Addition	Concentration	Activity (nmol/min/mg)	Activity%
Control	-	0.06	100%
-	1% (vol) methanol	0.052	87%
Miconazole	0.2 mM, 1% methanol	0.032	53%
DDC	0.1 mM	0.004	7%
4-MP	1.0 mM	0.005	8%
Ethanol	100 mM	0.012	18%

The oxidation of PNP by microsomes from clone MVh2E1-9 was determined as described under Materials and Methods in the presence of the indicated concentrations of the compounds. Numbers in parentheses refer to percent of control activity.

Table 4. Comparison of the Oxidation of Substrates by Microsomes from CYP2E1-transduced HepG2 Clones

		Enzyme Activity (nmol/min/mg)					
Substrate	Concentration	MV-3	MV-5	MV2E1-9	MV2E1-15	MV2E1-19	MV2E1-37
PNP	0.2 mM	0.002	0.004	0.059	0.025	0.032	0.066
DMN	4 mM	-	<0.002	0.018	-	-	-
Aniline	1 mM	-	<0.002	0.019	-	-	-
Ethanol	100 mM	-	<0.002	0.085	-	-	-

The oxidation of 0.2 mM PNP, 4 mM DMN, 1 mM aniline and 100 mM ethanol was carried out as described in Materials and Methods using microsomes from clones infected with virus lacking the P450-2E1 cDNA (MV-3 and -5) or containing the P450-2E1 cDNA (MVh2E1-9, 15, 19, 37).

The three other clones which were shown to express CYP2E1 protein (**Figure 4**) and RNA (**Figure 6**) were also catalytically active with PNP (**Table 4**). However, the control MV clones which were infected with virus lacking the 2E1 insert were essentially inactive in oxidizing PNP (**Table 4**). Control MV-5 clone was also ineffective in oxidizing DMN, aniline, and ethanol (**Table 4**).

A-6. In Situ Metabolism of PNP - The ability of intact transduced HepG2 clones to oxidize PNP was determined. Transduced clones MVh2E1-9 and -15 and control MV-3 and -5 were incubated with PNP for 24 hours and the medium was assayed for secreted p-nitrocatechol. Experiments were also carried out in the presence of 4-MP to inhibit any observable activity and thus link the activity to P450-2E1. Very low or non-detectable product was found in media from the control clones. 4-MP-sensitive PNP oxidation activity was at least an order of magnitude higher with the transduced clones (**Table 5**). This result forms the basis for *in vivo* metabolism of hepatotoxin and cytotoxicity studies with the transduced clones in the proposed experiments below.

Table 5. In Situ Metabolism of PNP and Effect of 4-MP in the Transduced Clones

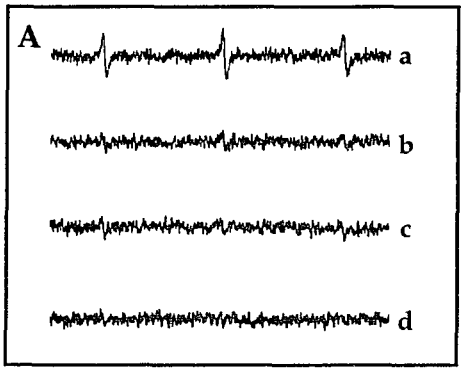
Experiment	Concentration (mM)	PNP Metabolism (nmol/24hr/dish)			
		MV-3	MV-5	MVh2E1-9	MVh2E1-15
Control	-	0	0.5	2.1	2.3
4-MP	2	0	0.3	0	0.2
4-MP sensitive		0	0.2	2.1	2.1

The oxidation of PNP by the control clones (MV-3 and -5) and the 2E1-transduced clones (MVh2E-9 and -15) was determined in situ as described under Materials and Methods. Effect of 4-MP was also evaluated and the 4-MP sensitive rates reflect the CYP2E1-dependent activity of the transduced P450.

B. Characterization of Generation of Reactive Oxygen Species and Initiation of Lipid Peroxidation in the Human CYP2E1-transduced HepG2 Cells

B-1. Superoxide Production - As described in Background and Methods and Experimental Design, chronic alcohol intake has been shown in rats to enhance the production of reactive oxygen species such as superoxide (Boveris et al., 1983; Ekström and Ingelman-Sundberg, 1989), hydrogen peroxide (Ekström and Ingelman-Sundberg, 1989; Lieber and DeCarli, 1970; Thurman, 1973), and hydroxyl radical (Dicker and Cederbaum, 1987; Klein et al., 1983), by isolated microsomes implying the ethanol-inducible microsomal enzyme CYP2E1 may be responsible for these increases. To evaluate this further, ESR was used to assay $\cdot O_2^-$ production by measuring stable nitroxide radicals formed from the reaction of $\cdot O_2^-$ with hydroxylamines (Rashba et al., 1990). Incubation of microsomes from clone MVh2E1-9 with NADPH plus the hydroxylamine detector resulted in a three line signal with identical splitting characteristics as the 4-oxo-TEMPO standard (**Figure 9A-a**). This signal was strongly inhibited (60 - 65%) by added SOD (**Figure 9A-b**) indicating that it was largely $\cdot O_2^-$-dependent. The control MV-5 clone displayed a weak signal, barely discernible above background levels (**Figure 9A-c**). This weak signal was substantially $\cdot O_2^-$-independent as SOD produced 10 to 20% inhibition (**Figure 9A-d**). By following the kinetics of the intensity of the middle peak as a function of time, rates of $\cdot O_2^-$ production were linear over a 10 minute time period (**Figure 9B**) and were calculated (SOD-sensitive portion) to be about 0.03 and 0.3 nmol/min/mg protein for microsomes from MV-5 and MVh2E1-9, respectively. Therefore, $\cdot O_2^-$ was indeed produced more in the CYP2E1 transduced MVh2E1-9 clone compared to the control MV-5 clone.

The redox cycling agent paraquat reacts with microsomes in the presence of NADPH with a resulting increase in $\cdot O_2^-$ production. This interaction of paraquat occurs



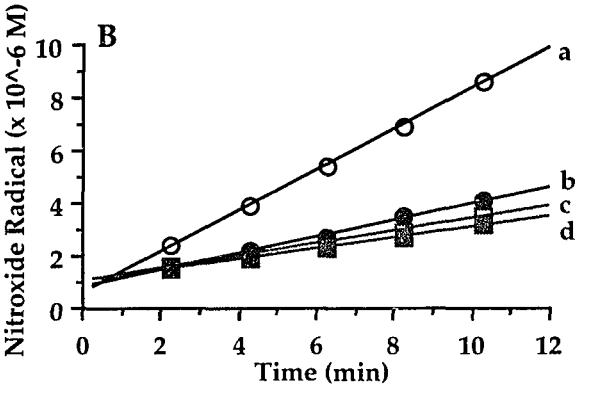


Figure 9. Nitroxide radical (NR) production from the interaction of 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine with superoxide radical from microsomes. (A) ESR spectra with microsomes (a) MV2E1-9 clone, (b) MV2E1-9 plus SOD, (c) Control MV-5 clone, and (d) MV-5 plus SOD. Results show spectra obtained after 10 minutes of reaction. (B) Time course for the production of nitroxyl radical.

primarily with NADPH-cytochrome P450 reductase. When 2 mM paraquat was added to the system, the rate of the nitroxide radical formation was increased dramatically, about 10 times for MVh2E1-9 clone and 100 times for MV-5 control clone. Rates of $\cdot O_2^-$ production in the presence of paraquat were similar for the two microsomal preparations, most likely reflecting the comparable activities of the reductase. (Paraquat can be catalyzed by the reductase to give rise to paraquat radical, which donates an electron to molecular oxygen to generate $\cdot O_2^-$). Conventional assays of the reductase by determining reduction of cytochrome C indicated similar reductase activity in MVh2E1-9 and MV-5 clones.

B-2. Hydroxyl Radical Production - Hydroxyl radical (·OH) production was measured by detecting POBN-HER adduct formation in the presence of Fe-EDTA (Gonthier et al., 1991; Pou et al., 1992). The signal for POBN-HER adduct has six lines (**Figure 10A**). In the absence of Fe-EDTA, very low rates were observed for both control (MV-5) and transduced (MVh2E1-9) clones indicating ·OH is not produced in the absence of iron catalyst in both microsomes. However, when 50 μM Fe-EDTA was added into the system, similar high rates were observed for both control (MV-5) and transduced (MVh2E1-9) clones (**Figure 10A and B**). These data indicate that ·OH production in the presence of Fe-EDTA, like the paraquat reaction above, is due to the P450 reductase and not CYP2E1. In contrast to paraquat, Fe-EDTA plays a dual function, i.e., production of $\cdot O_2^-$ and conversion of $\cdot O_2^-$ to ·OH via the iron-catalyzed Haber-Weiss reaction. The chelated ferrous iron, in the presence of P450 reductase, readily donates one electron to oxygen to make $\cdot O_2^-$, which can then undergo an iron-dependent Haber-Weiss reaction to become ·OH. The result of the experiment at 50 μM Fe-EDTA also confirms that NADPH-reductase activity is similar for control and transfected cells. Future experiments to study other more physiologically-relevant iron chelates e.g., Fe-ATP and Fe-histamine, remain to be conducted.

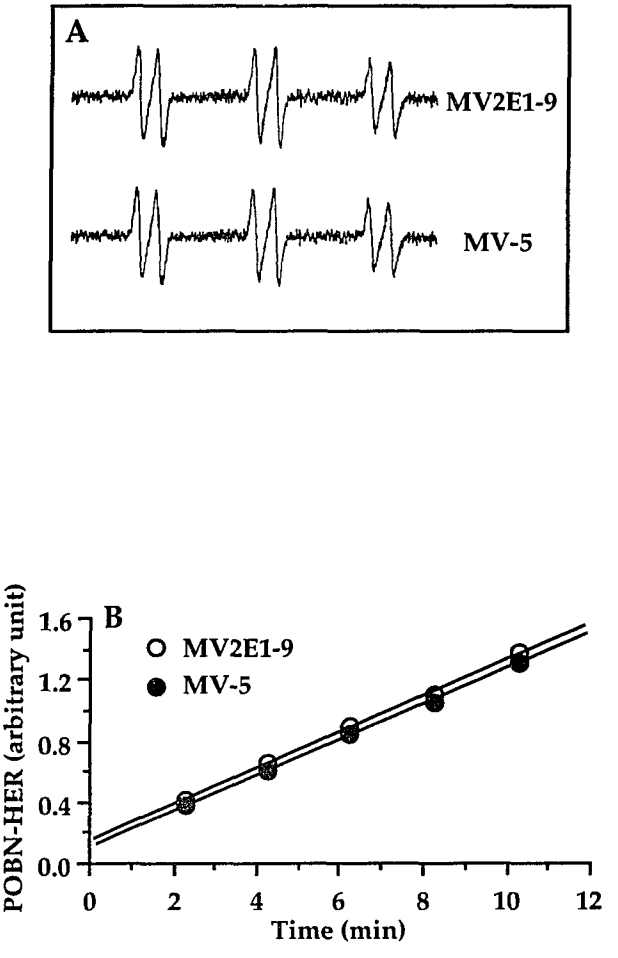


Figure 10. ESR spectra (A) and time course (B) of POBN-HER adducts by microsomes as an index of hydroxyl radical production.

B-3. Hydrogen Peroxide Production - H_2O_2 can be produced in microsomes from dismutation of $\cdot O_2^-$ or directly from the peroxygenated P450 complex. The production of H_2O_2 was determined by measuring the formation of formaldehyde from the oxidation of methanol by the catalatic mode of action of catalase compound I complex (Hildebrandt et al., 1978). Microsomes from clone MV-2E1-9 generated H_2O_2 in the presence of NADPH with a rate of about 0.25 nmol/min/mg protein (**Table 6**). Surprisingly, microsomes from the control clone MV-5 also produced H_2O_2 at a rate of about 0.12 nmol/min/mg (**Table 6**). Since the control clone did not produce significant amount of $\cdot O_2^-$ (**Figure 9**), most of the H_2O_2 produced is likely to arise from two electron reduction of oxygen rather than from dismutation of $\cdot O_2^-$. The difference between the two clones should reflect the contribution by the expressed CYP2E1. Addition of substrates for CYP2E1 did not stimulate H_2O_2 production in MV-5 clone (as expected) but also had no effect in MVh2E1-9. Inhibition by ethanol probably reflects the ability of ethanol to compete with methanol for catalase-H_2O_2.

B-4. Lipid Peroxidation from the Transduced Clones - Lipid peroxidation of the MVh2E1-9 clone was assayed by measuring production of thiobarbituric acid (TBA) reactive metabolites, e.g., malondialdehyde (MDA), which are breakdown products of lipid peroxides and an index of lipid peroxidation (Buege and Aust, 1978). Fe-ATP, which is very reactive in promoting microsomal lipid peroxidation, was used as the iron catalyst. When the concentration of Fe-ATP was high (25 μM), MDA production was identical in microsomes from MVh2E1-9 and MV-5 (**Figure 11**). Since chelated iron could interact with the reductase as well as P450, the comparable rates of production of TBA-reactive material with both microsomal preparations were interpreted to reflect primarily interaction of iron with the reductase when the concentration of iron was high. An iron titration curve was therefore carried out. As shown in **Figure 11** , little or no lipid peroxidation could be detected with Fe-ATP concentration up to 3 μM. Lipid peroxidation remained very low with the control MV-5 clone when the concentration of Fe-ATP was raised to 4 or 5 μM

Table 6. Production of Hydrogen Peroxide from the CYP2E1-transduced and Control Clones

Assay	Addition	Concentration	Results	
			MV-5	MVh2E1-9
O. D.412				
	Azide	1 mM	0.008	0.017
	Catalase	3000 U/ml	0.160	0.321
	Catalase - Azide	-	0.152	0.304
Activity (nmol/min/mg)				
	Control	-	0.127 (100%)	0.253 (100%)
	PNP	0.2 mM	0.136 (107%)	0.271 (107%)
	4-MP	1 mM	0.123 (97%)	0.225 (89%)
	Aniline	1 mM	0.201 (158%)	0.266 (105%)
	Ethanol	100 mM	0.046 (36%)	0.101 (40%)

Hydrogen peroxide production was determined as described under Materials and Methods using 2.5 mg of microsomal protein/ml and a 1-hour time course. Experiments were carried out in duplicate, in the absence and presence of the indicated additions. Results refer to the net activity found in the presence of catalase (3000 units/ml) minus that found in the presence of 1 mM sodium azide.

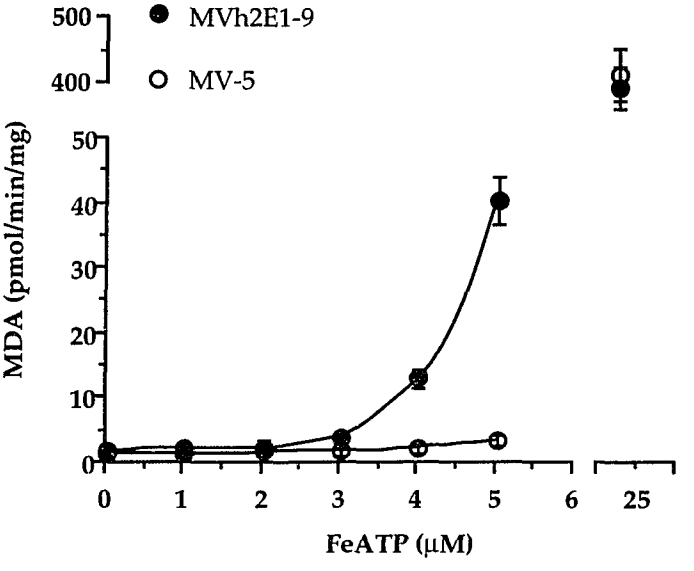


Figure 11. Lipid peroxidation in MVh2E1-9 and control MV-5 clones. Concentration curve for the stimulation for NADPH-dependent lipid peroxidation by ferric-ATP. Production of TBA-reactive material (malondialdehyde) was determined using microsomes from MV-5 and MVh2E1-9 clones

iron (**Figure 11**). However, a large increase in the production of TBA-reactive material was observed with microsomes from the transduced clone MVh2E1-9 at Fe-ATP concentrations of 4 and 5 μM (**Figure 11**). Thus a clear difference in lipid peroxidation between the two microsomal preparations can be found at low but not high concentrations of iron. Future experiments will be directed towards assays of other iron chelates and ferritin, and reduction of the iron chelates will be determined. The effect of inhibitors of CYP2E1 will also be determined.

To attempt to evaluate the nature of the reactive oxygen species responsible for the TBA production (i.e., ·X in lipid peroxidation described in Background) in clone MVh2E1-9, and that the elevated activity at 4 or 5 μM Fe-ATP indeed reflected peroxidation, the effect of antioxidants was determined. Production of TBA-reactive material in the presence of 5 μM Fe-ATP was completely inhibited by the antioxidants trolox, a vitamin E analog, and propylgallate, and strongly prevented by GSH (**Table 7**). There was no significant effect by SOD, catalase or ethanol suggesting that free or accessible $\cdot O_2^-$, H_2O_2 and ·OH did not play a major role in the overall mechanism of lipid peroxidation in the microsomes from MVh2E1-9 at a 5 μM iron concentration (**Table 7**). The possibility that SOD or catalase penetrate to hydrophobic membrane sites of $\cdot O_2^-$ or H_2O_2 production cannot be ruled out.

A comparison of activities by CYP2E1 and human liver microsomes is shown in **Table 8**. The significance of these activities will be presented further in Discussion.

Table 7. Inhibition of Lipid Peroxidation of MVh2E1-9 Cells

Addition	Concentration	Activity (pmol/min/mg)	Activity%
Control	-	54.5	100%
Trolox	0.1 mM	0	0%
PPG	0.05 mM	0	0%
GSH	5 mM	16.4	30%
SOD	100 U/ml	64.3	111%
Catalase	2600 U/ml	44.8	82%
Ethanol	50 mM	50.7	93%

The NADPH-dependent production of TBA-reactive material by microsomes from clone MVh2E1-9 was determined in the presence of 5 μM ferric-ATP and in the absence or presence of the indicated additives.

Table 8. Comparison of Catalytic Activities from MVh2E1-9 and Human Liver Microsomes

Activity	MVh2E1-9		Human Liver		MV2E1-9:HLM
	nmol/min/mg	nmol/min/nmol P450	nmol/min/mg	nmol/min/nmol P450	
PNP	0.059	5.9	0.87	2.56	2.3
DMN	0.018	1.8	0.25	0.74	2.4
Superoxide	0.26	26	0.49	1.44	18
Hydrogen peroxide	0.25	25	0.81	2.38	10
Lipid peroxidation	0.042	4.2	0.202	0.594	7.1

The indicated reactions were carried out using microsomes from clone MVh2E1-9 (P450-2E1 content of 0.01 nmol/mg of protein) or from human liver (P450 content of 0.34 nmol/mg protein). All reactions were carried out in duplicate. Results with the human liver microsomes are the average from three different samples. Lipid peroxidation was carried out with 0.005 mM ferric-ATP as the iron catalyst.

C. Cytotoxicity of Acetaminophen in Human CYP2E1-transduced Clones

C-1. Inhibition of Cell Proliferation by APAP in MV-5 and MVh2E1-9 Cells - Since high concentrations of APAP may have a direct toxic effect, e.g., inhibiting mitochondrial function (Nazareth et al., 1991), we first examined cell proliferation changes upon administration of APAP. A similar number of MV-5 and MVh2E1-9 cells were seeded in 35 mm 6-well plates and grown for 2 days. When 40% confluence was reached, the cells were treated with different concentrations of APAP, and harvested 48 hours later. The cells were counted using a hemacytometer. Surprisingly, at a relatively low concentration (1.25 mM), APAP inhibited about 50% of the cell proliferation as determined by cell number change. The inhibition of cell proliferation was nearly comparable in MV-5 and MVh2E1-9 cells although the latter were somewhat more affected (**Figure 12**). APAP at concentrations of 1.25 mM to 10 mM during the 48-hour incubation did not exert any cytotoxic effect as observed under the microscope. However, at 20 mM, APAP caused significant toxicity in both MV-5 and MVh2E1-9 cells as determined by the percentage of floating cells vs. attached cells (data not shown). This inhibition of cell growth by APAP appears to be independent of GSH since the anti-proliferative effect was identical in the absence or presence of treatment with BSO for both cell lines, e.g., 2.5 mM APAP inhibited cell replication 62% and 70% for MV-5 and MVh2E1-9 cells, respectively in the absence of BSO, and 66% and 72% respectively after treatment with 100 μM BSO.

