CYTOCHROME P450 PEROXIDASE/PEROXYGENASE-DEPENDENT METABOLIC ACTIVATION OF XENOBIOTICS

by

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ABSTRACT

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Cytochrome P450 (P450) peroxidase and peroxygenase activities have been well documented with microsomal enzymes and purified P450 preparations, however, the biological significance of these pathways in the bioactivation of xenobiotics and induction of oxidative stress in intact cells has not been demonstrated. The following summarizes our new findings regarding the role of P450 peroxidase/peroxygenase pathways in intact cells:

1. *tert*-butylhydroperoxide (tBHP) at non-toxic concentrations markedly enhanced up to 20 fold the cytotoxicity of various aromatic hydrocarbons and their phenolic metabolites towards isolated rat hepatocytes. The enhanced cytotoxicity was also accompanied by an increase in the hepatocyte O-demethylation of xenobiotics and xenobiotic reactive metabolites-GSH conjugates. An LC/MS analysis of the GSH conjugates identified hydroquinone:GSH and 4-methoxycatechol:GSH conjugates as the predominant adducts for 4-hydroxyanisole (4-HA). Pretreatment of hepatocytes with P450 inhibitors, e.g. phenylimidazole, prevented tBHP-enhanced 4-HA metabolism, GSH depletion and cytotoxicity. Hydroperoxides can therefore be used by intact

cells to support the bioactivation of xenobiotics through the P450 peroxygenase system.

2. The naturally occurring hydroperoxide, hydrogen peroxide, was found to be particularly effective at supporting cytochrome P450 1A2-catalyzed activation of the heterocyclic aromatic amine, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), to genotoxic metabolites. The addition of hydrogen peroxide or tBHP to rP4501A greatly enhanced the yield of histidine prototrophic (His^+) revertants, which was inhibited by α-naphthoflavone, a P450 1A inhibitor. Hydrogen peroxide was the most effective peroxygenase cofactor, particularly with human P450 1A2-containing microsomes (hP4501A2). The hydroperoxide-supported activation of IQ formed an adduct with 2'-deoxyguanosine similar to that of the well characterized DNA adduct formed (*in vivo* or *in vitro*) by the P450-catalyzed bioactivation system.

3. Organic hydroperoxides are believed to be primarily bioactivated to cytotoxic radical species by non heme iron. However, various P450 inhibitors were found to prevent cumene hydroperoxide (CumOOH) metabolism and subsequent cytotoxic effects including antimycin-A resistant respiration, lipid peroxidation, iron mobilization, ATP depletion, and cell membrane disruption. These results suggest that P450 enzymes in hepatocytes bioactivate CumOOH to form reactive radical metabolites or oxidants that cause lipid peroxidation and cytotoxicity.

DEDICATIONS

This thesis is dedicated to:

my children, Alireza and Diba
my wife, Afsoon
and my Mother & Father
who have provided unending
love and support

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TABLE OF CONTENTS

SUMMARY OF THESIS PUBLICATIONS

1. **M. Reza Anari,** S. Khan, Zhao Chao Liu and P. J. O'Brien (1995) Cytochrome P450 peroxidase/peroxygenase mediated xenobiotic metabolic activation and cytotoxicity in isolated hepatocytes. *Chemical Research in Toxicology* **8,** 997-1004. (Chapter 3 of the thesis)

2. **M. Reza Anari,** S. Khan, and P. J. O'Brien (1996) The involvement of cytochrome P450 in metabolic bioactivation of cumene hydroperoxide in isolated rat hepatocytes. *Chemical Research in Toxicology* **9,** 924-931. (Chapter 5 of the thesis)

3. **M. Reza Anari,** P. David Josephy, Tracey Henry, Peter J. O'Brien (1996) Hydrogen peroxide supports human cytochrome P450 1A2 catalyzed 2-amino-3-methylimidazo[4,5f]quinoline bioactivation to mutagenic metabolites: significance of cytochrome P450 peroxygenase. *Chemical Research in Toxicology,* submitted. (Chapter 4 of the thesis).

SUMMARY OF ABBREVIATIONS

ABBREVIATION	NAME
AA	Antimycin A
ATP	Adenosine triphosphate
BPS	Bathophenanthroline disulfonate
CID	Collisional-induced dissociation
CumOOH, CHP	Cumene hydroperoxide
DPPD	N,N'-Diphenyl-1,4-phenylenediamine
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GSH	Reduced glutathione
GSSG	Oxidized glutathione
4-HA	4-Hydroxyanisole
HAAs	Heterocyclic aromatic amines
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
His^+	histidine-prototrophic
hP4501A2	human P450 1A2 containing microsomes
HPLC	High Performance Liquid Chromatography
IQ	2-Amino-3-methylimidazo[4,5-f]quinoline