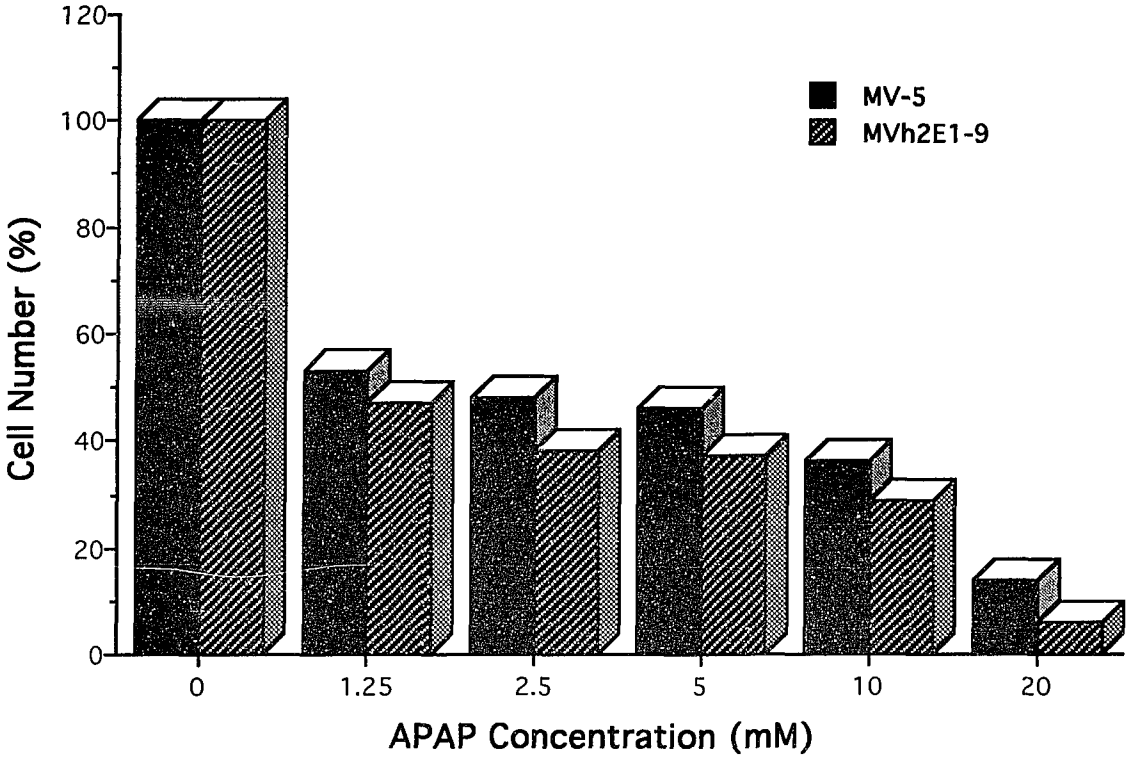


Figure 12. Effect of APAP on cell proliferation. MVh2E1-9 and the control MV-5 cells were seeded in 35 mm 6-well plates and grown for 2 days to reach about 40% confluence. Cells were treated with different concentrations of APAP and incubated for an additional 48 hours. Cells were then trypsinized, pelletted and resuspended in 1 ml PBS. Cells were counted twice using a hemacytometer and the average is shown as percentage of the non-treated control, which was taken as the 100% value.

C-2. Requirement of GSH Depletion for APAP Cytotoxicity - It has been shown by several groups that APAP-induced toxicity involves bioactivation of APAP by cytochrome P450 and that CYP2E1 contributes significantly to APAP bioactivation (Dahlin et al., 1984; Potter et al., 1973; Raucy et al., 1989). To evaluate this effect for human CYP2E1 in our culture model, MV-5 and MVh2E1-9 cells were treated with 5 mM APAP for four days in the absence or presence of BSO and LDH leakage was measured as an index of cell toxicity (**Figure 13**). BSO treatment (100 μM) caused an approximate 90% depletion of GSH in both cell lines. In the presence of 100 μM BSO, APAP showed a toxic effect on MVh2E1-9 cells whereas no significant LDH leakage was found with MV-5 cells. In the presence of 100 μM BSO, APAP caused about a 5-fold increase in the LDH leakage index compared to the non-BSO treated control for the MVh2E1-9 cell line whereas LDH leakage was similar in the absence or presence of BSO with the MV-5 cells. In the absence of BSO, APAP caused a comparable low LDH leakage in both MV-5 and MVh2E1-9 cells. Therefore, GSH depletion is required for APAP toxicity in MVh2E1-9 cells whereas no such toxicity is observed in MV-5 cells.

Cytotoxicity by APAP in the presence of BSO in MVh2E1-9 cells also correlated with morphological changes observed under the phase contrast microscope. As shown in **Figure 14**, treatment with 5 mM APAP and 100 μM BSO for four days caused significant cell death in MVh2E1-9 cells but not in MV-5 cells.

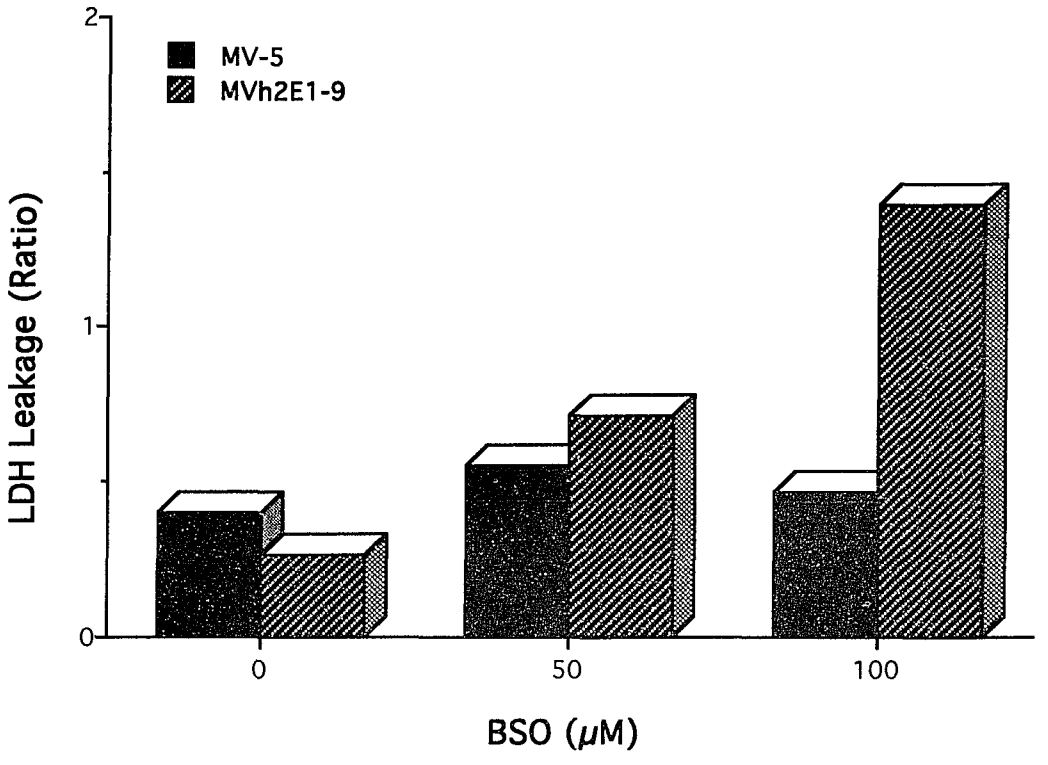


Figure 13. Requirement for GSH depletion for APAP cytotoxicity in MVh2E1-9 cells. MVh2E1-9 and the control MV-5 cells were seeded in 35 mm 6-well plates and grown to about 40% confluence. Cells were treated with 5 mM APAP in the absence or presence of 50 or 100 μM BSO for four days. LDH activity was determined in both the culture medium and the total cell extract. The ratio of LDH activity in the culture medium to that of the extract is expressed as LDH leakage. In the absence of APAP, there was little LDH leakage even after BSO treatment.

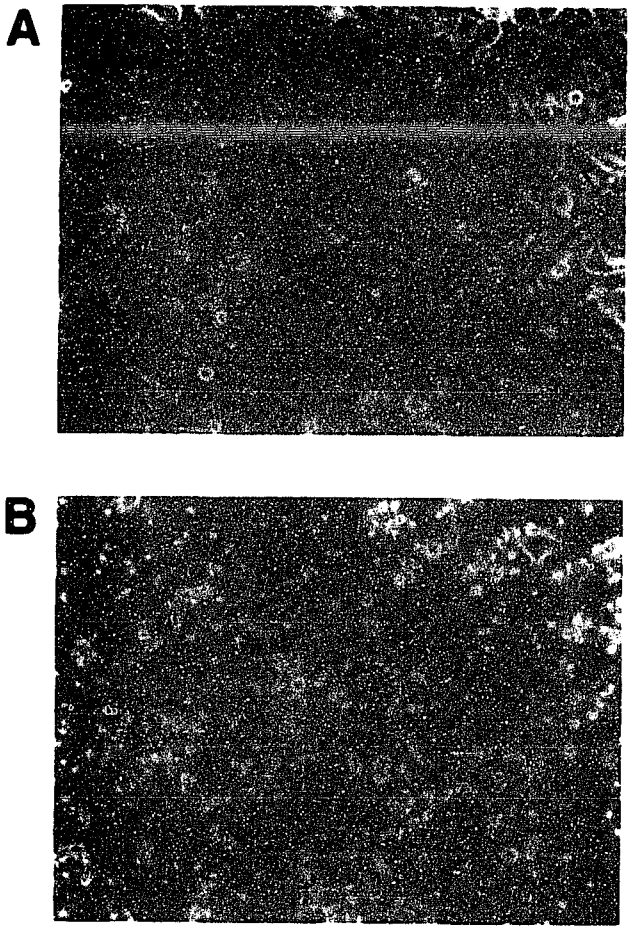


Figure 14. Morphological changes in MVh2E1-9 cells upon treatment with APAP. MVh2E1-9 and the control MV-5 cells were treated with 5 mM APAP and 100 μM BSO as described in Figure 2. Pictures were taken at day four under a phase contrast microscope (200x). A) MV-5 cells. B) MVh2E1-9 cells.

C-3. Characterization of APAP Cytotoxicity in MVh2E1-9 Cells - To characterize the cytotoxic effect of APAP, time course and dose-dependent experiments were conducted. The APAP-induced cytotoxicity as determined by LDH leakage was observed in MVh2E1-9 cells three days after the treatment with 5 mM APAP in the presence of 100 μM BSO whereas no significant toxicity was seen in MV-5 cells even after four days (**Figure 15A**). At day 5, APAP toxicity in MVh2E1-9 cells was doubled compared to days 3 and 4; however, toxicity in MV-5 cells also increased to a significant level (**Figure 15A**) at this time point.

The cytotoxic effect of various concentrations of APAP added in culture in the presence of 100 μM BSO for three days was also studied (**Figure 15B**). At low concentrations (below 2.5 mM) there was no significant toxicity in either cell line. At 5 mM, APAP caused significant toxicity in MVh2E1-9 cells but not in MV-5 cells. When the APAP concentration was increased to 10 mM, toxicity in MVh2E1-9 cells was doubled compared to 5 mM treatment, whereas toxicity in MV-5 cells was only slightly increased (**Figure 15B**). Therefore, based upon these time course and dose response studies, treatment of the cells with 5 or 10 mM APAP in the presence of 100 μM BSO for three days was the condition chosen for subsequent experiments in the present study.

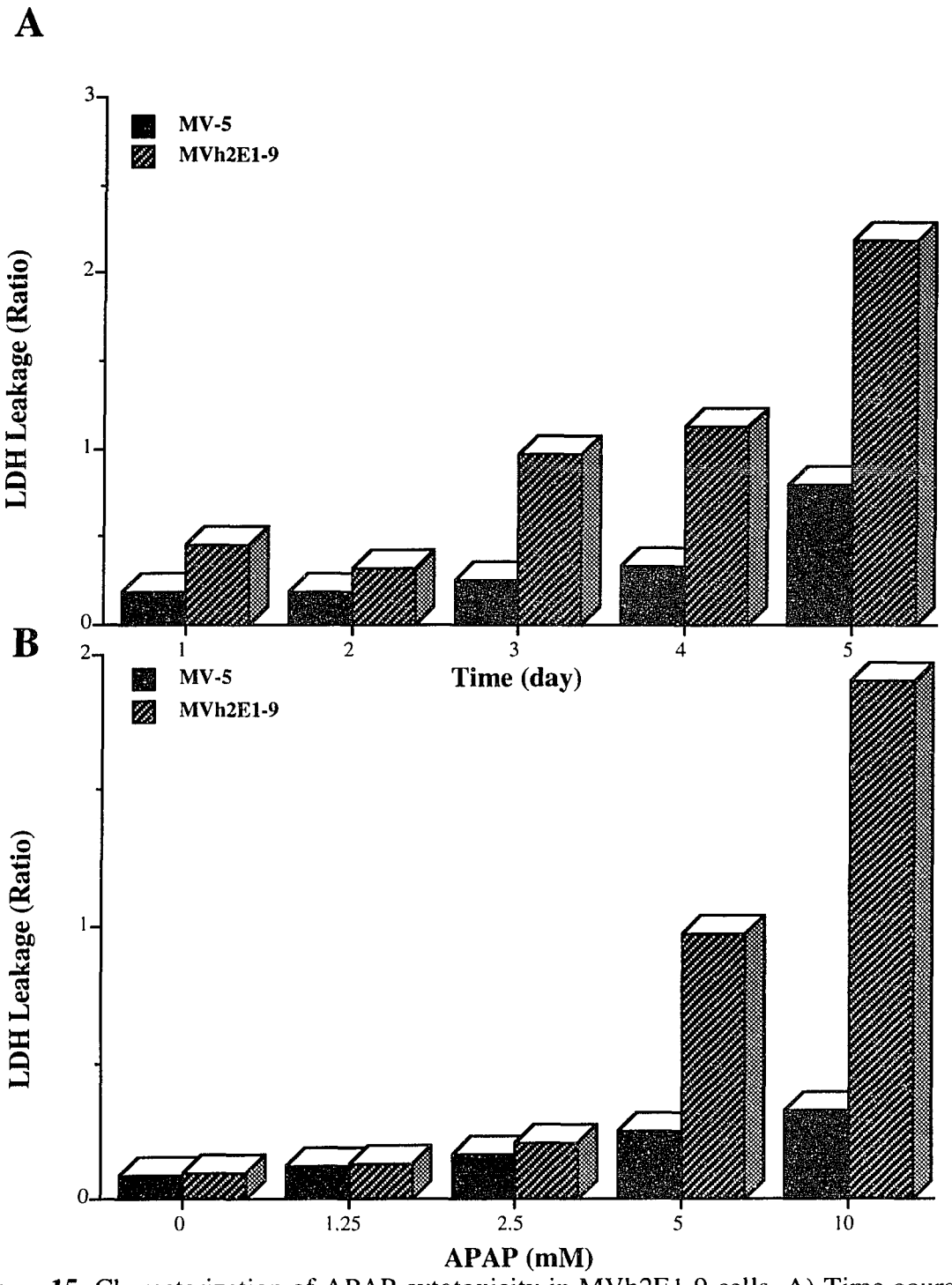


Figure 15. Characterization of APAP cytotoxicity in MVh2E1-9 cells. A) Time course. MV-5 and MVh2E1-9 cells were grown to about 30% confluence, treated with 5 mM APAP in the presence of 100 μM BSO and grown for the indicated periods of time. LDH activity was determined on the medium and the total cell extract. B) Dose-dependence. Cells were grown and treated with the indicated concentrations of APAP in the presence of 100 μM BSO for four days. LDH leakage was then determined.

C-4. Role of CYP2E1 in APAP Cytotoxicity - Since the only apparent difference between MVh2E1-9 and MV-5 cells is the expression of CYP2E1 in the former, but not the latter, it would appear that the cytotoxicity caused by APAP in MVh2E1-9 is due to the presence of CYP2E1 in these cells. To validate the role of CYP2E1 in APAP cytotoxicity in the MVh2E1-9 cells, several 2E1 ligands or inhibitors were added to MV-5 and MVh2E1-9 cells along with 10 mM APAP and 100 μM BSO. Results are shown in **Table 9**. 4-MP, a good ligand of 2E1 and an effective inhibitor of CYP2E1-catalyzed oxidation of substrates (Feierman and Cederbaum, 1986), completely prevented the cytotoxicity produced by APAP in the MVh2E1-9 cells when added to a final concentration of 2 mM. 4-MP at a concentration of 0.5 mM was also effective. Ethanol, another ligand and substrate of 2E1 inhibited APAP toxicity 71% at 25 mM and 75% at 100 mM. These two agents did not evoke LDH leakage nor cause any change in MV-5 cells. DAS, a mechanism-based 2E1 inhibitor (Brady et al., 1991), completely prevented APAP toxicity when added at a concentration of 0.1 mM, but had a somewhat less protective effect at a higher (0.4 mM) concentration, perhaps because DAS itself is toxic to the cells at this concentration. However, DAS was not toxic to MV-5 cells at this concentration implying that the potential toxic effect of DAS may be mediated by its interaction with CYP2E1 or with the bioactivation of APAP by the P450.

C-5. Effect of Antioxidants on APAP Cytotoxicity - To further characterize the nature of APAP-induced cytotoxicity, several antioxidants were added with 5 mM APAP and 100 μM BSO. As shown in **Table 10**, *N*-acetylcysteine produced a concentration-dependent inhibitory effect on the APAP-induced cytotoxicity in MVh2E1-9 cells; *N*-acetylcysteine was also protective against the low LDH leakage produced by APAP in MV-5 cells. At concentrations of 2 or 8 mM, *N*-acetylcysteine reduced the APAP-induced LDH leakage by 50% and 80%, respectively. PBN, a spin trapping agent, which has been shown to protect against toxicity caused by reactive radicals (Paracchini et al., 1993), had a protective effect

Table 9. Effect of CYP2E1 Ligands on APAP Cytotoxicity

Addition	Concentration (mM)	LDH Leakage (ratio)	
		MV-5	MVh2E1-9
None	-	0.24	7.74
4-MP	0.5	0.23	0.24
	2.0	0.26	0.25
DAS	0.1	0.24	0.24
	0.4	0.24	1.06
Ethanol	25.0	0.21	2.23
	100.0	0.25	1.90

MVh2E1-9 cells and the control MV-5 cells were grown in 35 mm 6-well plates to about 30% confluence. Cells were then treated with 5 mM APAP in the presence of 100 μM BSO and the indicated additions for three days. LDH activity was measured in both culture medium and the cell extract, and the ratio of the activity in the medium to that in the cell extract was taken as the index of LDH leakage.

Table 10. Effect of Antioxidants on APAP Cytotoxicity

Addition	Concentration (mM)	LDH Leakage (ratio)	
		MV-5	MVh2E1-9
None	-	0.15	1.95
N-Acetylcysteine	0.5	0.11	1.60
	1.0	0.09	1.25
	2.0	0.10	0.86
	4.0	0.08	0.67
	8.0	0.07	0.43
Propylgallate	0.025	0.11	2.35
	0.050	0.09	2.87
	0.100	0.09	4.69
PBN	0.25	0.10	1.22
	0.50	0.09	1.02
	1.00	0.07	0.88

MVh2E1-9 cells and the control MV-5 cells were grown in 35 mm 6-well plates to about 30% confluence. Cells were then treated with 5 mM APAP in the presence of 100 μM BSO and the indicated addition for three days. LDH activity was measured in both the culture medium and the cell extract.

against the APAP toxicity in MVh2E1-9 cells. At 1 mM, PBN inhibited about 50% of the APAP-induced toxicity. Propylgallate, a classical inhibitor of lipid peroxidation, did not prevent the APAP toxicity in MVh2E1-9 cells; in fact some increase in LDH leakage was observed.

C-6. Inhibition of Microsomal PNP Oxidation by APAP - The ability of 4-MP, ethanol and DAS to prevent APAP-induced cytotoxicity in MVh2E1-9 cells suggests that CYP2E1-mediated oxidation of APAP to toxic metabolites which can be scavenged by GSH, *N*-acetylcysteine or PBN is involved in the overall pathway of APAP cytotoxicity. To show that APAP was interacting with CYP2E1 expressed in MVh2E1-9 cells, the effect of APAP on the oxidation of PNP by microsomes isolated from these cells was compared to its effect on PNP oxidation by rat liver microsomes. Although rates of PNP oxidation differ between the two microsomal preparations, the APAP inhibition curve of PNP oxidation is identical (**Figure 16**). At 10 mM, APAP produced about 40% inhibition of the PNP oxidation by both microsomal preparations. PNP oxidation by microsomes from MVh2E1-9 cells was previously shown to be sensitive to inhibition by 4-MP, ethanol, diethyldithiocarbamate and antibody against CYP2E1 (**Table 2** and **3**) indicating its dependence on CYP2E1.

C-7. APAP Adduct Formation in MVh2E1-9 Cells - Radioactive ^{14}C-labeled APAP at a final concentration of 2 μCi/ml was added to MVh2E1-9 and MV-5 cells in the absence or presence of 100 μM BSO. Cells were harvested 24 hours later and total protein adducts were determined by counting radioactivity in the TCA-precipitable fraction. As shown in **Table 11**, a small amount of labeled adducts was found in MV-5 cells which was comparable in both non-BSO-treated and BSO-treated cells (9.3 and 11.9 pmol/mg of cell protein, respectively). Adduct formation in MV-5 cells was not affected by 4-MP, GSH, *N*-acetylcysteine, ethanol or PBN suggesting that these low counts may reflect nonspecific

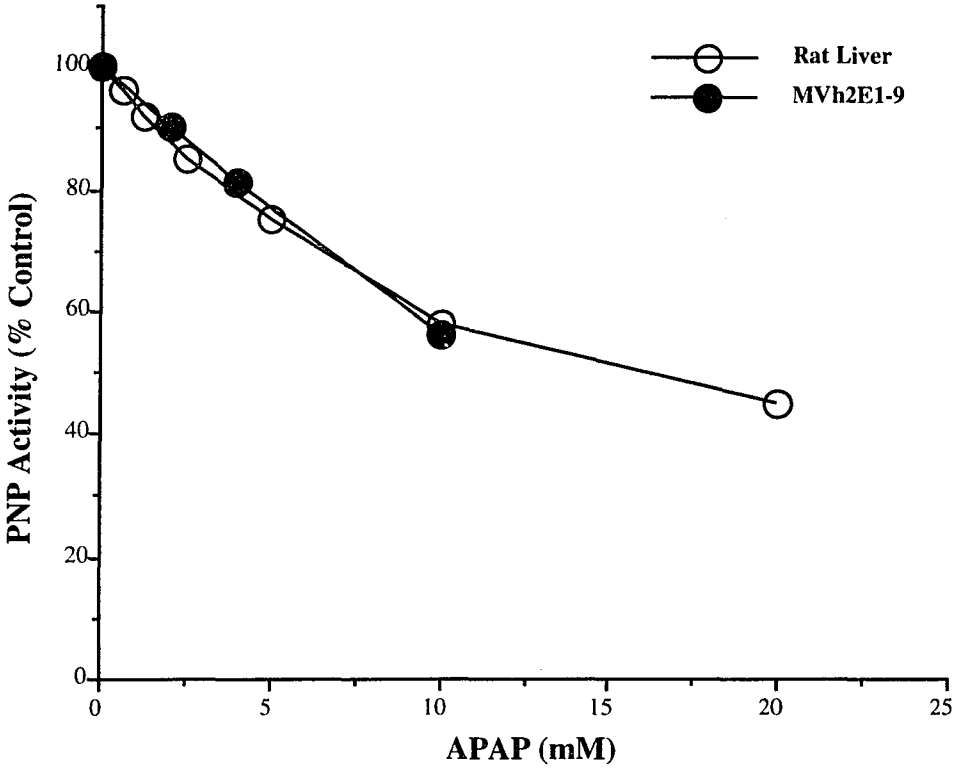


Figure 16. Inhibition of microsomal oxidation of PNP by APAP. PNP oxidation was assayed with microsomes from rat liver or MVh2E1-9 cells treated with 0.1 μg/ml PMA for 24 hours in the presence of different concentrations of APAP. Protein concentration was 1 mg/ml for rat liver microsomes and 2.5 mg/ml for MVh2E1-9 microsomes. Each point represents results from duplicate experiments. Activity was expressed as percentage of the non-APAP-treated control; control values in the absence of APAP were 2.2 and 0.15 nmol/min/mg microsomal protein for rat liver microsomes and MVh2E1-9 microsomes, respectively.

Table 11. APAP Adduct Formation and Effect of Various Additions

Addition	Concentration (mM)	BSO (100μM)	APAP Adduct (pmol/mg protein)		
			MV-5	MVh2E1-9	MVh2E1-9 - MV-5
None	-	-	9.3	20.4	11.1
4-MP	2	-	5.9	9.8	3.9
None	-	+	11.9	46.4	34.5
4-MP	2	+	10.1	8.5	-1.6
N-acetylcysteine	2	+	8.9	18.9	9.9
Ethanol	100	+	8.6	14.8	6.2
PBN	1	+	13.2	16.4	3.2

MVh2E1-9 and the control MV-5 cells were grown to about 80% confluence in 35 mm 6-well plates. Cells were treated with 2 μCi/ml 14C-labeled APAP in the absence or presence of 100 μM BSO plus the indicated compounds and grown for an additional 24 hours. Cells were then trypsinized, pelletted and sonicated. Total cellular radioactively-labeled protein adduct was measured as decribed in Materials and Methods.

binding of ^{14}C-labeled APAP to the cellular protein. Increased adduct formation (20.4 pmol/mg of cell protein) was detected in MVh2E1-9 cells compared to MV-5 cells (9.3 pmol/mg of cell protein) in the absence of BSO; and this increase was intensified when BSO was present (46.4 compared to 11.9 pmol/mg of cell protein) (**Table 11**). A 2.5-fold increase in adduct formation was observed when GSH was depleted from the MVh2E1-9 cells confirming the ability of GSH to bind reactive APAP metabolites such as NAPQI (Albano et al., 1985; Moore et al., 1985).

Importantly, the addition of various chemicals which were effective in preventing APAP cytotoxicity proved to be effective in inhibition of the APAP adduct formation in MVh2E1-9 cells (**Table 11**). 4-MP at a 2 mM concentration lowered the adduct formation in MVh2E1-9 cells to the level found in MV-5 cells, i.e., 4-MP completely prevented the elevated production of APAP adducts in the MVh2E1-9 cells. *N*-acetylcysteine at 2 mM, ethanol at 100 mM, and PBN at 1 mM produced about 71%, 82% and 91% inhibition of the CYP2E1-dependent adduct formation respectively. None of these agents caused significant changes in MV-5 cells.

C-8. Intracellular Localization of APAP Adduct - To investigate where inside the cell APAP-protein adducts were produced, the total cell extracts from MVh2E1-9 cells and the control MV-5 cells that were treated with ^{14}C-labeled APAP for 24 hours were fractionated by differential centrifugation. The difference in counts of the TCA precipitated radioactivity of various fractions isolated from the MVh2E1-9 and MV-5 cells was assumed to reflect APAP-protein adducts produced by the CYP2E1-dependent pathway. As shown in **Table 12**, this net difference was 552 cpm/mg of protein for the total cell extract. While there appeared to be a slight enrichment of adducts in the nuclear and mitochondrial fractions, all fractions displayed counts at least equivalent to the starting extract, suggesting that APAP-protein adducts were formed to similar extents in all subcellular compartments.

Table 12. Subcellular Fractionation of 14C-labeled APAP-Protein Adducts

Fraction	APAP-Protein Adducts (pmol/mg protein)		
	MV-5	MVh2E1-9	MVh2E1-9 - MV-5
Total extract	22.1	55.5	33.4
Nuclear	22.1	72.0	49.9
Post-nuclear	22.5	52.8	30.3
Mitochondrial	32.5	75.4	42.9
Post-mitochondrial	25.0	55.3	30.3
Microsomal	24.0	56.5	32.5
Cytosolic	13.6	45.6	32.0

MV-5 and MVh2E1-9 cells were treated with 2 μCi/ml 14C-labeled APAP in the presence of 100 μM BSO for 24 hours. Cell extracts were fractionated, precipitated with TCA, washed and counted as decribed in Materials and Methods. The counts of MVh2E1-9 minus MV-5 were calculated and represent the CYP2E1-dependent APAP-protein adducts.

C-9. Role of Calcium on Anti-proliferation and Cytotoxicity of APAP in MVh2E1-9 Cells

- Since loss of calcium regulation has been proposed to be the final common pathway in many models of cell death, experiments were carried out to evaluate the effects of a calcium channel blocker, verapamil, and a calcium/calmodulin antagonist, chlorpromazine, on APAP toxicity in MVh2E1-9 cells. First, we examined whether these two agents altered the anti-proliferative effect of APAP on MV-5 and MVh2E1-9 cells. Cells were treated with either 5 μM verapamil or 1 μM chlorpromazine in the presence of 1 mM APAP for three days. 1 mM APAP produced a 35% decrease in MV-5 cell counts and a 47% decrease in MVh2E1-9 cell counts after 3 days of treatment in the absence of BSO. Neither verapamil nor chlorpromazine protected against the APAP inhibition of proliferation of both cell lines (**Table 13**). Under more drastic conditions, e.g., incubation with 10 mM APAP in the presence of BSO, cell proliferation was inhibited by about 85% with both cell lines and verapamil or chlorpromazine again did not prevent this effect of APAP (**Table 13**). Higher concentration of these agents caused significant toxicity in both MV-5 and MVh2E1-9 cells and could not be used.

Secondly, the MV-5 and MVh2E1-9 cells were treated with verapamil or chlorpromazine in the presence of 10 mM APAP and 100 μM BSO for three days to evaluate the effect of the two agents on the cytotoxic effect of APAP. APAP increased LDH leakage by 3.5- and 8-fold for MV-5 and MVh2E1-9 cells, respectively. Verapamil and chlorpromazine produced a small non-significant protective effect against the APAP-induced increase in LDH leakage of MVh2E1-9 cells but not MV-5 cells (**Table 13**).

Table 13. Effect of Calcium Antagonists on the Antiproliferative and Cytotoxic Effect of APAP

BSO	Addition	Cell Count (x1000)				LDH Leakage (ratio)	
		MV-5		MVh2E1-9		MV-5	MVh2E1-9
A							
0	None	1300	(100%)	1341	(100%)		
	1 mM APAP	869	(65%)	716	(53%)		
	1 mM APAP + 5 μM Verapamil	621	(46%)	725	(54%)		
	1 mM APAP + 1 μM Chlorpromazine	603	(45%)	670	(50%)		
B							
100 μM	None	1368	(100%)	1530	(100%)	0.075	0.082
	10 mM APAP	209	(15%)	199	(13%)	0.263	0.624
	10 mM APAP + 5 μM Verapamil	245	(18%)	233	(15%)	0.279	0.488
	10 mM APAP + 1 μM Chlorpromazine	231	(17%)	216	(14%)	0.261	0.531

MV-5 and MVh2E1-9 cells were incubated with the indicated additions in the absence (A) or presence (B) of 100 μM BSO for 3 days. Cells were counted with a hemocytometer or LDH leakage was determined (B).

D. Inactivation and Degradation of the Expressed CYP2E1 in the Transduced HepG2 Cells

D-1. Induction of CYP2E1 Expression by PMA in MVh2E1-9 Cells - Since the expression of the transfected CYP2E1 is relatively low (15 pmol/mg microsomal protein) in MVh2E1-9 cells, a variety of chemicals were tested to determine if any these compounds could increase the 2E1 expression in this system. As shown in **Table 14**, ethanol, all trans-retinoic acid, dibutyryl cAMP and interleukin 6 increased the 2E1 expression by 60% to 100% as determined by an increase in the oxidation of PNP by isolated microsomes. Increased activity by retinoic acid, dibutyryl cAMP and IL-6 is presumably mediated through transcriptional activation of the viral LTR promoter while that of ethanol is probably due to stabilization of the P450 2E1 itself against degradation (Song et al., 1986). PMA added in culture increased the 2E1 expression by about 250% at concentrations ranging between 0.04 μg/ml to 1 μg/ml (**Table 14**). As will be discussed below, the increased enzyme activity was correlated with the increase in 2E1 protein content. In most of the following experiments, PMA at a concentration of 0.1 μg/ml was added to the culture medium in order to stimulate 2E1 expression in the system. Treatment of MV-5 cells, which lack the cDNA for 2E1, with the agents shown in **Table 14** did not result in an induction of the endogenous CYP2E1 gene.

Table 14. Induction of Expression of the Transfected P450-2E1 by Compounds Added in Culture

Addition	Concentration	PNP Activity (pmol/min/mg)	Stimulation by addition (%)
Control	-	39	-
Ethanol	30 mM	62	60
Retinoic Acid	0.1 mM	79	104
db-cAMP	0.5 mM	82	110
IL-6	10 U/ml	79	104
PMA	0.04 µg/ml	133	240
	0.2 µg/ml	141	262
	1 µg/ml	134	244
	5 µg/ml	111	185

MVh2E1-9 cells were treated with the indicated additions to attempt to find the condition that maximizes the 2E1 expression. Treatment was carried out in situ for 24 hours. Microsomes were isolated for each treatment and PNP activity was assayed with 2.5 mg microsomal protein/ml, 0.2 mM PNP, and 1 mM NADPH in 0.1 M phosphate buffer for 60 minutes.

D-2. Inactivation and Degradation of CYP2E1 by CCl_4 Added in Culture - MVh2E1-9 cells were treated with 2 mM CCl_4 in the absence or presence of 0.1 μg/ml PMA for 24 hours. Microsomes were isolated and oxidation of PNP was determined. In the absence of PMA, CCl_4 treatment caused an approximate 35% loss of enzyme activity (**Figure 17**, columns 5 and 6). In the presence of PMA, CCl_4 caused a more prominent (~50%) loss of 2E1 enzyme activity (**Figure 17**, columns 7 and 8). These results indicated that treatment with CCl_4 caused an inactivation of CYP2E1. To evaluate whether the inactivated CYP2E1 was degraded, Western blot analysis to assay the content of CYP2E1 protein was carried out. In the MVh2E1-9 cells, anti-human CYP2E1 IgG recognized a 54,000 kD protein (**Figure 18**, lane 5). Treatment with PMA increased the intensity of this band (**Figure 18**, lane 7) analogous to the increase in PNP oxidation caused by PMA. Treatment with CCl_4 caused a decrease in CYP2E1 protein (**Figure 18**, lane 6 compared to lane 5, or lane 8 compared to lane 7); the decrease in CYP2E1 protein was similar to the decrease in PNP oxidation and more pronounced after the PMA treatment.

Since the experiments were carried out in continuous culture, new 2E1 protein is being synthesized during the 24 hour treatment with PMA and CCl_4; this newly-synthesized protein may mask the true effect of CCl_4 on 2E1 inactivation and degradation. To evaluate the net effect of CCl_4 on 2E1 degradation, cycloheximide was used to block new protein synthesis. Under this condition, 2E1 enzyme activity and protein were lowered in the MVh2E1-9 cells (**Figures 17 and 18**, lanes 1 and 3 compared to lanes 5 and 7). Inactivation and degradation of CYP2E1 by CCl_4 was more pronounced in the presence of cycloheximide as PNP oxidation and the content of CYP2E1 were decreased by ~70% in the absence of PMA (**Figures 17 and 18**, lanes 1 and 2) and by ~90% in the presence of PMA (**Figures 17 and 18**, lanes 3 and 4) after the treatment with CCl_4. Since PNP hydroxylase activity and 2E1 protein content measured by Western blot were lowered to comparable levels by CCl_4, it appears that

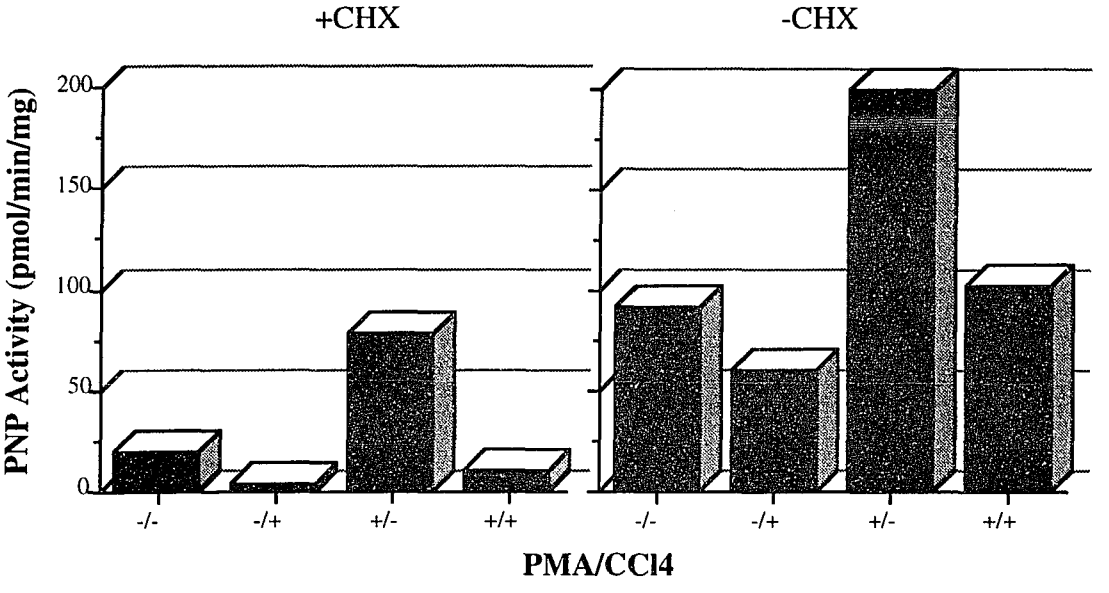
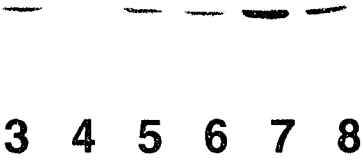


Figure 17. Effect of treatment of MVh2E1-9 cells with CCl_4 on the oxidation of PNP. MVh2E1-9 cells were grown in the absence (lanes 5 to 8) or presence (lanes 1 to 4) of 10 μM cycloheximide under the following conditions: no other addition (lanes 1 and 5); 2 mM CCl_4 (lanes 2 and 6); 0.1 μg/ml PMA (lanes 3 and 7); PMA plus CCl_4 (lanes 4 and 8). Cycloheximide was added at the same time to the cultures as was PMA and CCl_4. After 24 hours, microsomes were isolated and the oxidation of PNP was determined.



1 2 3 4 5 6 7 8

Figure 18. Effect of treatment of MVh2E1-9 cells with CCl_4 on the content of CYP2E1. MVh2E1-9 cells were grown in the absence (lanes 5 to 8) or presence (lanes 1 to 4) of 10 μM cycloheximide under the following conditions: no other addition (lanes 1 and 5); 2 mM CCl_4 (lanes 2 and 6); 0.1 μg/ml PMA (lanes 3 and 7); PMA plus CCl_4 (lanes 4 and 8). After 24 hours, microsomes were isolated and the content of 2E1 was determined using Western blot analysis. 50 μg microsomal protein was loaded in each lane.

2E1 protein labilized by CCl_4 is rapidly removed by an intracellular proteolytic system present in the HepG2 cells.

D-3. Lack of Inactivation of CYP2E1 in situ by APAP - We have observed in the above section that microsomal oxidation of PNP *in vitro* was also inhibited by APAP, analogous to the CCl_4 inhibition. We therefore investigated whether APAP, like CCl_4, could inactivate CYP2E1 *in situ*. MVh2E1-9 cells were incubated with 10 mM APAP in the absence and presence of BSO for 2 days. Microsomes were isolated, and PNP hydroxylase activity determined. As shown in **Figure 19**, *in situ* incubation of MVh2E1-9 cells with APAP, in contrast to CCl_4, did not result in inhibition of PNP oxidation suggesting that APAP did not inactivate CYP2E1. These results contrast the effects of two hepatotoxins on labilizing and inactivating CYP2E1 *in situ*.

D-4. Inhibition of Microsomal PNP Oxidation In Vitro by CCl_4 - The effect of CCl_4 on 2E1 degradation requires initial bioactivation of CCl_4 by the P450. To show that CCl_4 was interacting with CYP2E1 expressed in the MVh2E1-9 cells, the effect of CCl_4 on PNP oxidation in the isolated microsomes of MVh2E1-9 cells was evaluated. As shown in **Figure 20**, CCl_4 caused a dose-dependent decrease of microsomal PNP oxidation in MVh2E1-9 cells. At a concentration of 0.25 mM, CCl_4 inhibited about 50% of microsomal PNP oxidation. To test whether the *in vitro* incubation with CCl_4 caused degradation of CYP2E1, Western blots were carried out with samples incubated in the absence and presence of CCl_4 (**Figure 21**). In contrast to the degradation found *in situ*, *in vitro* addition of CCl_4 did not cause CYP2E1 degradation (lanes 1 and 2) even after addition of ATP plus Mg^{2+} (lane 4). Under conditions of extensive lipid peroxidation, e.g., addition of ferric-ATP, there was degradation of CYP2E1 (**Figure 21,** lane 3). The ability of CCl_4 to promote lipid peroxidation by the isolated microsomes was evaluated by studying the formation of thiobarbituric acid-reactive components. Control rates of microsomal lipid

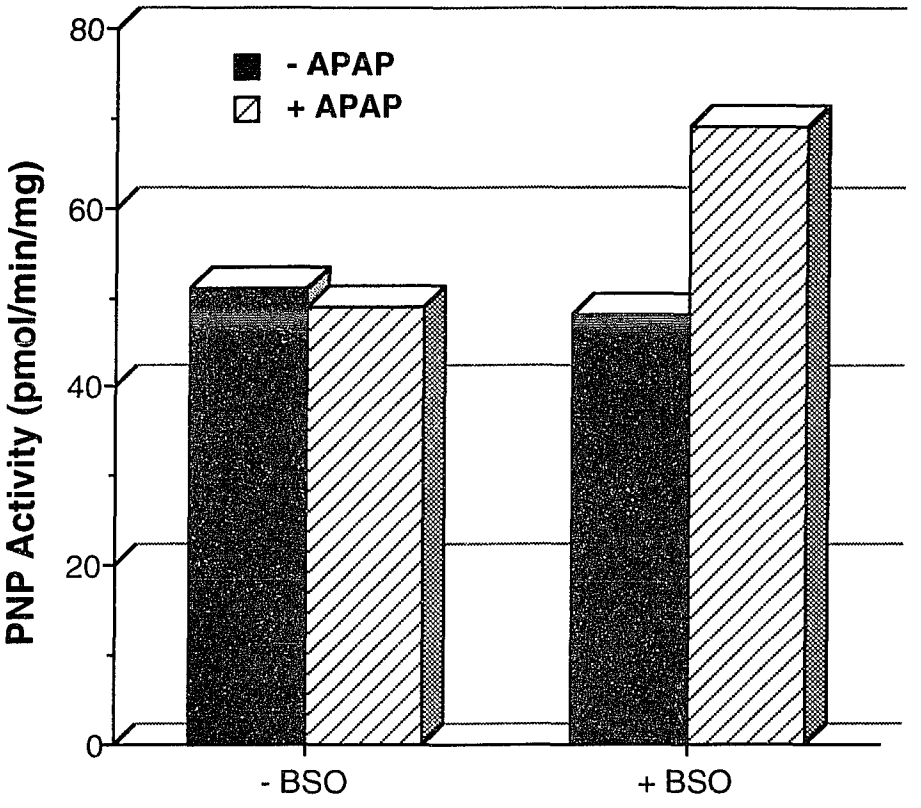


Figure 19. Effect of APAP on the CYP2E1 enzyme activity. MVh2E1-9 cells were treated with 10 mM APAP in the absence or presence of 100 μM BSO for 2 days. Microsomes were isolated for each treatment. PNP assay was done with 2.5 mg/ml microsomal protein, 0.2 mM PNP, 1 mM NADPH in 0.1 M potassium phosphate buffer for 60 minutes.

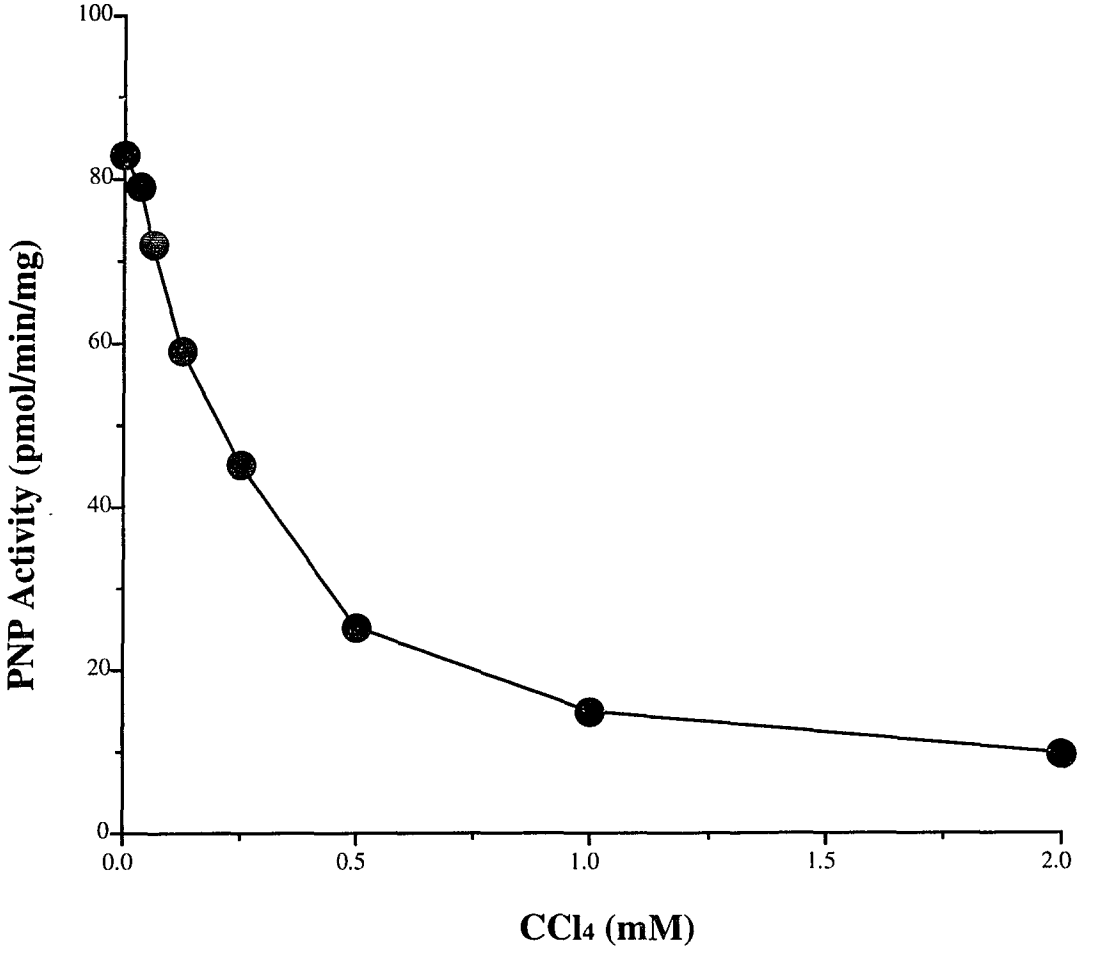


Figure 20. Effect of *in vitro* addition of CCl_4 on the oxidation of PNP by microsomes isolated from MVh2E1-9 cells. The oxidation of 0.2 mM PNP was determined in the presence of the indicated concentrations of CCl_4.

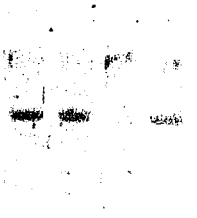


Figure 21. Effect of CCl_4 and ferric-ATP on CYP2E1 content in MVh2E1-9 microsomes. Microsomes were prepared from the MVh2E1-9 cells that were grown to confluence followed by treatment with 0.1 μg/ml PMA for 24 hours. Microsomes were incubated in phosphate buffer (lane 1), plus 2 mM CCl_4 (lane 2), 50 μM ferric-ATP (lane 3), or 2 mM CCl_4 and 10 mM Mg/ATP, and reacted for 4 hours in the presence of NADPH. Western blot with anti-human CYP2E1 antibody was then carried out to determined the content of 2E1.

peroxidation were low, and these low rates, in contrast to ferric-ATP, were not elevated by CCl_4 (data not shown).

D-5. Characterization of CCl_4-induced Inactivation and Degradation of 2E1 in MVh2E1-9 Cells - To further characterize the relationship between the necessity for bioactivation of CCl_4 by human CYP2E1 and the CCl_4-enhanced inactivation and degradation of 2E1 itself, several 2E1 substrates which would compete with CCl_4 for CYP2E1 were added along with 2 mM CCl_4 and PMA to the medium and the cells were cultured for 24 hours. In the absence of substrates, the treatment with CCl_4 caused a 40% loss of PNP activity and 2E1 content (**Table 15** and **Figure 22**). This loss in PNP oxidation was prevented by the competitive substrates; if compared to the corresponding no CCl_4 control, the loss in enzyme activity could be prevented about 66% by 4 mM DMN, 68% by 2 mM 4-MP and 74% by 100 mM ethanol (**Table 15**). The degradation of CYP2E1 as reflected by the loss in CYP2E1 content was also prevented to similar levels by these agents (**Figure 22**). **Table 19** shows the arbitrary units of the densitometry scans, CCl_4 produced about 54% degradation of CYP2E1 and almost complete protection was afforded by 4-MP, ethanol and DMN. Under the same condition, *N*-acetylcysteine did not prevent degradation of the CCl_4-labilized CYP2E1.

Table 15. Effect of 2E1 Ligands and Substrates on CCl4-induced Loss of PNP Activity in MVh2E1-9 Cells

Addition	Concentration	PNP Activity (pmol/min/mg)		Ratio of PNP Activity
		(-) CCl4	(+) CCl4	(+)CCl4/(-)CCl4
Control	-	130	81	0.62
DMN	4 mM	126	110	0.87
4-MP	2 mM	194	171	0.88
Ethanol	100 mM	171	154	0.90

MVh2E1-9 cells were treated with the indicated additions in the absence or presence of CCl4 for 24 hours with 0.1 μg/ml PMA. Microsomes were isolated from each treatment. The assay for PNP oxidation was carried out with 2.5 mg/ml microsomal protein, 0.2 mM PNP and 1 mM NADPH for 60 minutes.

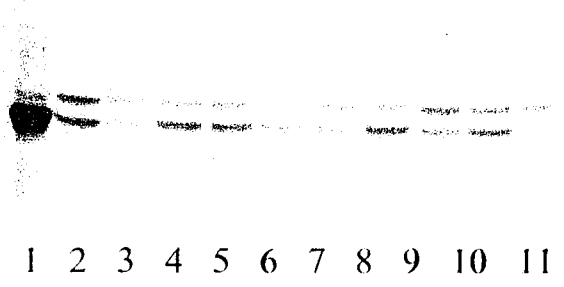


Figure 22. Effect of CYP2E1 ligands and an antioxidant on the CCl_4-induced degradation of CYP2E1 in MVh2E1-9 cells. MVh2E1-9 cells were incubated in the absence and presence of 2 mM CCl_4 (lanes 2 and 3) along with either 2 mM 4-MP (lanes 4 and 5), 4 mM DMN (lanes 6 and 7), 100 mM ethanol (lanes 8 and 9), or 2 mM *N*-acetylcysteine (lanes 10 and 11) for 24 hours. Microsomes were prepared and the content of CYP2E1 was determined by Western blot analysis as described in Materials and Methods. 50 μg of microsomal protein was loaded to each lane. Lane 1, human liver microsomes (5 μg protein).

Since it has been reported that labilization of CYP2E1 by oxidative stress is required for the subsequent inactivation and degradation of the enzyme, the effect of several antioxidants on the actions by CCl_4 on 2E1 was evaluated. *N*-acetylcysteine, a sulfhydryl compound, at a concentration of 2 mM appeared to prevent the loss of 2E1 enzyme activity by about 75% (**Table 16**). However, it lowered the control PNP oxidation rate. *N*-Acetylcysteine did not prevent the loss of CYP2E1 protein produced by CCl_4 (**Figure 22**). PBN (1.25 mM), a spin trapping agent, and propylgallate (0.1 mM), a classical inhibitor of lipid peroxidation, did not have any protective effect (**Table 16**). Although CCl_4 did not stimulate microsomal production of TBA-reactive components, incubation of the microsomes with NADPH plus ferric-ATP did. Under such conditions of microsomal lipid peroxidation, there was a loss in CYP2E1 content (**Figure 21**)

D-6. CCl_4 Adduct Formation in MVh2E1-9 Cells - To show further that CCl_4 is indeed metabolized by 2E1 and can interact with intracellular macromolecules, radioactive ^{14}C-labeled CCl_4 at a final concentration of 1 mM was added to MVh2E1-9 and the control MV-5 cells and adduct formation was determined after TCA precipitation. As shown in **Table 17**, MV-5 cells produced 27.8 pmol/mg cell protein, and these counts were completely insensitive to 4-MP, indicating they were not dependent on CYP2E1. Either these counts reflect non-specific counts or adduct formation via CCl_4 bioactivation by CYP2E1-independent pathways. There was an increase in adduct formation in the MVh2E1-9 cells to 40.4 pmol/mg cell protein, and importantly, this increase was completely prevented by 4-MP. The inhibition by 4-MP links the increase in CCl_4 adduct formation to CYP2E1-dependent activity. When microsomes isolated from MV-5 and MVh2E1-9 cells were reacted with ^{14}C-labeled CCl_4 *in vitro*, the adduct formation in the MV-5 microsomes was insensitive to 4-MP and ethanol indicating that it was not CYP2E1-dependent. There was an increase in adduct formation with the microsomes from the MVh2E1-9 cells, and the net increase (5.8

Table 16. Effect of Antioxidants on CCl4-induced Loss of PNP Activity in MVh2E1-9 Cells

Addition	Concentration	PNP Activity (pmol/min/mg)		Ratio of PNP Activity
		(-) CCl4	(+) CCl4	(+)CCl4/(-)CCl4
Control	-	58	42	0.72
PBN	1.25 mM	85	58	0.68
PPG	0.1 mM	59	35	0.60

MVh2E1-9 cells were treated in situ with the indicated agents including the spin trapping agent PBN and the antioxidants N-acetylcysteine and PPG for 24 hours in the absence or presence of CCl4 with 0.1 µg/ml PMA. Microsomes were isolated for each treatment and oxidation of PNP was determined.

Table 17. CCl4 Adduct Formation in HepG2 Cells and in Isolated Microsomes

Reations	Addition	CCl4 adducts (pmol/mg protein)		CYP2E1-dependent adducts (cpm/mg protein)
		MV-5	MVh2E1-9	
A. Intact cells	CCl4	27.8	40.4	12.6
	CCl4+4-MP	29.8	25.8	-4.0
B. Microsomes	CCl4	23.8	29.6	5.8
	CCl4+4-MP	21.8	20.2	-1.6
	CCl4+ethanol	25.1	22.9	-3.2

A. MVh2E1-9 cells were treated in situ with 1 mM 14C-labeled CCl4 in the absence or presence of 2 mM 4-MP for 24 hours. Cells were harvested and cell extracts were prepared. 0.5 mg of cell extracts were precipitated with TCA, washed and counted as described in Materials and Methods.

B. Microsomes from MVh2E1-9 cells were treated in vitro with 1 mM 14C-labeled CCl4 or CCl4 plus 2 mM 4-MP or CCl4 plus 100 mM ethanol for 4 hours. The reactions were then TCA-precipitated, washed and counted as above.

The difference in counts of MVh2E1-9 and MV-5 cells were calculated as CYP2E1-dependent CCl4 adduct formation.

pmol/mg cell protein) was completely blocked by 4-MP and ethanol, indicating its dependence on CYP2E1 (**Table 17**)

D-7. Effect of Protease Inhibitors on CCl_4-induced degradation of CYP2E1. -To attempt to identify the degradative system that hydrolyzes 2E1 after labilization by CCl_4, a variety of protease inhibitors were used. As shown in **Table 18**, E-64 and leupeptin, two representative serine protease inhibitors, were not effective against the CCl_4-induced loss of PNP hydroxylase activity, suggesting these protease inhibitors did not affect the inactivation of CYP2E1 by the activated CCl_4. However, the ~ 50% loss of 2E1 protein, as measured by Western blot (**Figure 23**), was lowered to 17% and 15% by E-64 and leupeptin respectively. This loss of 2E1 protein was also significantly prevented to 28% and 23% by NH_4Cl and chloroquine, two other lysosomal protease inhibitors, respectively (**Figure 23** and **Table 19**), suggesting that CCl_4-induced degradation of CYP2E1 may at least in part be dependent on lysosomal proteases.

Table 18. Effect of Proteas Inhibitors on CCl4-induced Loss of PNP Activity in MVh2E1-9 Cells

Addition	Concentration	PNP Activity (pmol/min/mg)		Ratio of PNP Activity
		(-) CCl4	(+) CCl4	(+)CCl4/(-)CCl4
Control	-	94	54	0.57
E-64	0.1 mM	87	57	0.66
Leupeptin	0.1 mM	92	43	0.47

MVh2E1-9 cells were treated in situ with the indicated proteas inhibitors in the absence or presence of 2 mM CCl4 for 24 hours with 0.1 μg/ml PMA. Microsomes were then prepared and PNP oxidation was determined.

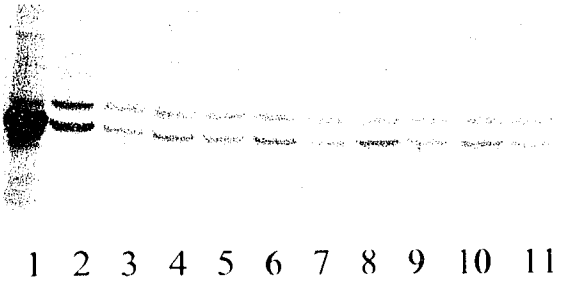


Figure 23. Effect of protease inhibitors on the CCl_4-induced degradation of CYP2E1 in MVh2E1-9 cells. MVh2E1-9 cells were incubated in the absence and presence of 2 mM CCl_4 (lanes 2 and 3) along with either 0.1 mM E-64 (lanes 4 and 5), 0.1 mM leupeptin (lanes 6 and 7), 5 mM NH_4Cl (lanes 8 and 9), or 50 μM chloroquine (lanes 10 and 11) for 24 hours. Microsomes were prepared and the content of CYP2E1 was determined by Western blot analysis as described in Materials and Methods. 50 μg of microsomal protein was loaded to each lane. Lane 1, human liver microsomes (5 μg protein).

Table 19. Effect of 2E1 Ligands and Proteas Inhibitors on CCl4-induced Loss 2E1 Content in MVh2E1-9 Cells

Addition	Concentration	Density (arbitrary unit)		Ratio
		(-) CCl4	(+) CCl4	(+)CCl4/(-)CCl4
A.				
(-)	-	1.659	0.76	0.46
4-MP	2 mM	1.933	1.609	0.83
DMN	4 mM	0.811	0.86	1.06
Ethanol	100 mM	1.462	1.264	0.86
N-Acetylcysteine	2 mM	1.45	0.56	0.39
B.				
(-)	-	2.002	0.986	0.49
E-64	0.1 mM	1.577	1.311	0.83
Leupeptin	0.1 mM	1.574	1.332	0.85
NH4Cl	5 mM	1.377	0.985	0.72
Chloroquine	50 μM	1.231	0.948	0.77

MVh2E1-9 cells were treated in situ with the indicated additions in the absence or presence of 2 mM CCl4 for 24 hours with 0.1 μg/ml PMA. Microsomes were then prepared and 2E1 protein was assayed by Western blot and densitometry scanning.

DISCUSSION

A. Stable Expression of Human CYP2E1 in HepG2 Cells and Characterization of Catalytic Activities

Human cytochrome P450-2E1 has been transiently expressed in two culture systems, COS cells through transfection (Umeno et al., 1988) and HepG2 cells via recombinant *Vaccinia* virus infection (Patten et al., 1992; Waxman et al., 1991), as well as in *E. Coli* (Winters and Cederbaum, 1992) in a catalytic active form. A stable cell line constitutively expressing P450-2E1 has been reported in which human CYP2E1 was successfully transduced into rat liver epithelial cells and mouse NIH3T3 cells using recombinant retroviral infection. The expressed CYP2E1 oxidized DMN, producing covalent adducts of the carcinogen with cellular DNA (Nouso et al., 1992). HepG2 cells were selected as the target cell line in this study to stably express the human CYP2E1. This replicating cell line originates from human neoplastic liver tissue (Aden et al., 1979), expresses several liver-specific cell functions (Javitt, 1990), was shown to catalyze oxidation of certain drug substrates (Sassa et al., 1987), contains NADPH-cytochrome P450 reductase and cytochrome b5, and has a very low or nondetectable level of endogenous P450-2E1 (Aoyama et al., 1990; Patten et al., 1992; Waxman et al., 1991).

Human cytochrome P450-2E1 was stably transduced into HepG2, a human hepatoma cell line, by a recombinant retrovirus expression system. Southern blot analysis showed a successful integration of a single copy of unaltered viral DNA into the genome of each transduced clone tested. Western blot analysis indicated that the transduced clones produced a protein band at 54 kD, which is the same molecular weight as CYP2E1 in human liver microsomes. Northern blot analysis detected the CYP2E1 RNA transcripts from the exogenously transduced copy of the CYP2E1 gene. In both Northern and Western

blot analyses, HepG2, as well as the control clones, showed no CYP2E1 RNA transcripts or protein indicating CYP2E1 is not expressed in HepG2 cells. Southern blot analysis for HepG2 cells and the control clones, however, showed an unaltered endogenous copy of CYP2E1 suggesting that non-expression of CYP2E1 in HepG2 cells is due to an apparent lack of certain factors required for the basal transcription of the endogenous 2E1 gene rather than alteration of the gene itself.

The successfully CYP2E1-transduced HepG2 clones showed varying degrees of catalytic activities towards classic 2E1 substrates. The control clones or HepG2 cells did not express such activities. It is noticed that PNP hydroxylase activity is about 3-fold higher than DMN activity. This difference was also observed in a separate experiment with human liver microsomes, 0.87 and 0.25 nmol/min/mg microsomal protein for PNP and DMN activities respectively (**Table 8**), whereas in rat liver microsomes these two activities are about the same. This 0.25 nmol/min/mg DMN activity by human liver microsomes is in agreement with other reports with normal human samples (Wrighton et al., 1986). Since anti-human CYP2E1 antibody almost completely wiped out PNP activity in human liver microsomes (data not shown) and 2E1 was the predominant, if not sole, catalyst of DMN oxidation in rat liver microsomes (Yang et al., 1985), it is highly suggested that the differential activities for PNP and DMN in the two species indicate that human CYP2E1 is a better PNP catalyst than rat 2E1 and the quantitative difference in catalytic activities may be due to the qualitative differences of the 2E1 structure in the two species. The structural differences could also, at least partially, explain the differential inhibitory effects of PNP activity by anti-2E1 antibodies from the two species, i.e., anti-human 2E1 inhibits PNP activity from MVh2E1-9 clone better than anti-rat 2E1 antibody (**Table 2 and 3**). This was also in agreement with the result from a separate PNP inhibitory experiment with human and rat liver microsomes by the two antibodies. Therefore, the expressed human CYP2E1 had the general 2E1 characteristics observed in human liver microsomes and, as

expected, was more like human 2E1 than rat 2E1 especially in the enzyme activities. The differences in the molecular structure and biochemical properties of CYP2E1 among different species should be further investigated.

In summary, a human liver derived cell line has been established which stably and constitutively expresses the human CYP2E1 isoform. This cell line should prove valuable in studies designed to evaluate the oxidation of potential substrates for 2E1 in a native microsomal environment and to assess the potential toxicity associated with the oxidation of these substrates, as described below for acetaminophen. Current studies in our laboratory are assessing the cytotoxicity produced by ethanol (D. Wu), halothane (D. Feierman) and mitomycin (Q. Chen) in these cell lines. The cell line will also be of value in studying the ability of ligands to stabilize the expressed CYP2E1 against degradation, e.g., as shown for ethanol and 4-MP (**Table 14** and **15**) and which is shown in more detail in (Carroccio et al., 1994), and in determining the half-life of the expressed protein under a variety of conditions. These cell lines may also be used as starting points for attempts to increase the level of CYP2E1, e.g., by re-infecting with the retrovirus in order to obtain more copies integrated into the cells, or by infecting with a second retrovirus containing the CYP2E1 cDNA plus a different marker for antibiotic resistance.

B. Characterization of Generation of Reactive Oxygen Species in MVh2E1-9 Cells

Chronic alcohol consumption has been shown in rats to enhance the production of reactive oxygen species such as superoxide (Boveris et al., 1983; Ekström and Ingelman-Sundberg, 1989), hydrogen peroxide (Ekström and Ingelman-Sundberg, 1989; Lieber and DeCarli, 1970; Thurman, 1973), and hydroxyl radical (Dicker and Cederbaum, 1987; Klein et al., 1983), by isolated microsomes. Since CYP2E1 is the major ethanol-inducible enzyme found in microsomal systems, and is a loosely coupled enzyme which produces

oxygen radicals such as $\cdot O_2^-$ and H_2O_2 (see catalytic mechanism of P450), it may by itself contribute to the alcohol liver injury. Indeed, we detected a 2-fold elevated H_2O_2 production in CYP2E1-transduced clone than in the control MV-5 clone. Interestingly, the net difference in H_2O_2 production between the two microsomal preparations (about 0.13 nmol/min/mg protein) is about one half the net difference in $\cdot O_2^-$ production between the two microsomal preparations (about 0.27 nmol/min/mg protein). This suggests that most of the increase in H_2O_2 production in MVh2E1-9 microsomes is due to the increase in $\cdot O_2^-$ production, i.e., most of the elevated production of H_2O_2 is derived from dismutation of $\cdot O_2^-$ (reaction 5 in Background). When these results were expressed on a total P450 basis, the total H_2O_2 production from the MVh2E1-9 clone was about 10-fold more than normal human liver microsomes (**Table 8**). The elevated $\cdot O_2^-$ production in the MVh2E1-9 clone detected by the sensitive ESR technique was 18-fold higher than that detected in human liver microsomes in terms of nmol 2E1 (**Table 8**). Oxidation of PNP and DMN by microsomes from MVh2E1-9 cells was 2- to 3-fold higher than human liver microsomes when expressed on a total P450 basis. This was expected since all the P450 in the MVh2E1-9 cells is CYP2E1, a good catalyst for the oxidation of these drugs, whereas several P450 isoforms are present in human liver microsomes, some of which may not be good catalysts for PNP and DMN oxidation. Since drug oxidation was only 2 to 3 fold higher, the 10-fold higher H_2O_2 production and 18-fold higher $\cdot O_2^-$ production indicate the effectiveness of CYP2E1 in generation of reactive oxygen species relative to a mixed population of P450 enzymes found in human liver microsomes. The $\cdot O_2^-$ generated from MVh2E1-9 microsomes should produce ·OH through the iron-catalyzed Haber-Weiss reaction (reaction 3 and 4 in Background). We did not detect ·OH production without addition of iron. But, when Fe-EDTA was added, ·OH production was so high that there was no difference between the transduced and control clones. As mentioned in Introduction, iron or other redox cycling agents plays an important role in generation of reactive oxygen species and initiation of lipid peroxidation (Aust et al., 1985; Halliwell,

1978; McCord and Day, 1978). This iron redox balance may be regulated by iron-reducing enzymes such as P450 and/or P450 reductase. It is also known that P450 reductase is an excellent reductant of Fe-EDTA, at least at high concentration of iron. Therefore, addition of high concentrations of iron to the system can largely reflect information about P450 reductase. The effect of lower concentrations of iron, and use of other iron chelates will be evaluated in future studies.

Similarly, we observed a high activity in lipid peroxidation in both CYP2E1-transduced and control clones with a high concentration of added iron, which indicates that the peroxidative activity in both systems was already saturated at this concentration of iron. Therefore, we did a titration to study the effect of low concentrations of iron on lipid peroxidation to see if any difference could be picked up under unsaturating conditions. Indeed, a significant difference was observed between the two clones when the added iron was around a few μM Fe-ATP (**Figure 11**). This result suggests that at a more physiological low iron concentration P450-2E1 is a better catalyst for lipid peroxidation than P450 reductase. This suggests that chronic alcohol consumption may cause tissue, especially liver, injury through induction of CYP2E1, the ethanol-inducible form of P450 in the presence of physiologically relevant concentration of nonheme iron.

The production of $\cdot O_2^-$ and H_2O_2 in MVh2E1-9 microsomes was not affected by the addition of effective substrates for the catalytic activity of CYP2E1. With cytochrome $P450_{cam}$ from *Pseudomonas putida*, the redox potential of the heme iron becomes more positive when substrate binds (Poulos and Raag, 1992; Sligar and Gunsalus, 1976); reduction of the P450 becomes thermodynamically more favorable when substrate is bound which ensures that consumption of reducing equivalents and oxygen activation occur only in the presence of substrate (Poulos and Raag, 1992; Sligar and Gunsalus, 1976). Mammalian liver microsomal P450s do not appear to be as tightly coupled as the $P450_{cam}$

(Gorsky et al., 1984; Porter and Coon, 1991). The lack of effect of substrates on $\cdot O_2^-$ and H_2O_2 production by microsomes containing the expressed CYP2E1 could be a reflection of the spin state of the CYP2E1; when isolated, CYP2E1 is in high spin state (Koop et al., 1982), which apparently results in transfer of electrons from the reductase even in the absence of substrates. We have not yet been able to assess the spin-state of the expressed human CYP2E1 in MVh2E1-9 microsomes because of its low content.

For lipid peroxidation, it is still unclear and controversial regarding the immediate initiator(s) of the reaction. However, chelated iron, especially Fe-ATP, and reducing agent NADPH (Hochstein and Ernster, 1963) as well as NADPH reductase (Pederson and Aust, 1972) are required. Cytochrome P450 has also been shown to enhance lipid peroxidation in reconstitutive systems (Ekström and Ingelman-Sundberg, 1984; Morehouse and Aust, 1988). The oxidizing species responsible for initiation of lipid peroxidation in microsomal systems has been suggested to be ·OH, ferryl, or perferryl types of oxidants, or ferrous-ferric oxygenated complexes with maximal effectiveness when ferrous-ferric redox states are equivalent (Braughler et al., 1986; Minotti and Aust, 1987; Ryan and Aust, 1992). We showed in this thesis that cytochrome P450-2E1, the most leaky P450 enzyme, could initiate a high lipid peroxidation in the microsomes from CYP2E1-transduced human hepatoma cells in the presence of low iron concentrations. This lipid peroxidation could not be inhibited by SOD or catalase implying $\cdot O_2^-$, ·OH and H_2O_2 are not required in this reaction. Therefore, the mechanism for CYP2E1-enhanced lipid peroxidation, e.g., reduction of the iron catalyst, degradation of lipid hydroperoxides, still remains to be answered.

C. Cytotoxicity of Acetaminophen in MVh2E1-9 Cells

The primary goal of this series of study was to investigate the toxicity of APAP in a human liver cell line where the major or only significant cytochrome P450 isoform is human CYP2E1. The previous extensive studies on APAP toxicity were mostly carried out in animal models where many P450 isozymes are present, several of which are capable of metabolizing APAP, e.g., 2E1, 1A2 and 3A4 (Patten et al., 1993; Raucy et al., 1989; Thummel et al., 1993). Direct linkage between APAP metabolism by human CYP2E1 and APAP cytotoxicity has not been conclusively demonstrated in a controlled culture system with a human liver-derived cell line. Recently, Mapoles et al. established a stable cell line, DB-7, that expresses human CYP2E1 in neuroblastoma PC-12 cells and showed that APAP could cause cytotoxicity in the transfected cells but not in the parental PC-12 cells (Mapoles et al., 1993). We utilized our human hepatoma HepG2 subline, MVh2E1-9, that was transduced with human CYP2E1 cDNA using a retrovirus shuttle vector and constitutively expresses the human CYP2E1 to study APAP cytotoxicity in a human liver derived cell line. An advantage of this model is the stable, constitutive expression of CYP2E1, in contrast to the rapid decline in this isoform in cell culture. APAP induced cytotoxicity in MVh2E1-9 cells but not in the control MV-5 cells. Since the only difference between these two cell lines is the expression of functionally active CYP2E1, these results indicate that human CYP2E1 indeed plays an important role in APAP-induced cytotoxicity. The APAP-induced toxicity in this system requires GSH depletion (**Figure 12**). This differs from the PC-12 model, in which GSH depletion was not essential to reveal APAP toxicity (Mapoles et al., 1993). This could be explained by the fact that PC-12 cells have low GSH contents relative to HepG2 cells and therefore are considerably more vulnerable than HepG2 cells to reactive species produced inside the cell.

CYP2E1 has been shown to metabolize APAP to a reactive intermediate, NAPQI, that can covalently bind to thiol groups of cellular proteins and cause cytotoxicity (Vermeulen et al., 1992). Proof that the cytotoxicity produced by APAP is due to the presence of CYP2E1 in MVh2E1-9 cells is the fact that CYP2E1 ligands such as 4-MP, competitive substrates such as ethanol and inhibitors such as DAS prevented APAP-induced toxicity in MVh2E1-9 cells (**Table 9**). The effect of ligands and substrates on CYP2E1-catalyzed activities in cell cultures is complex; the same compound, under certain conditions, may inhibit or may display an enhancing effect on APAP toxicity. For example, in one study, isoniazid inhibited APAP toxicity (Anundi et al., 1993), but in another study, it potentiated the toxicity (Burk et al., 1990). This can probably be explained by the dual effect of 2E1 ligands on the content or activity of CYP2E1 and hence on the bioactivation of APAP by CYP2E1. One effect by ligands is to stabilize the P450 against degradation; this should enhance the APAP toxicity. The second effect is to compete with and thereby inhibit the metabolism of substrates like APAP by CYP2E1; this should block APAP toxicity. The final balance between the two would determine the direction of the ligand effect on the APAP toxicity *in vivo* or in cell culture. Although 4-MP and ethanol were found to increase the content of CYP2E1 in the MVh2E1-9 cells probably by stabilizing the 2E1 against degradation, these agents, nevertheless, prevented the APAP cytotoxicity in our system (**Table 9**) presumably due to their competitive inhibition of APAP metabolism, as validated by studies on adduct formation (**Table 11**).

The molecular mechanism underlying the hepatotoxic effect of APAP has been extensively studied since the initial studies by Mitchell and co-workers (Jollow et al., 1973; Mitchell et al., 1973; Potter et al., 1973). It has been generally accepted that covalent binding of the reactive metabolites of APAP to thiol groups of proteins following GSH depletion initiate the APAP-induced hepatotoxicity (Vermeulen et al., 1992). Recently, Pumford et al. established an immunochemical method to detect 3-(cysteine-S-yl)-

paracetamol adducts to serum protein and demonstrated a good correlation between adduct formation and hepatotoxicity *in vivo* (Pumford et al., 1989). In the present study, depletion of GSH increased APAP adduct formation in MVh2E1-9 cells (**Table 11**), which correlates with the increased cytotoxicity produced upon GSH depletion (**Figure 13**). *N*-acetylcysteine by either promoting GSH production or by its own reactivity decreased APAP adduct formation and also significantly prevented the APAP toxicity. Similarly, 4-MP and ethanol prevented or decreased APAP adduct formation, and also prevented APAP toxicity. Albano et al. (Albano et al., 1985) found that treatment of hepatocytes with NAPQI in the presence of BCNU, a GSH depleting agent, produced significantly more toxicity than the non-BCNU-treated control, however covalent binding was unaffected by the BCNU treatment. This is probably because the high concentration of NAPQI used (250 μM) already saturated the system under their experimental condition. Besides covalent binding to cytosolic GSH and proteins, APAP metabolites were also shown to bind to mitochondrial GSH and proteins which also play a role in the induction of APAP cytotoxicity (Tirmenstein and Nelson, 1989). Indeed, the mitochondrial fraction of the MVh2E1-9 cells contained labeled APAP adducts. Cytosolic APAP adducts appear to be more prominent in the toxicity produced by APAP (Pumford et al., 1989). Upon fractionation of the MVh2E1-9 cells, APAP adducts were evenly distributed in each subcellular fraction of the cell extract (**Table 12**).

Apart from covalent binding to intracellular GSH and proteins, APAP metabolites have also been proposed to be able to induce cellular oxidative stress after GSH depletion, resulting in lipid peroxidation and protein carbonyl formation. Lipid peroxidation has been shown to occur in various model systems after administration of high concentration of APAP (Albano et al., 1983; Wendel and Feuerstein, 1981; Younes et al., 1986). It has also been shown that induction of cytochrome P450 3A4 and depletion of GSH after APAP administration correlated with increased lipid peroxidation (Wendel and Feuerstein, 1981).

However, chelation of endogenous iron by desferioxamine was shown to prevent APAP-induced lipid peroxidation without affecting hepatic necrosis *in vivo* (Younes and Siegers, 1985) or in freshly-isolated hepatocytes (Devalia et al., 1982). In another study, the APAP toxicity in rat hepatocytes could be prevented by dithiothreitol without affecting lipid peroxidation (Tee et al., 1986). APAP and four derivatives of APAP all displayed equal toxicity in hepatocytes, but only APAP and its 3-methyl derivative induced lipid peroxidation (van de Straat et al., 1987). These data suggest that lipid peroxidation accompanying APAP-induced toxicity reflects the consequence rather than the cause of the cytotoxicity. In the present study, APAP-induced toxicity was not prevented by propylgallate, a classical antioxidant (**Table 10**), or by trolox, a vitamin E analog (data not shown). Therefore, it appears that lipid peroxidation is not a major factor in the APAP cytotoxicity to MVh2E1-9 cells, although further studies with antioxidant enzymes and reactive oxygen scavengers are required to further evaluate the role of oxidative stress in the APAP toxicity.

It was observed that APAP at a concentration as low as 1 mM inhibited cell proliferation in both MVh2E1-9 and MV-5 cells about 50% compared to the untreated control (**Figure 12**). This concentration of APAP did not cause any toxicity as determined by LDH leakage in either cell line even in the presence of BSO. The fact that a significant anti-proliferative effect by APAP is observed in MV-5 cells suggests that this effect by APAP does not require CYP2E1. A recent abstract (Navarro et al., 1994) also reported that as little as 0.5 mM APAP resulted in a significant reduction in the rate of replication of Hepa 1-6 cells. These cells lack P450 enzymes capable of activating APAP and the authors concluded that APAP is capable of blocking cell growth by a mechanism that does not require P450 activation. The anti-proliferative effect of APAP may be due to the direct action of APAP on mitochondrial function, that is independent of cytochrome P450 (Hongslo et al., 1988; Nazareth et al., 1991). Nazareth et al. (Nazareth et al., 1991)

showed that the mitochondrial phosphorylation potential decreased within 30 minutes after administration of 10 mM APAP to rat hepatocytes using $TPMP^+$ (triphenylmethylphosphonium) as a probe and that treatment with a P450 inhibitor did not diminish this effect of APAP. Addition of cysteine to the system did not alter the decrease by APAP indicating that this early effect on mitochondrial function was not GSH-dependent. This is in agreement with observations that low concentrations of APAP did not decrease the GSH content in the MVh2E1-9 cells (data not shown) although these concentrations inhibited cell growth (**Figure 12**). In addition, the anti-proliferative effect of APAP is identical in the absence or presence of BSO for both cell lines. Therefore, it seems that APAP has a dual toxic effect on hepatocytes. One is a P450-dependent effect in which APAP is activated to form reactive intermediates such as NAPQI that can deplete intracellular GSH and oxidize protein thiol groups eventually resulting in a cytotoxic action on the cells. The second is a direct, P450-independent anti-proliferative effect which may be due to mitochondrial malfunction, resulting in suppression of oxidative phosphorylation and of ATP synthesis, which subsequently leads to an inhibition of macromolecular synthesis and, thus, of cell proliferation. This does not role out the possibility that APAP may directly inhibit key components of the cell replication machinery. Under our experimental conditions, the anti-proliferative effect of APAP is not in itself sufficient to cause cell death since the remaining MV-5 cells are morphologically intact and show little LDH leakage. Whether the anti-proliferative effect of APAP is required to reveal the cytotoxic effect of APAP in MVh2E1-9 remains to be determined though NAPQI itself was shown to have a direct toxic effect in hepatocytes (Albano et al., 1985).

Calcium has been implicated as playing a key role in cell toxicity. Loss of calcium regulation is proposed to be the final common pathway to cell death in a variety of systems (Farber, 1990; Nicotera et al., 1990; Schanne et al., 1979). APAP administration has been shown to cause a rise in total calcium and free intracellular calcium in the liver (Corcoran et

al., 1988; Corcoran et al., 1987). Studies concerning the relationship between calcium and toxicity suggest that calcium plays a key role in APAP-induced hepatic necrosis *in vivo* and in cytotoxicity in cultured hepatocytes (Landon et al., 1986; Ray et al., 1993). Experiments were carried out to evaluate the role of calcium in MVh2E1-9 cells in the anti-proliferative effect of APAP that inhibits cell growth and division without cytotoxicity and the cytotoxic effect of APAP which occurs through the metabolism of APAP by the transfected CYP2E1. The calcium channel blocker verapamil and the calcium/calmodulin antagonist chlorpromazine did not prevent the anti-proliferative effect of APAP and only slightly but not significantly prevented the cytotoxic effect of APAP on MVh2E1-9 cells (**Table 13**). Verapamil at a concentration as high as 250 μM did not inhibit acetaminophen adduct formation *in vivo* or in cultured mouse hepatocytes, but did prevent calcium accumulation and DNA damage (Ray et al., 1993). While it is intriguing to speculate that calcium metabolism may play a key role in either the anti-proliferative effect or the cytotoxic effect, or both effects, by APAP, neither verapamil nor chlorpromazine were protective in our culture model. One possible problem with these experiments concerns the low concentrations of verapamil and chlorpromazine which had to be used in the system; unfortunately, higher concentrations of these agents caused significant cytotoxicity in the culture system in the absence of APAP. Further studies using other types of calcium modulators will be required to evaluate the relationship between calcium and the APAP toxicity in these HepG2 cell lines.

In summary, these results indicate that following treatment with APAP, MVh2E1-9 and the control MV-5 cells display a P450-independent suppression of cell proliferation which may be due to a direct inhibitory effect of APAP on cellular function. Treatment with APAP in the presence of BSO causes a cytotoxic effect in MVh2E1-9 cells but not control MV-5 cells. This cytotoxic effect is prevented by ligands and inhibitors of CYP2E1 indicating that it is dependent on the transduced CYP2E1. Cytotoxicity appears to correlate

with APAP-induced adduct formation and not lipid peroxidation since agents which prevent adduct formation also prevent cytotoxicity. This suggests a dual toxic effect of APAP on MVh2E1-9 cells: an anti-proliferative effect which is P450-independent and a cytotoxic effect which is P450-dependent. The MVh2E1-9 cells appears to represent a useful and convenient culture model for studying CYP2E1-dependent vs. 2E1-independent drug metabolism and drug-induced toxicity, and the molecular mechanisms involved in cytotoxicity.

D. Degradation of Human CYP2E1 in MVh2E1-9 Cells by CCl_4

The hepatotoxicity associated with carbon tetrachloride has been extensively studied in various model systems. Centrilobular necrosis and fat accumulation after CCl_4 exposure characterize the hepatic damage (Ishak et al., 1991; Jauhonen et al., 1982). Although the mechanism of CCl_4 toxicity is still not clear, generation of $\cdot CCl_3$, initiation of lipid peroxidation and covalent binding of macromolecules by CCl_4 metabolites have been implicated in the process. $\cdot CCl_3$ is generated by a reductive metabolism of CCl_4 via P450-mediated one electron transfer (Rechnagel and Glende, 1973). $\cdot CCl_3$ may dismutate to give chloroform, form covalent adducts to cellular macromolecules, and attack unsaturated fatty acids resulting in lipid peroxidation (Brattin et al., 1985; Kornbrust and Mavis, 1980; Villarruel et al., 1977). The toxicity of CCl_4 was shown to be elevated after ethanol treatment, probably due to the induction of CYP2E1 (Johansson and Ingelman-Sundberg, 1985; Strubelt et al., 1978; Teschke et al., 1984). Production of chloroform from CCl_4 was increased three-fold in imidazole treated rabbits compared to untreated controls suggesting that CYP2E1 plays a key role in CCl_4 metabolism (Johansson and Ingelman-Sundberg, 1985). Rabbit CYP2E1 has also been shown in a reconstituted system to be 100-fold more active than CYP1A2 and CYP2B4 in initiating CCl_4-dependent lipid

peroxidation (Johansson and Ingelman-Sundberg, 1985). Therefore, CYP2E1 seems to be a key component for bioactivation and hepatotoxicity of CCl_4.

The content of CYP2E1 which controls the rate of CCl_4 metabolism and hence the degree of toxicity is regulated by a balance of continual synthesis and degradation of the enzyme. Bioactivation of CCl_4 by CYP2E1 has been reported to cause destruction and loss of 2E1 *in vivo* and in cultured hepatocytes (Sohn et al., 1991; Tierney et al., 1992). Early studies suggested that a rapid decrease in P450 after CCl_4 treatment was due to destruction of the heme moiety of cytochrome P450 (English and Anders, 1985). Sohn et al. demonstrated that the rapid decrease of rat CYP2E1 by CCl_4 was due to post-translational destruction of CYP2E1 protein (Sohn et al., 1991). Tierney et al., using an *in vivo* mouse model, showed that CCl_4 treatment resulted in a complete loss of CYP2E1-dependent PNP hydroxylation and a 75% loss of immunochemically detectable protein within 1 hour of administration (Tierney et al., 1992). Ingelman-Sundberg and co-workers using rabbit liver microsomes showed that CCl_4-induced lipid peroxidation was CYP2E1-dependent and that CCl_4 also caused degradation of the enzyme (Eliasson et al., 1990; Johansson and Ingelman-Sundberg, 1985). Whether human CYP2E1 is also post-translationally decreased after treatment with CCl_4 has not been studied or characterized.

The human hepatoma subline, MVh2E1-9 cells, in which human CYP2E1 cDNA under the control of the viral LTR promoter, constitutively and stably expresses CYP2E1. This cell line, unlike most *in vivo* animal models, expresses only the transduced copy of the integrated P450 and, therefore, can be used for studies which avoid complication from interactions with other isoforms of P450. This cell line produces catalytically active human CYP2E1 that oxidizes PNP, DMN, aniline and ethanol, and can also display CYP2E1-dependent acetaminophen cytotoxicity (described above). Although the half-life of the transduced CYP2E1 in MVh2E1-9 cells has not been definitively determined, CYP2E1

produced in this cell line can also be post-translationally stabilized by its substrates such as 4-MP and ethanol (Carroccio et al., 1994).

Intracellular protein degradation has been of great interest in the past few years since the discovery of ATP- and ubiquitin-dependent proteolytic systems in eukaryotic cells (Hershko and Ciechanover, 1982). In contrast to intracellular degradation of abnormal proteins, oxidatively modified proteins undergo degradation by a nonlysosomal ATP- and ubiquitin-independent proteolytic pathways in both eukaryotes and prokaryotes (Davies, 1986). CYP2E1, like other oxidatively modified proteins (Stadtman, 1992), is proposed to be degraded by a two-step mechanism. In the first (labilization) step, the enzyme is labilized to a catalytically inactive form, which is subsequently rapidly degraded by intracellular proteases in the second (degradation) step. Treatment of MVh2E1-9 cells with CCl_4 *in situ* for 24 hours caused loss of both 2E1 enzyme activity and 2E1 protein to 50% (**Figure 17** and **18**). The fact that the loss of 2E1 protein and the decrease in 2E1 enzyme activity were closely correlated suggests that almost all the labilized 2E1 protein was immediately degraded and removed from the endoplasmic reticulum.

In the first step, labilization of 2E1 by CCl_4 appears to involve bioactivation of CCl_4 by the P450 followed by covalent modifications of the enzyme by CCl_4 metabolites. The fact that 2E1 ligands such as 4-MP and competitive substrates such as DMN and ethanol significantly prevented the loss of enzyme activity (**Table 15**) and 2E1 protein (**Figure 22**) indicates that the CCl_4-induced inactivation and degradation of 2E1 is due to interaction of CCl_4 with CYP2E1 in MVh2E1-9 cells. The ability of CCl_4 to inhibit PNP oxidation *in vitro* and the increased formation of CCl_4 adducts in *situ* and *in vitro* are reflections of the interaction of CCl_4 with CYP2E1. CYP2E1-dependent CCl_4 adduct formation *in situ* and in the microsomal fraction is completely blocked by ethanol and/or 4-MP (**Table 17**). $\cdot CCl_3$ is a key reactive product of CCl_4 metabolism by P450 isoforms

(Rechnagel and Glende, 1973). $\cdot CCl_3$ radical can react with molecular oxygen to form more reactive species such as the trichloromethylperoxyl radical. Both of these products may directly attack intracellular macromolecules especially at the active sites where they are generated e.g., proteins such as 2E1, thereby causing labilization and covalent modification of the enzyme. These radicals are also able to attack unsaturated fatty acids to initiate lipid peroxidation, which generates a variety of lipid radicals. Therefore, these secondary radicals may attack CYP2E1, oxidatively modify and mark the enzyme for further degradation. N-acetylcysteine and PBN react with a variety of reactive species. The failure of these two agents to prevent degradation of CYP2E1 suggests that the reactive CCl_4 metabolites are generated at the active site of CYP2E1 where they are not accessible to scavenging by these agents.

Lipid peroxidation is known to cause degradation of P450 isoforms (Guengerich, 1978; Levin et al., 1973; Wills, 1971) and incubation of microsomes from MVh2E1-9 cells with NADPH plus ferric/ATP indeed promoted lipid peroxidation and degradation of CYP2E1. Although extensive studies have been reported regarding the ability of CCl_4 to initiate lipid peroxidation, TBA-reactive materials were not detected after incubating microsomes of MVh2E1-9 cells with CCl_4. The absence of a significant CCl_4-catalyzed formation of TBA-reactive materials is surprising and may reflect a variety of factors. A comparison of production of TBA-reactive materials by rat and human liver microsomes indicated that human microsomes were only 10% as reactive as rat microsomes in forming TBA-reactive materials under the same experimental conditions (Rashba-Step and Cederbaum, 1994), suggesting that the fatty acid composition may be different in rat liver and human liver (especially HepG2) microsomes, or the content of membranous antioxidants such as vitamin E may be higher in human microsomes, or the 3- to 4-fold lower content of total human P450 and activity of NADPH-cytochrome P450 reductase may be responsible for the lower rates of the formation of TBA-reactive materials. A lower

content of P450 would be especially notable in the MVh2E1-9 cells where the content of CYP2E1, the major P450 present, is still less than 15% that of human liver microsomes and less than 5% that of rat liver microsomes even after treatment with PMA (CYP2E1 content of about 30 to 40 pmol/mg microsomal protein). Another possibility may be the susceptibility of human CYP2E1 to CCl_4 metabolites, i.e., the rapid inactivation of CYP2E1 by CCl_4 metabolites, would not leave a sufficient residual amount of radicals to initiate lipid peroxidation. The inability of CCl_4 to promote lipid peroxidation is in agreement with the lack of protection by antioxidants such as *N*-acetylcysteine, PBN or propylgallate against the loss of the enzyme activity or enzyme content in MVh2E1-9 cells (**Table 16**). We recently demonstrated that in human liver microsomes CCl_4-induced inactivation of CYP2E1 could not be prevented by the potent antioxidant boldine, whereas ferric-ATP catalyzed inactivation of CYP2E1 was prevented by boldine (Kringstein and Cederbaum, 1994). Therefore, although lipid peroxidation is sufficient for marking CYP2E1 for degradation *in vitro*, it does not appear to play a significant role in the CCl_4-mediated marking of CYP2E1 in human liver microsomes or in MVh2E1-9 cells; rather direct attack by CCl_4 metabolites would appear to play the major role in labilizing CYP2E1.

Phosphorylation of rat CYP2E1 at a serine residue has recently been reported by Eliasson et al. to be important for recognition of the marked enzyme by a protease degradation system (Eliasson et al., 1990), suggesting labilization of 2E1 may also occur outside the active site of the enzyme. This may be relevant under physiological rather than toxicological conditions. *In vitro* proteolysis experiments showed that the phosphorylated 2E1 is rapidly degraded by ATP-dependent proteases in the microsomal fraction, suggesting the proteases for degrading the marked 2E1 is present in or tightly associated with the endoplasmic reticulum (Eliasson et al., 1992). Two such proteases have recently been purified from rat liver microsomes. Tierney et al. using an *in vivo* mouse model showed that 2E1 inactivated by CCl_4 and 3-aminotriazole was rapidly removed from the

endoplasmic reticulum while the 1-aminobenzotriazole inactivated enzyme was not rapidly degraded (Tierney et al., 1992). In this model they detected production of high molecular weight ubiquitin-conjugated microsomal proteins following CCl_4 treatment (Tierney et al., 1992). These data imply that the second step of degradation may involve more than one proteolytic pathway. In the present study, we used protease inhibitors added to MVh2E1-9 culture along with CCl_4 to attempt to identify that protease system in the HepG2 cells which degrades the CCl_4-labilized CYP2E1. A variety of inhibitors of lysosomal proteases including E-64, leupeptin, chloroquine and NH_4Cl were found to protect against the CCl_4-induced degradation of CYP2E1 (**Figure 23**). This suggests a role for the lysosomes in degrading the CCl_4-labilized human CYP2E1 in the HepG2 cells, which is in contrast to the role of microsomal ATP-dependent protease or the ubiquitin system found to be important in degrading CCl_4-labilized CYP2E1 in rat liver studies. Under conditions in which CCl_4 inhibited oxidation of PNP by isolated microsomes, we did not find CCl_4-induced degradation of CYP2E1 even after addition of ATP plus Mg^{2+} (**Figure 21**). These results suggest the absence of ATP-dependent proteases in HepG2 microsomes which can degrade CCl_4-labilized CYP2E1 or a difference in labilization by CCl_4 of human versus rat CYP2E1, or an important role for CCl_4-catalyzed lipid peroxidation with rat liver microsomes in enhancing the susceptibility of labilized CYP2E1 to the microsomal proteases.

Under conditions in which CCl_4 caused inactivation of CYP2E1, there was no loss of viability of the MVh2E1-9 cells as assayed by release of lactic dehydrogenase into the medium (data not shown). Another hepatotoxic agent known to be activated by CYP2E1, acetaminophen, did cause a loss of viability of the MVh2E1-9 cells; acetaminophen, however, did not inactivate or cause degradation of CYP2E1 (**Figure 19**). These differences between CCl_4 and acetaminophen may reflect the disposition of the primary metabolites produced from these two hepatotoxins. The benzoquinone imine produced

from acetaminophen activation by P450 is known to be released from the endoplasmic reticulum into the cellular environment where it forms adducts in various cellular fractions, especially the cytosol (Pumford et al., 1989). Indeed, we found that the cytotoxicity and protein adducts produced by acetaminophen in the MVh2E1-9 cells were prevented by GSH, *N*-acetylcysteine and PBN. Release of benzoquinone imine, in contrast to CCl_4 metabolites, from the active site of CYP2E1 probably explains why CCl_4 but not acetaminophen causes inactivation of CYP2E1 in this experimental model. Studies to measure the distribution of ^{14}C-labeled CCl_4 protein adducts would be helpful to test this suggestion.

In summary, these results indicate that following treatment *in situ* with CCl_4, both enzyme activity and protein content of CYP2E1 in MVh2E1-9 cells were decreased by about 50%. This loss of enzyme activity and protein was magnified by the simultaneous addition of cycloheximide in culture to prevent new protein synthesis suggesting that the process was due to post-translational protein degradation. The fact that CYP2E1 ligands and substrates such as 4-MP, DMN and ethanol prevented both the loss of enzyme activity and 2E1 protein indicate that bioactivation of CCl_4 by CYP2E1 may be required as an initial step in the degradation of the P450. Inhibitors of lipid peroxidation did not prevent the 2E1 degradation by CCl_4 implying that lipid peroxidation is not required for 2E1 degradation in this model, although it may be sufficient to induce 2E1 degradation *in vitro*. Antioxidants such as N-acetylcysteine and PBN were not protective, suggesting that the reactive CCl_4 species responsible for labilizing CYP2E1 were probably generated at the active site and not released for scavenging by these agents. The formation of radioactive CCl_4 adducts in both cultured MVh2E1-9 cells,

as well as its microsomes, suggests that reactive metabolites were produced which are able to attack intracellular macromolecules such as CYP2E1, and that the labilized human

CYP2E1 is degraded by proteases, including lysosomal proteases, present in the HepG2 cells.

BIBLIOGRAPHY

Aden, D. P., Fogel, A., Plotkin, S., Damjanov, I. and Knowles, B. B. (1979) Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. *Nature* 282, 615-616.

Albano, E., Poli, G., Chiarpotto, E., Brasi, F. and Dianzani, M. (1983) Paracetamol-stimulated lipid peroxidation in isolated rat and mouse hepatocytes. *Chem. Biol. Interact.* 47, 249-262.

Albano, E., Rundgren, M., Harvison, P. J., Nelson, S. D. and Moldeus, P. (1985) Mechanisms of *N*-acetyl-*p*-benzoquinone imine cytotoxicity. *Mol. Pharmacol.* 28, 306-312.

Albano, E., Tomasi, A., Goria-Gatti, L. and Dianzani, M. U. (1988) Spin-trapping of free radical species produced during the microsomal metabolism of ethanol. *Chem. Biol. Interact.* 65, 223-234.

Albano, E., Tomasi, A., Persson, J. O., Terelius, Y., Goria-Gatti, L., Ingelman-Sundberg, M. and Dianzani, M. U. (1991) Role of ethanol-inducible cytochrome P-450 in catalyzing free radical activation of aliphatic alcohols. *Biochem. Pharmacol.* 41, 1895-1902.

Anundi, I., Lahteenmaki, T., Rundgren, M., Moldeus, P. and Lindros, K. O. (1993) Zonation of acetaminophen metabolism and cytochrome P450 2E1-mediated toxicity studied in isolated periportal and perivenous hepatocytes. *Biochem. Pharmacol.* 45, 1251-1259.

Aoyama, T., Nagata, K., Yamazoe, Y., Kato, R., Matsunaga, E., Gelboin, H. and Gonzalez, F. J. (1990) Cytochrome b_5 potentiation of cytochrome P-450 catalytic activity demonstrated by a vaccinia virus-mediated *in situ* reconstitution system. *Proc. Natl. Acad. Sci. USA* 87, 5425-5429.

Argeles, J. M. (1986) Has acetone a role in the conversion of fat to carbohydrate in mammals? *Trends Biochem. Sci.* 11, 61-63.

Atherton, N. M. (1979) *Electron Spin Resonance: Theory and Applications,* Halsted Press, City.

Aust, S. D., Morehouse, L. A. and Thomas, C. E. (1985) Role of metals in oxygen radical reactions. *Free Radical Biol. Med.* 1, 3-25.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1990) *Currrent protocols in molecular biology,* Green Publishing Associates and Wiley-Interscience, New York, NY.

Battula, N. (1989) Transduction of cytochrome P3-450 by retroviruses: Constitutive expression of enzymatically active microsomal hemoprotein in animal cells. *J. Biol. Chem.* 264, 2991-2996.

Black, M. (1984) Acetaminophen hepatotoxicity. *Ann. Rev. Med.* 35, 577-593.

Boveris, A., Fraga, C. G., Varsavsky, A. I. and Koch, O. R. (1983) Increased chemiluminescence and superoxide production in the liver of chronically ethanol-treated rats. *Arch. Biochem. Biophys.* 227, 534-541.

Brady, J. F., Ishizaki, H., Fukuto, J. M., Lin, M. C., Fadel, A., Gapac, J. M. and Yang, C. S. (1991) Inhibition of cytochrome P-450IIE1 by diallyl sulfide and its metabolites. *Chem. Res. Toxicol.* 4, 642-647.

Brady, J. F., Li, D., Ishizaki, H., Lee, M., Ning, S. M., Xiao, F. and Yang, C. S. (1989) Induction of cytochromes P450IIE1 and P450IIB1 by secondary ketones and the role of P450IIE1 in chloroform metabolism. *Toxicol. Appl. Pharmacol.* 100, 342-349.

Brattin, W. J., Glende, E. A. and Rechnagel, R. O. (1985) Pathological mechanisms in carbon tetrachloride hepatotoxcity. *J. Free. Rad. Biol. Med.* 1, 27-38.

Braughler, J. M., Duncan, L. A. and Chase, A. L. (1986) *J. Biol. Chem.* 261, 10282-10289.

Buege, J. A. and Aust, S. D. (1978) Microsomal lipid peroxidation. *Methods Enzymol.* 52, 302-310.

Bühler, R., Lindros, K. O., Nordling, A., Johansson, I. and Ingelman-Sundberg, M. (1992) Zonation of cytochrome P450 isozyme expression and induction in rat liver. *Eur. J. Biochem.* 204, 407-412.

Bullock, W. O., Fernandez, J. M. and Short, J. M. (1987) XL1-Blue: A high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. *Biotechniques* 5, 376-378.

Burk, R. F., Hill, K. E., Hunt, R. W., Jr. and Martin, A. E. (1990) Isoniazid potentiation of acetaminophen hepatotoxicity in the rat and 4-methylpyrazole inhibition of it. *Res. Commun. Chem. Pathol. Pharmacol.* 69, 115-118.

Carroccio, A., Wu, D. and Cederbaum, A. I. (1994) Ethanol increases content and activity of human cytochrome P450-2E1 in a transduced HepG2 cell line. *Biochem. Biophys. Res. Commun.* In press.

Casazza, J. P., Yun, Y. P., Veech, R. L. and Song, B.-J. (1990) Serum acetone and P450IIE1 levels in pregnant rats. *FASEB J.* 4, A2244.

Caszza, J. P., Felver, M. E. and Veech, R. L. (1984) The metabolism of acetone in rat. *J. Biol. Chem.* 259, 231-236.

Cederbaum, A. I. (1987) Microsomal generation of hydroxyl radicals: Its role in microsomal ethanol oxidizing system (MEOs) activity and requirement for iron. *Annals N. Y. A. S.* 492, 35-49.

Cederbaum, A. I. (1989) Introduction: role of lipid peroxidation and oxidative stress in alcohol toxicity. *Free Rad. Biol. Med.* 7, 537-539.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18, 5294-5299.

Clark, B. J. and Waterman, M. R. (1991) Heterologous expression of mammalian P450 in COS cells. *Methods Enzymol.* 206, 100-108.

Clejan, L. A. and Cederbaum, A. I. (1990) Oxidation of pyrazole by reconstituted system containing P-450IIE1. *Biochem. Biophys. Acta* 1034, 233-237.

Clejan, L. A. and Cederbaum, A. I. (1992) Role of cytochrome P450 in the oxidation of glycerol by reconsitituted systems and microsomes. *FASEB J.* 6, 765-770.

Coon, M. J., Ding, X., Pernecky, S. J. and Vaz, A. D. N. (1992) Cytochrome P450: progress and predictions. *FASEB J.* 6, 669-673.

Corcoran, G. B., Bauer, J. A. and Lau, D. T.-W. (1988) Immediate rise in intracellular calcium and glycogen phosphorylase a activities upon acetaminophen covalent binding leading to hepatotoxicity in mice. *Toxicology* 50, 157-167.

Corcoran, G. B., Wong, B. K. and Neese, B. L. (1987) Early sustained rise in total liver calcium during acetaminophen hepatotoxicity in mice. *Res. Commun. Chem. Pathol. Pharmacol.* 58, 291-305.

Crespi, C. L. (1991) Expression of cytochrome P450 cDNAs in human B lymphoblastoid cells: Applications to toxicology and metabolite analysis. *Methods. Enzymol.* 206, 123-130.

Dahlin, D. C., Miwa, G. T., Lu, A. Y. H. and Nelson, S. D. (1984) N-acetyl-p-benzoquinone imine: A cytochrome P-450 mediated oxidation product of acetaminophen. *Proc. Natl. Acad. Sci. USA* 81, 1327-1331.

Davies, K. J. A. (1986) *Free Radical Biol. Med.* 2, 155-173.

Dawson, J. H. (1988) Probing structure-function relations in heme containing oxygenases and peroxidases. *Science* 240, 433-439.

Devalia, J. L., Ogilvie, R. L. and Mclean, A. E. M. (1982) Dissociation of cell death from covalent binding of paracetamol by flavones in hepatocyte system. *Biochem. Pharmacol.* 31, 3745-3749.

Diamond, L., Kruszewski, F., Aden, D. P., Knowles, B. and Baird, W. M. (1980) Metabolic activation of benzo(a)pyrene by a human hepatoma cell line. *Carcinogenesis* 1, 871-875.

Dianzani, M. U. (1985) Lipid peroxidation in ethanol poisoning: A critical reconsideration. *Alcohol Alcoholism* 20, 161-173.

Dicker, E. and Cederbaum, A. I. (1987) Hydroxyl radical generation by microsomes after chronic ethanol consumption. *Alcoholism: Clin. Exp. Res.* 11, 309-314.

Diluzio, N. R. (1964) Prevention of the acute ethanol-induced fatty liver by the simultaneous administration of antioxidants. *Life Sci.* 3, 113-118.

Diluzio, N. R. (1968) The role of lipid peroxidation and antioxidants in ethanol-induced lipid alterations. *Exp. Mol. Pathol.* 8, 394-402.

Doehmer, J. and Oesch, F. (1991) V79 Chinese hamster cells genetically engineered for stable expression of cytochromes P450. *Methods. Enzymol* 206, 117-123.

Ekström, G., Cronholm, T. and Ingelman-Sundberg, M. (1986) Hydroxyl-radical production and ethanol oxidation by liver microsomes isolated from ethanol-treated rats. *Biochem. J.* 233, 755-761.

Ekström, G. and Ingelman-Sundberg, M. (1984) Cytochrome P450-dependent lipid peroxidation in reconstituted membrane vesicles. *Biochem. Pharmacol.* 33, 2523-2525.

Ekström, G. and Ingelman-Sundberg, M. (1989) Rat liver microsomal NADPH-supported oxidase activity and lipid peroxidation dependent on ethanol-inducible cytochrome P-450 (P-450IIE1). *Biochem. Pharmacol.* 38, 1313-1319.

Eliasson, E., Johansson, I. and Ingelman-Sundberg, M. (1990) Substrate-, hormone-, and cAMP-regulated cytochrome P450 degradation. *Proc. Natl. Acad. Sci., USA* 87, 3225-3229.

Eliasson, E., Johansson, I. and Ingelman-Sundberg, M. (1988) Ligand-dependent maintenance of ethanol-inducible cytochrome P-450 in primary rat hepatocyte cell culture. *Biochem. Biophys. Res. Comm.* 150, 436-443.

Eliasson, E., Mkrtchian, S. and Ingelman-Sundberg, M. (1992) Hormone- and substrate-regulated intracellular degradation of cytochrome P450 (2E1) involving MgATP-activated rapid proteolysis in the endoplasmic reticulum membranes. *J. Biol. Chem.* 267, 15765-15769.

English, J. C. and Anders, M. W. (1985) Evidence for the metabolism of N-nitrosodimethylamine and carbon tetrachloride by a common isozyme of cytochrome P-450. *Drug Metab. Dispos.* 13, 449-452.

Farber, J. L. (1990) The role of calcium in lethal cell injury. *Chem. Res. Toxicol.* 3, 503-508.

Farber, J. L., Kyle, M. E. and Coleman, J. B. (1990) Biology of disease: Mechanism of cell injury by activated oxygen species. *Lab. Invest.* 62, 670-679.

Feierman, D. and Cederbaum, A. I. (1986) In vitro inhibition of microsomal oxidation of ethanol by pyrazole and 4-methylpyrazole. *Biochem. J.* 239, 671-677.

Feierman, D. E. and Cederbaum, A. I. (1989) Role of cytochrome P-450 IIE1 and catalase in the oxidation of acetonitrile to cyanide. *Chem. Res.Toxicol.* 2, 359-366.

Feinberg, A. P. and Vogelstein, B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132, 6-13.

Finkelstein, E., Rosen, G. M. and Rauckman, E. J. (1980) Spin trapping of superoxide and hydroxyl radical: Practical aspects. *Arch. Biochem. Biophys.* 200, 1-16.

Freeman, J. E., Stirling, D., Russell, A. L. and Wolf, C. R. (1992) cDNA sequences, deduced amino acid sequence, predicted gene structure and chemical regulation of mouse Cyp2e1. *Biochem. J.* 281, 689-695.

Frezza, M., DiPadova, C., Pozzato, G., Terpin, M., Baraona, E. and Lieber, C. S. (1990) High blood alcohol levels in women: the role of decreased gastric alcohol dehydrogenase activity and first-pass metabolism. *N. Engl. J. Med.* 322, 95-99.

Garfinkle, D. (1958) Studies on pig liver microsomes: I. Enzyme and pigment composition of different microsomal fractions. *Arch. Biochem. Biophys.* 77, 493-509.

Goedde, H. W. and Agarwal, D. P. (1989) *Alcoholism: Biomedical and Genetics Aspects,* Pergamon Press, New York.

Gonthier, O. B., Jeunet, A. and Barret, L. (1991) Electron spin resonance study of free radicals produced from ethanol and acetaldehyde after exposure to a Fenton system or to the brain and liver microsomes. *Alcohol* 8, 369-375.

Gonzalez, F. J. (1989) The molecular biology of cytochrome P450s. *Pharmacological Review* 40, 243-288.

Gonzalez, F. J. (1992) Human cytochromes P450: Problems and prospects. *Trends Pharmacol. Sci.* 13, 346-352.

Gonzalez, F. J., Aoyama, T. and Gelboin, H. V. (1991) Expression of mammalian cytochrome P450 using vaccinia virus. *Methods Enzymol.* 206, 85-92.

Gorsky, L. D., Koop, D. R. and Coon, M. J. (1984) On the stoichiometry of the oxidase and monooxygenase reactions catalyzed by liver microsomal cytochrome P-450. *J. Biol. Chem.* 259, 6812-6817.

Grant, B. F., Dufour, M. C. and Harford, T. C. (1988) Epidemiology of alcoholic liver disease. *Semin. Liver Dis.* 8, 12-25.

Grant, M. H., Duthie, S. J., Gray, A. G. and Burke, D. (1988) Mixed function oxidase and UDP-glucuronyltransferase activities in the human HepG2 hepatoma cell line. *Biochem. Pharmacol.* 37, 4111-4116.

Gruenke, L. D., Konopka, K., Koop, D. R. and Waskell, L. (1988) Characterization of halothane oxidation by hepatic microsomes and purified cytochrome P-450 using gas chromatographic mass spectrometric assay. *J. Pharmacol. Exp. Ther.* 246, 454-459.

Guengerich, F. P. (1978) Destruction of heme and hemoproteins mediated by liver microsomal NADPH cytochrome P450 reductase. *Biochemistry* 17, 3633-3639.

Guengerich, F. P. (1983) Oxidation-reduction properties of rat liver cytochromes P-450 and NADPH-cytochrome P-450 reductase related to catalysis in reconstitued systems. *Biochemistry* 22, 2811-2820.

Guengerich, F. P. (1987) *Mammalian cytochrome P450,* CRC Press, Boca Raton, FL.

Guengerich, F. P. (1990) Enzymatic oxidation of xenobiotic chemicals. *Crit. Rev. Biochem. Mol. Biol.* 25, 97-153.

Guengerich, F. P., Kim, D.-H. and Iwasaki, M. (1991) Role of human cytochrome P-450 IIE1 in the oxidation of many low molecular weight cancer suspects. *Chem. Res. Toxicol.* 4, 168-179.

Hall, P. (1985) *Alcoholic liver disease: Pathobiology, epidemiology and clinical aspects,* John Wiley & Sons, New York.

Halliwell, B. (1978) Superoxide dependent formation of hydroxyl radicals in the presence of iron chelates. *FEBS Lett.* 92, 321.

Hanahan, D. (1983) Studies on transformation of Escherichia coli with plasmids. *J. Mol. Biol.* 166, 557-580.

Hershko, A. and Ciechanover, A. (1982) Mechanisms of intracellular protein breakdown. *Annu. Rev. Biochem.* 51, 335-364.

Hildebrandt, A. G., Roots, I., Tjoe, M. and Heinemeyer, G. (1978) Hydrogen peroxide in hepatic microsomes. *Methods Enzymol.* 52, 342-350.

Hinson, J. A. (1980) Biochemical toxicology of acetaminophen. *Rev. Biochem. Toxicol.* 2, 103-130.

Hochstein, P. and Ernster, L. (1963) ADP-activated lipid peroxidation coupled to the TPNH oxidase system of microsomes. *Biochem. Biophys. Res. Commun.* 12, 388-394.

Hoffman, J., Konopka, K., Buckhorn, C., Koop, D. R. and Waskell, L. (1989) Ethanol-inducible cytochrome P450 in rabbits metabolizes enflurane. *Br. J. Anaesth.* 63, 103-108.

Hongslo, J. K., Christensen, T., Brunborg, G., Björnstad, C. and Holme, J. A. (1988) Genotoxic effects of paracetamol in V79 Chinese hamster cells. *Mutat. Res.* 204, 333-341.

Horton, A. A. and Fairhurst, S. (1987) Lipid peroxidation and mechanisms of toxicity. *CRC Crit. Rev. Toxicol.* 18, 27-79.

Housey, G. M., Johnson, M. D., Hsiao, W. L. W., O'Brian, C., A., Murphy, J. P., Kirschmeier, P. T. and Weinstein, I. B. (1988) Overproduction of protein kinase C causes disordered growth control in rat fibroblasts. *Cell* 52, 343-354.

Hu, J. J., Lee, M.-J., Vapiwala, M., Reuhl, K., Thomas, P. E. and Yang, C. S. (1993) Sex-related differences in mouse renal metabolism and toxicity of acetaminophen. *Toxicol. Appl. Pharmacol.* 122, 16-26.

Ingelman-Sundberg, M. and Johansson, I. (1984) Mechanisms of hydroxyl radical formation and ethanol oxidation by ethanol-inducible and other forms of rabbit liver microsomal cytochromes P-450. *J. Biol. Chem.* 259, 6447-6458.

Ingelman-Sundberg, M., Johansson, I., Penttila, K. E., Glaumann, H. and Lindros, K. O. (1988) Centribular expression of ethanol-inducible cytochrome P-450 (IIE1) in rat liver. *Biochem. Biophys. Res. Commun.* 157, 55-60.

Ingelman-Sundberg, M. and Jörnvall, H. (1984) Induction of the ethanol-inducible form of rabbit liver microsomal cytochrome P-450 by inhibitors of alcohol dehydrogenase. *Biochem. Biophys. Res. Commun.* 124, 375-382.

Ishak, K. G., Zimmerman, H. J. and Ray, M. B. (1991) Alcoholic liver disease: pathologic pathogenic and clinic aspects. *Alc. Clin. Exp. Res.* 15, 45-66.

Jauhonen, V. P., Baraona, E., Miyakawa, H. and Lieber, C. S. (1982) Mechanism for selective perivenular hepatotoxicity of ethanol. *Alcoholism: Clin. Esp. Res.* 6, 350-357.

Javitt, N. B. (1990) *FASEB J.* 4, 161-168.

Johansson, I., Eliasson, E. and Ingelman-Sundberg, M. (1991) Hormone controlled phosphorylation and degradation of CYP2B1 and CYP2E1 in isolated rat hepatocytes. *Biochem. Biophys. Res. Comm.* 174, 37-42.

Johansson, I. and Ingelman-Sundberg, M. (1985) Carbon tetrachloride-induced lipid peroxidation dependent on an ethanol-inducible form of rabbit liver micrsomal cytochrome P-450. *FEBS Lett.* 183, 265-269.

Johansson, I. and Ingelman-Sundberg, M. (1988) Benzene metabolism by ethanol-, acetone-, and benzene-inducible cytochrome P450 (IIE1) in rat and rabbit liver microsomes. *Cancer Res.* 48, 5387-5390.

Jollow, D. J., Mitchell, J. R., Potter, W. Z., Gillette, J. R., Davis, D. and Brodie, B. B. (1973) Acetaminophen-induced necrosis II. Role of covalent binding *in vivo*. *J. Pharmacol. exp. Ther.* 187, 195-202.

Joly, J. G., Ishii, H., Teschke, R., Hasamura, Y. and Lieber, C. S. (1973) Effect of chronic alcohol feeding on the activities and submicrosomal distribution of reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase and demethylase for aminopyrine and ethylmorphine. *Biochem. Pharmacol.* 22, 1532-1535.

Jones, S. M., Boobis, A. R., Moore, G. E. and Stanier, P. M. (1992) Expression of CYP2E1 during human fetal development: Methylation of the CYP2E1 gene in human fetal and adult liver samples. *Biochem. Pharmacol.* 43, 1876-1879.

Khani, S. C., Porter, T. D., Fujita, V. S. and Coon, M. J. (1988) Organization and differential expression of two highly similar genes in the rabbit alcohol-inducible cytochrome P-450 subfamily. *J. Biol. Chem.* 263, 7170-7175.

Khani, S. C., Zaphiropoulos, P. G., Fujita, V. S., Porter, T. D., Koop, D. R. and Coon, M. J. (1987) cDNA and derived amino acid sequence of ethanol-inducible rabbit liver cytochrome P-450. *Proc. Natl. Acad. Sci., USA* 84, 638-642.

Kieschmeier, P. T., Housey, G. M., Johnson, M. D., Perkins, A. S. and Weinstein, I. B. (1988) Laboratory methods: Construction and characterization of a retroviral vector demonstrating efficient expression of cloned cDNA sequences. *DNA* 7, 219-225.

Kim, S. G. and Novak, R. F. (1990) Induction of rat hepatic P450IIE1 (CYP2E1) by pyridine: Evidence for a role of protein synthesis in the absence of transcriptional activation. *Biochem. Biophys. Res. Commun.* 166, 1072-1079.

Kim, S. G. and Novak, R. F. (1990) Role of P450IIE1 in the metabolism of 3-hydroxypyridine, a constituent of tobacco smoke: Redox cycling and DNA strand scission by the metabolite 2,5-dihydroxypyridine. *Cancer Res.* 50, 5333-5339.

Kim, S. G., William, D. E., Schuetz, E. G., Guzelian, P. S. and Novak, R. F. (1988) Pyridine induction of cytochrome P-450 in the rat: Role of P-450j (alcohol-inducible form) in pyridine N-oxidation. *J. Pharmacol. Exp. Ther.* 246, 1175-1182.

Klein, S. M., Cohen, G., Lieber, C. S. and Cederbaum, A. I. (1983) Increased microsomal oxidation of hydroxyl radical scavenging agents and ethanol after chronic consumption of ethanol. *Arch. Biochem. Biophys* 223, 425-432.

Klingenberg, M. (1958) Pigments of rat liver microsomes. *Arch. Biochem. Biophys.* 75, 376-386.

Koop, D. R. (1986) Hydroxylation of p-nitrophenol by rabbit ethanol-inducible cytochrome P450 isozyme 3a. *Mol. Pharmacol.* 29, 399-404.

Koop, D. R. (1992) Oxidative and reductive metabolism by cytochrome P450 2E1. *FASEB J.* 6, 724-730.

Koop, D. R. and Casazza, F. P. (1985) Identification of ethanol inducible P450 isozyme 3a as the acetone and acetol monooxygenase of rabbit microsomes. *J. Biol. Chem.* 260, 13607-13612.

Koop, D. R. and Coon, M. J. (1984) Purification of liver microsomal cytochrome P-450 isozymes 3a and 6 from imidazole-treated rabbits: Evidence for the identify of isozyme 3a with the form obtained by ethanol treatment. *Mol. Pharmacol.* 25, 494-501.

Koop, D. R. and Coon, M. J. (1986) Ethanol oxidation and toxicity: Role of alcohol P-450 oxygenase. *Alcohol. Clin. Exp. Res.* 10, 44s-49s.

Koop, D. R., Crump, B. L., Nordblom, G. D. and Coon, M. J. (1985) Immunochemical evidence for induction of the alcohol-oxidizing cytochrome P-450 or rabbit liver microsomes by diverse agents: Ethanol, imidazole, trichloroethylene, acetone, pyrazole, and isoniazid. *Proc. Natl. Acad. Sci. USA* 82, 4065-4069.

Koop, D. R., Laethem, C. L. and Schnier, G. G. (1989) Identification of ethanol-inducible P450 isozyme 3a (P450IIE1) as a benzene and phenol hydroxylase. *Toxicol. Appl. Pharmacol.* 98, 278-288.

Koop, D. R., Morgan, E. T., Tarr, G. E. and Coon, M. J. (1982) Purification and characterization of a unique isozyme of cytochrome P450 from liver microsomes of ethanol-treated rabbits. *J. Biol. Chem.* 257, 8472-8480.

Koop, D. R. and Tierney, D. J. (1990) Multiple mechanisms in the regulation of ethanol-inducible cytochrome P450IIE1. *BioEssays* 12, 429-435.

Kornbrust, D. J. and Mavis, R. D. (1980) Microsomal lipid peroxidation II: stimulation by carbon tetrachloride. *Mol. Pharmacol.* 17, 408-414.

Kringstein, P. and Cederbaum, A. I. (1994) Boldine prevents human liver microsomal lipid peroxidation and inactivation of cytochrome P450-2E1. Free Rad. Biol. Med.

Krinitskaya, L. A. and Volodarskii, L. B. (1982) 3-Monohaloderivatives of triacetone amine, 1-hydroxy-2,2,6,6-tetramethyl-4-oxopiperidine, and 2,2,6,6,-tetramethyl-4-oxopiperidine-1-oxyl. *Bull. Acad. Sci. USSR, Div. Chem. Sci.* 31, 401-404.

Kuthan, H. and Ullrich, V. (1982) Function of the microsomal cytochrome P-450 monooxygenase system. *Eur. J. Biochem.* 126, 583-588.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Landon, E. J., Neukam, R. J. and Rama Sastry, B. V. (1986) Effects of calcium channel blocking agents on calcium and centrilobular necrosis in the liver of rats treated with hepatotoxic agents. *Biochem. Pharmacol.* 33, 697-705.

Larson, J. R., Coon, M. J. and Porter, T. D. (1991) Purification and properties of a shortened form of cytochrome P-450 2E1: deletion of the NH_2-terminal membrane insertion signal peptide does not alter the catalytic activities. *Proc. Natl. Acad. Sci., USA* 88, 9141-9145.

Lasker, J. M., Raucy, J., Kubota, S., Bloswick, B. P., Black, M. and Lieber, C. S. (1987) Purification and characterization of human liver cytochrome P-450 Alc. *Biochem. Biophys. Res. Commun.* 148, 232-238.

Levin, W., Lu, A. Y. H., Jacobson, H. and Kuntzman, R. (1973) Lipid peroxidation and the degradation of cytochrome P450 heme. *Arch. Biochem. Biophys.* 158, 842-852.

Lieber, C. S. (1988) Metabolic effects of ethanol and its interaction with other drugs, hepatotoxic agents, vitamins and carcinogens. *Semin. Liver Dis.* 8, 47-68.

Lieber, C. S. (1990) Mechanism of ethanol induced hepatic injury. *Pharmacol. Ther.* 46, 1-41.

Lieber, C. S. and DeCarli, L. M. (1968) Ethanol oxidation by hepatic microsomes: Adaptive increase after ethanol feeding. *Science* 162, 917-918.

Lieber, C. S. and DeCarli, L. M. (1970) Hepatic microsomal ethanol oxidizing system: In vitro characteristics and adaptive properties in vivo. *J. Biol. Chem.* 245, 2505-2512.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurements with the folin phenol reagent. *J. Biol. Chem.* 193, 265-275.

Lu, A. Y. H. and West, S. B. (1979) Multiplicity of mammalian microsomal cytochromes P-450. *Pharmcol. Rev.* 31, 277-295.

Mapoles, J., Berthou, F., Alexander, A., Simon, F. and Ménez, J.-F. (1993) Mammalian PC-12 cell genetically engineered for human cytochrome P450 2E1 expression. *Eur. J. Biochem.* 214, 735-745.

McCord, J. M. and Day, E. D. (1978) Superoxide-dependent production of hydroxyl radical catalyzed by iron-EDTA complex. *FEBS Lett.* 86, 139.

McCoy, G. D. and Koop, D. R. (1988) Reconsitution of rabbit liver microsomal N-nitrosopyrrolidine a-hydroxylase activity. *Cancer Res.* 48, 3987-3992.

Mieyal, J. J. and Blumer, J. L. (1976) Acceleration of the autooxidation of human oxyhemoglobin by aniline and its relation to hemoglobin-catalyzed aniline hydroxylation. *J. Biol. Chem.* 251, 3442-3446.

Miller, A. D. and Buttimore, C. (1986) Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol. Cell. Biol.* 6, 2895-2902.

Minotti, G. and Aust, S. D. (1987) The requirement for iron(II) in the initiation of lipid peroxidation by iron(II) and hydrogen peroxide. *J. Biol. Chem.* 262, 1098-1104.

Minowa, O., Sogawa, K., Higashi, Y. and Fujii-Kuriyama, Y. (1990) Functional expression of microsomal and mitochondrial cytochrome P-450 (d and ssc) in COS-7 cells from cloned cDNA. *Cell Struct. Funct.* 15, 21-30.

Mishra, L., Sharma, S., Potter, J. J. and Mezey, E. (1989) More rapid elimination of alcohol in women as compared to their male siblings. *Alcohol Clin. Exp. Res.* 13, 752-754.

Mitchell, J. R. and Horning, M. J. (1984) *Drug metabolism and drug toxicity,* Raven Press, New York.

Mitchell, J. R., Jollow, D. J., Potter, W. Z., Gilllette, J. R. and Brodie, B. B. (1973) Acetaminophen-induced hepatic necrosis I. Role of drug metabolism. *J. Pharmacol. Exp. Ther.* 187, 185-194.

Miwa, G. T., West, S. B. and Lu, A. Y. H. (1978) Studies on the rate-limiting enzyme component in the microsomal monooxygenase system: incorporation of purified NADPH-cytochrome C reductase and cytochrome P-450 into rat liver microsomes. *J. Biol. Chem.* 253, 1921-1929.

Moore, M., Thor, H., Moore, G., Nelson, S. D., Moldeus, P. and Orrenius, S. (1985) The toxicity of acetaminophen and *N*-acetyl-*p*-benzoquinone imine in isolated hepatocytes is associated with thiol depletion and increased calcium. *J. Biol. Chem.* 260, 13035-13040.

Morehouse, L. A. and Aust, S. D. (1988) Reconstituted microsomal lipid peroxidation: ADP-Fe^{3+}-dependent peroxidation of phospholipid vesicles containing NADPH-cytochrome P450 reductase and cytochrome P450. *Free Radical Biol. Med.* 4, 269-277.

Morgan, E. T., Koop, D. R. and Coon, M. J. (1982) Catalytic activity of cytochrome P450 isozyme 3a isolated from liver microsomes of ethanol-treated rabbits. *J. Biol. Chem.* 257, 13951-13957.

Morgan, E. T., Koop, D. R. and Coon, M. J. (1983) Comparison of six rabbit liver cytochrome P450 isozymes in formation of a reactive metabolite of acetaminnophen. *Biochem. Biophys. Res. Commun.* 112, 8-13.

Narashimhulu, S., Cooper, D. Y. and Rosenthal, O. (1965) Spectrophotometric properties of a triton-clarified steroid 21-hydroxylase system of adrenocortical microsomes. *Life Sci.* 4, 2101-2107.

Nash, T. (1953) The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.* 55, 416-421.

Navarro, C., Cohen, S. D. and Khairallah, E. A. (1994) Acetaminophen administration alters rate of cell replication in hepatoma cells without activation of drugs by cytochrome P450. *FASEB J.* 9, Abstract 1216.

Nazareth, W. M. A., Sethi, J. K. and McLean, A. E. M. (1991) Effect of paracetamol on mitochondrial membrane function in rat liver slices. *Biochem. Pharmacol.* 42, 931-936.

Nebert, D. W., Nelson, D. R., Coon, M. J., Estabrook, R. W., Feyereisen, R., Fujii-Kuriyama, Y., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Loper, J. C., Sato, R., Waterman, M. R. and Waxman, D. J. (1991) The P450 superfamily: Update on new sequences, gene mapping, and recommended nomenclature. *DNA and Cell Biology* 10, 1-14.

Nicotera, P., Bellomo, G. and Orrenius, S. (1990) The role of Ca2+ in cell killing. *Chem. Res. Toxicol.* 3, 484-494.

Niehaus, W. G. J. and Jamuelson, B. (1968) Formationof malonaldehyde from phospholipid arachidonate during microsomal lipid peroxidation. *Eur. J. Biochem.* 6, 126-130.

Nouso, K., Thorgeirsson, S. S. and Battula, N. (1992) Stable expression of human cytochrome P450IIE1 in mammalian cells: Metabolic activation of nitrosodimethylamine and formation of adducts with cellular DNA. *Cancer Res.* 52, 1796-1800.

Olson, M. J., Kim, S. G., Reidy, C. A., Johnson, J. T. and Novak, R. F. (1990) Oxidation of 1,1,2,2-tetrafluoroethane in rat liver microsomes is catalized primarily by cytochrome P-450IIE1. *Drug Metab. Dispos.* 19, 298-303.

Omura, T. and Sato, R. (1961) A new cytochrome in liver microsomes. *J. Biol. Chem.* 327, PC1375-PC1376.

Omura, T. and Sato, R. (1964a) The carbon-monoxide binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* 239, 2370-2378.

Omura, T. and Sato, R. (1964b) The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification, and properties. *J. Biol. Chem.* 239, 2379-2385.

Ortiz de Montellano, P. R. (1986) *Cytochrome P-450: Structure, mechanism, and biochemistry,* Plenum Press, New York.

Palakodety, R. B., Clejan, L. A., Krikun, G., Feierman, D. E. and Cederbaum, A. I. (1988) Characterization and identification of a pyrazole-inducible form of cytochrome P450. *J. Biol. Chem.* 263, 878-884.

Paracchini, L., Jotti, A., Bottiroli, G., Prosperi, E., Supino, R. and Piccinini, F. (1993) The spin trap PBN protects against myelotoxicity and cardiotoxicity of adriamycin while preserving the cytotoxic activity. *Anti Cancer Res.* 13, 1607-1612.

Patten, C. J., Ishizaki, H., Aoyama, T., Lee, M., Ning, S. M., Huang, W., Gonzalez, F. J. and Yang, C. S. (1992) Catalytic properties of the human cytochrome P450 2E1 produced by cDNA expression in mammalian cells. *Arch. Biochem. Biophys.* 299, 163-171.

Patten, C. J., Ning, S. M., Lu, A. Y. H. and Yang, C. S. (1986) Acetone inducible cytochrome P450: Purification, catalytic activity and interaction with cytochrome b_5. *Arch. Biochem. Biophys.* 251, 629-638.

Patten, C. J., Thomas, P. E., Guy, R. L., Lee, M., Gonzalez, F. J., Guengerich, F. P. and Yang, C. S. (1993) Cytochrome P450 enzymes involved in acetaminophen activation by rat and human liver microsomes and their kinetics. *Chem. Res. Toxicol.* 6, 511-518.

Pederson, T. C. and Aust, S. D. (1972) NADPH-dependent lipid peroxidation catalyzed by purified NADPH-cytochrome C reductase from rat liver microsomes. *Biochem. Biophys. Res. Commun.* 48, 789-795.

Peter, R., Bocker, R., Beaune, P. H., Iwasaki, M., Guengerich, F. P. and Yang, C. S. (1990) Hydroxylation of chlorzoxazone as a specific probe for human liver cytochrome P-450IIE1. *Chem. Res. Toxicol.* 3, 566-573.

Pezzano, M., Richard, C., Lampl, E., Pelletier, G., Fabre, M., Rimailho, A. and Auzepy, P. (1988) Hepatic and renal toxicity of paracetamol in chronic alcoholic subjects. *Presse Med.* 17, 21-24.

Porter, T. D. and Coon, M. J. (1991) Cytochrome P450: Multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms. *J. Biol. Chem.* 266, 13469-13472.

Potter, W. Z., Thorgeirsson, S. S., Jollow, D. J. and Mitchell, J. R. (1973) Acetaminophen-induced hepatic necrosis III. Cytochrome P-450-mediated covalent binding *in vitro*. *J. Pharmacol. Exp. Ther.* 187, 203-210.

Pou, S., Ramos, C. L., Renks, E., Centra, M., Young, D., Kang, G., Cohen, M. S. and Rosen, G. M. (1992) *Arch. Biochem. Biophys.* In press.

Poulos, T. L. and Raag, R. (1992) Cytochrome $P450_{cam}$: Crystallography, oxygen activation, and electron transfer. *FASEB J.* 6, 674-679.

Prasad, J. S., Chen, N. Q., Liu, Y. X., Goon, D. J. and Holtzman, J. L. (1990) Effects of ethanol and inhibitors on the binding and metabolism of acetaminophen and *N*-acetyl-*p*-benzoquinone imine by hepatic microsomes from control and ethanol-treated rats. *Biochem. Pharmacol.* 40, 1989-1995.

Prescott, L. F. (1983) Paracetamol overdosage-pharmacological considerations and clinical management. *Drugs* 25, 290-314.

Pumford, N. R., Hinson, J. A., Potter, D. W., Rowland, K. L., Benson, R. W. and Roberts, D. W. (1989) Immunochemical quantitation of 3-(cystein-S-yl) acetaminophen adducts in serum and liver proteins of acetaminophen-treated mice. *J. Pharmacol. Exp.Ther.* 248, 190-196.

Rashba, Y. E., Chernikov, V. A., Baider, L. M. and Vartanyan, L. S. (1990) Application of piperidine and imidazoline hydroxylamines for ESR quantitation of superoxide radicals in biochemical systems. *Biol. Membranes (USSR)* 3, 1307-1320.

Rashba-Step, J. and Cederbaum, A. I. (1994) Generation of reactive oxygen intermediates by human liver microsomes in the presence of NADPH or NADH. *Mol. Pharmacol.* 45, 150-157.

Raucy, J., Fernandes, P., Black, M., Yang, S. L. and Koop, D. R. (1987) Identification of a human liver cytochrome P-450 exhibiting catalytic and immunochemical similarities to cytochrome P-450 3a of rabbit liver. *Biochem. Pharmacol.* 36, 2921-2926.

Raucy, J. L., Lasker, J. M., Lieber, C. S. and Black, M. (1989) Acetaminophen activation by human liver cytochromes P-450IIE1 and P-450IA2. *Arch. Biochem. Biophys.* 271, 270-283.

Ray, S. D., Kamendulis, L. M., Gurule, M. W., Yorkin, R. D. and Corcoran, G. B. (1993) Calcium antagonists inhibit DNA fragmentation and toxic cell death induced by acetaminophen. *FASEB J.* 7, 453-463.

Rechnagel, R. O. and Glende, E. A. (1973) Carbon tetrachloride toxicity: an example of lethal cleavage. *Crit. Rev. Toxicol.* 2, 263-297.

Reichard, G. A. J., Haff, A. C., Skutches, C. L., Paul, P., Holroyde, C. P. and Owen, O. E. (1979) Plasma acetone metabolism in the fasting human. *J. Clin. Invest.* 63, 619-626.

Reinke, L. A. and Moyer, M. J. (1985) *p*-Nitrophenol hydroxylation: A microsomal oxidation which is highly inducible by ethanol. *Drug Metab. Dispos.* 13, 548-552.

Remmer, H., Schenkman, J. B., Estabrook, R. W., Sasame, H., Gillette, J. R., Cooper, Y., Narashimhulu, S. and Rosenthal, O. (1966) Drug interaction with hepatic microsomal cytochrome. *Mol. Pharmacol.* 2, 187-190.

Ryan, D. E., Koop, D. R., Thomas, P. E., Coon, M. J. and Levin, W. (1986) Evidence that isoniazid and ethanol induce the same microsomal cytochrome P-450 in rat liver, an isozyme 3a. *Arch. Biochem. Biophys.* 246, 633-644.

Ryan, D. E., Ramanathan, L., Iida, S., Thomas, P. E., Haniu, M., Shively, J. E., Lieber, C. S. and Levin, W. (1985) Characterization of a major form of rat hepatic microsomal cytochrome P-450 induced by isoniazid. *J. Biol. Chem.* 260, 6385-6393.

Ryan, T. P. and Aust, S. D. (1992) The role of iron in oxygen-mediated toxicities. *Crit. Rev. Toxicol.* 22, 119-141.

Sambrook, S., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A laboratory manual,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sassa, S., Sugita, O., Galbraith, R. A. and Kappas, A. (1987) Drug metabolism by the human hepatoma cell, HepG2. *Biochem. Biophys. Res. Commun.* 143, 52-57.

Sato, R. and Omura, T. (1978) *Cytochrome P450,* Kodansha Academic Press, Tokyo/New York.

Schanne, F. A. X., Kane, A. B., Young, E. E. and Farber, J. L. (1979) Calcium dependence of toxic cell death: a final common pathway. *Science* 206, 700-702.

Schenkman, J. B., Remmer, H. and Estabrook, R. W. (1967) Spectral studies of drug interaction with hepatic microsomal cytochrome. *Mol. Pharmacol.* 3, 113-123.

Schuetz, E. G., Schuetz, J. D., Strom, S. C., Thompson, M. T., Fisher, R. A., Molowa, D. T., Li, D. and Guzelian, P. S. (1993) Regulation of human liver cytochrome P450 in family 3A in primary and continuous culture of human hepatocytes. *Hepatology* 18, 1254-1262.

Sligar, S. G. and Gunsalus, I. C. (1976) A thermodynamic model of regulation: Modulation of redox equilibria in camphor monoxygenase. *Proc. Natl. Acad. Sci. USA* 73, 1078-1982.

Sohn, D. H., Yun, Y. P., Park, K. S., Veech, R. L. and Song, B. J. (1991) Post-translational reduction of cytochrome P450IIE1 by CCl_4, its substrate. *Biochem. Biophys. Res. Comm.* 179, 449-454.

Sohn, O. S., Ishizaki, H., Yang, C. S. and Fiala, E. S. (1991) Metabolism of azoxymethane, methylazoxymethanol and N-nitrosodimethylamine by cytochromme P450IIE1. *Carcinogenesis* 12, 127-131.

Song, B.-J., Matsunaga, T., Hardwick, J. P., Park, S. S., Veech, R. L., Yang, C. S., Gelboin, H. V. and Gonzalez, F. J. (1987) Stabilization of cytochrome P450j messenger ribonucleic acid in the diabetic rat. *Mol. Endocrinol.* 1, 542-547.

Song, B.-J., Veech, R. L., Park, S. S., Gelboin, H. V. and Gonzalez, F. J. (1989) Induction of rat hepatic N-nitrosodimethylamine demethylase by acetone is due to protein stabilization. *J. Biol. Chem.* 264, 3568-3572.

Song, B. J., Gelboin, H. V., Park, S. S., Yang, C. S. and Gonzalez, F. J. (1986) Complementary DNA and protein sequences of ethanol-inducible rat and human P-450s: transcriptional and post-transcriptional regulation of the rat enzyme. *J. Biol. Chem.* 261, 16689-16697.

Stadtman, E. R. (1992) Protein Oxidation and Aging. *Science* 257, 1220-1224.

Streeter, A. G., Dahlin, D. C., Nelson, S. D. and Baillie, T. A. (1984) The covalent binding of acetaminophen to protein. Evidence for cysteine residues as major sites of arylation *in vitro*`. *Chem. Biol. Interact.* 48, 348-366.

Strubelt, O., Obermeier, F., Siegers, C. P. and Volpel, M. (1978) Increased carbon tetrachloride hepatotoxicity after low-level ethanol consumption. *Toxicology* 10, 261-270.

Takahashi, M., Akiyama, I., Tsujita, N. and Yoshida, A. (1989) The effect of alchol on the vestibulo-ocular reflex and gaze regulation. *Arch. Otorhinolaryngol.* 246, 195-199.

Tee, L. B. G., Boobis, A. R., Huggett, A. C. and Davies, D. S. (1986) Reversal of acetaminophen toxicity in isolated hamster hepatocytes by dithiothreitol. *Toxicol. Appl. Pharmacol.* 83, 294-314.

Terelius, Y. and Ingelman-Sundberg, M. (1986) Metabolism of n-pentane by ethanol-inducible cytochrome P-450 in liver microsomes and reconsitituted membranes. *Eur. J. Biochem.* 161, 303-308.

Teschke, R., Vierke, W. and Gellert, J. (1984) Effect of ethanol on carbon tetrachloride levels and hepatotoxicity after acute carbon tetrachloride poisoning. *Arch. Toxicol.* 56, 78-82.

Thummel, K. E., Lee, C. A., Kunze, K. L., Nelson, S. D. and Slattery, J. T. (1993) Oxidation of acetaminophen to N-acetyl-p-aminobenzoquinone imine by human CYP3A4. *Biochem. Pharmacol.* 45, 1563-1569.

Thurman, R. G. (1973) Induction of hepatic microsomal NADPH-dependent production of hydrogen peroxide by chronic prior treatment with ethanol. *Mol. Pharmacol.* 9, 670-675.

Tierney, D. J., Haas, A. L. and Koop, D. R. (1992) Degradation of cytochrome P450 2E1: selective loss after labilization of the enzyme. *Arch. Biochem. Biophys.* 293, 9-16.

Tirmenstein, M. A. and Nelson, S. D. (1989) Subcellular binding and effects on calcium homeostasis produced by acetaminophen and a nonhepatotoxic regioisomer, 3'-hydroxyacetanilide, in mouse liver. *J. Biol. Chem.* 264, 9814-9819.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheet: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76, 4350-4355.

Tribble, D. L., Aw, T. Y. and Jones, D. P. (1987) The pathophysiological significance of lipid peroxidation in oxidative cell injury. *Hepatology* 7, 377-386.

Tsutsumi, M., Lasker, J. M., Shimizu, M., Rosman, A. S. and Lieber, C. S. (1989) The intralobular distribution of ethanol-inducible P450 IIE1 in rat and human liver. *Hepatology* 10, 437-446.

Umeno, M., McBride, W., Yang, C. S., Gelboin, H. V. and Gonzalez, F. J. (1988) Human ethanol-inducible P450IIE1: Complete gene sequence, promoter characterization, chromosome mapping, and cDNA-directed expression. *Biochemistry* 27, 9006-9013.

Umeno, M., Song, B. J., Kozak, C., Gelboin, H. V. and Gonzalez, F. J. (1988) The rat P450IIE1 gene: Complete intron and exon sequence, chromosome mapping,

and correlation of developmental expression with specific 5' cytosine demethylaiton. *J. Biol. Chem.* 263, 4956-4962.

van de Straat, R., Bijloo, G. J. and Vermeulen, N. P. E. (1987) Paracetamol, 3-monoalkyl- and 3,5-dialkyl-substituted derivatives: antioxidant activity and relationship between lipid peroxidation and cytotoxicity. *Biochem. Pharmacol.* 37, 3473-3476.

Vaz, A. D. N., Roberts, E. S. and Coon, M. J. (1990) Reductive β-scission of the hydroperoxides of fatty acids and xenobiotics: Role of alcohol-inducible cytochrome P-450. *Proc. Natl. Acad. Sci. USA* 87, 5499-5503.

Vermeulen, N. P. E., Bessems, J. G. M. and van de Straat, R. (1992) Molecular aspects of paracetamol-induced hepatotoxicity and its mechanism-based prevention. *Drug Metab. Rev.* 24, 367-407.

Villarruel, M. D. C., de Toranzo, E. G. D. and Castro, J. A. (1977) Carbon tetrachloride activation, lipid peroxidation and the mixed function oxygenase activity of various rat tissues. *Toxicol. Appl. Pharmacol.* 17, 337-344.

Vogelstein, B. and Gillespie, D. (1979) Preparative and analytical purification of DNA from agarose. *Proc. Natl. Acad. Sci. USA* 76, 615-619.

Vore, M. L., A., Kuntzman, R. and Conney, A. J. (1974) Organic solvent extraction of liver microsomal lipid II. Effect on the metabolism of substrates and binding spectra of cytochrome P-450. *Mol. Pharmacol.* 10, 963-974.

Waterman (1991) *Methods in Enzymology: Cytochrome P450,* Academic Press, New York, NY.

Waterman, M. R., Ullrich, V. and Estabrook, R. W. (1973) Effects of substrate on the spin state of cytochrome P-450 in hepatic microsomes. *Arch. Biochem. Biophys.* 155, 355-360.

Waxman, D. J., Lapenson, D. P., Aoyama, T., Gelboin, H. V., Gonzalez, F. J. and Korzekwa, K. (1991) Steroid hormone hydroxylase specificities of eleven cDNA-expressed human cytochrome P450s. *Arch. Biochem. Biophys.* 290, 160-166.

Wendel, A. and Feuerstein, S. (1981) Drug-induced lipid peroxidation in mice-I: modulation by monooxygenase activity, glutathione and selenium status. *Biochem. Pharmacol.* 30, 2513-2520.

Werringloer, J. (1978) Assay for formaldehyde generated during microsomal oxidation reactions. *Methods Enzymol.* 52, 297-302.

Wertz, J. and Bolton, J. R. (1974) *Electron Spin Resonance: Elementary Theory and Applications,* McGraw-Hill, City.

Wills, E. D. (1969) Lipid peroxide formation in microsomes: General consideration. *Biochem. J.* 113, 315-324.

Wills, E. D. (1971) Effects of lipid peroxidation on membrane-bound enzymes of the endoplasmic reticulum. *Biochem. J.* 123, 983-991.

Winters, D. K. and Cederbaum, A. I. (1992) Expression of a catalytically active human cytochrome P450-2E1 in *Escherichia coli*. *Biochem. Biophys. Acta* 1117, 15-24.

Winters, D. K., Clejan, L. A. and Cederbaum, A. I. (1988) Oxidation of glycerol to formaldehyde by rat liver microsomes. *Biochem. Biophys. Res. Commun.* 153, 612-617.

Wrighton, S. A., Thomas, P. E., Molowa, D. T., Haniu, N., Shively, J. E., Maines, S. L., Watkins, P. B., Parker, G., Mendez-Picon, G., Levin, W. and Guzelian, P. S. (1986) Characterization of ethanol-inducible human liver *N*-nitrosodimethylamine demethylase. *Biochemistry* 25, 6731-6735.

Yamazaki, L., Piette, L. A. and Grover, T. A. (1990) Kinetic studies on spin trapping of superoxide or hydroxyl radicals generated in NADPH-cytochrome P-450 reductase-paraquat systems. Effect of iron chelates. *J. Biol. Chem.* 265, 652-659.

Yang, C. S., Tu, Y. Y., Koop, D. R. and Coon, M., J, (1985) Metabolism of nitrosamines by purified rabbit liver cytochrome P-450 isozymes. *Cancer Res.* 45, 1140-1145.

Yang, C. S., Yoo, J.-S. H., Ishizaki, H. and Hong, J. (1990) Cytochrome P450IIE1: Roles in nitrosamine metabolism and mechanisms of regulation. *Drug Metab. Rev.* 22, 147-159.

Yoshida, Y. and Kumaoka, H. (1975) Studies on the substrate-induced spectral change of cytochrome P-450 in liver microsomes. *J. Biochem. (Tokyo)* 78, 455-468.

Younes, M., Cornelius, S. and Siegers, C. P. (1986) Ferrous iron supported *in vivo* lipid peroxidation induced by paracetamol. *Res. Commun. Chem. Pathol. Pharmacol.* 51, 89-90.

Younes, M. and Siegers, C.-P. (1985) The role of iron in the paracetamol- and CCl_4-induced lipid peroxidation and hepatotoxicity. *Chem. Biol. Interac.* 55, 327-334.

Zhou, Z., Casciano, D. A. and Heflich, R. H. (1986) Use of the human liver cell line HepG2 in a modified salmonella reversion assay. *Cancer Lett.* 32, 327-334.