LC_{50}	Lethal concentration required to kill 50% of the cell population at 2 hr
LC/MS	Liquid chromatography-tandem mass spectrometry
3-MC	3-Methylcholanthrene
MDA	Malondialdehyde
MROD	Methoxyresorufin O-demethylation
4-NA	4-Nitroanisole
NAT	Acetyl-CoA: arylamine N-acetyltransferase
NDGA	Nordihydroguaiaretic acid
P450	Cytochrome P450
PNPH	*p*-nitrophenol hydroxylase
ROS	Reactive oxygen species
rP4501A	3-MC-induced rat liver microsomes
SIM	Selective ion monitoring
TBARS	Thiobarbituric acid reactive substances
tBHP	*tert*-Butylhydroperoxide
TEMPO	4-Hydroxy-2,2,6,6-Tetramethylpiperidine-N-oxyl

SUMMARY OF TABLES

SUMMARY OF FIGURES

Chapter 1

INTRODUCTION

A wide variety of enzymes metabolize xenobiotic substrates through the various oxidation reactions including hydride abstraction (e.g. dehydrogenases), electron abstraction (e.g. oxidoreductases), and incorporation of oxygen into the substrate, a reaction characterizing oxygenases (e.g. cytochrome P450). The later is known as the most important reaction of oxidation in the metabolism of xenobiotics and endogenous compounds. Depending on whether one or both atoms of molecular oxygen are transferred to the substrate, the enzymes are categorized as monooxygenases and dioxygenases, respectively. Monooxygenation reactions are mediated by various enzymes which differ markedly in their structure and properties. Among these, the most important as far as xenobiotic metabolism is concerned—and most likely also endobiotic metabolism—are the **cytochromes P450**, a very large group of enzymes encoded by the P450 gene superfamily and belonging to heme-coupled monooxygenases (Gonzalez, 1989; Gonzalez, 1990; Nebert *et al.*, 1987; Nebert and Gonzalez, 1987; Nebert *et al.*, 1989a; Nebert *et al.*, 1991). Other monooxygenases whose role in xenobiotic metabolism is well recognized are the flavin-containing monooxygenases [EC 1.14.13.8] and dopamine β-monooxygenase [EC 1.14.17.1], a copper-dependent monooxygenase.

1.1. CYTOCHROME P450

1.1.1. A First Look at Cytochrome P450

Cytochromes P450 are entered in the Enzyme Nomenclature mainly as unspecific monooxygenase [substrate, reduced-flavoprotein:oxygen oxidoreductase (RH-hydroxylating or -epoxidizing); microsomal P-450; EC 1.14.14.1] (IUBMB, International Union of Biochemistry and Molecular

Table 1-1: Cytochrome P450 Activities Coded by the International Union of Biochemistry and Molecular Biology, 1992.

P450 Activity	IUBMB Enzyme Code
Cholesterol 7α-monooxygenase	[EC 1.14.13.17]
Leukotriene-B_4 20-monooxygenase	[EC 1.14.13.30]
Methyltetrahydroprotoberberine 14-monooxygenase	[EC 1.14.13.37]
Tyrosine N-monooxygenase	[EC 1.14.13.41]
(-)-Limonene 3-monooxygenases	[EC 1.14.13.47]
(-)-Limonene 6-monooxygenases	[EC 1.14.13.48]
(-)-Limonene 7-monooxygenases	[EC 1.14.13.49]
Camphor 5-monooxygenase	[EC 1.14.15.1]
Steroid 11ß-monooxygenase	[EC 1.14.15.4]
Cortisone 18-monooxygenase	[EC 1.14.15.5]
Cholesterol monooxygenase	[EC 1.14.15.6]
Steroid 17α–monooxygenase	[EC 1.14.99.9]
Steroid 21-monooxygenase	[EC 1.14.99.10]

Biology, 1992). In addition, many other P450 activities have been recognized that are listed in Table 1-1.

Cytochrome P450 is a hemoprotein of unusual properties found in almost all living organisms such as bacteria, yeast, plants, and animals (e.g. insects and vertebrates), but interestingly not in helminths (Holton *et al.*, 1993; Miners *et al.*, 1988; Ortiz de Montellano, 1986; Precious and Barrett, 1986). Mammalian cytochromes P450 are found in almost all organs and tissues examined (Nebert *et al.*, 1989a; Sinal *et al.*, 1995). These enzymes are membrane-bound, located mostly in the endoplasmic reticulum but also in mitochondria. In fact, they are present in almost all membranes and cells (Coon *et al.*, 1992; Guengerich, 1991; Kapke and Baron, 1980; LaBella, 1991; Soucek and Gut, 1992; Waterman and Johnson, 1991). It must also be noted, however, that soluble (i.e. cytosolic) cytochromes P450 are not unknown; besides the cytochrome P450cam studied widely, a mammalian soluble cytochrome P450 designated H450 has been reported (Hasegwa, 1983; Omura *et al.*, 1984).

The appearance and evolution of cytochrome P450 may be traced back to the early days of biological evolution (more than 3.5 billion years ago), before the advent of the oxygen atmosphere when its presence must have been associated with reductive reactions (Gonzalez, 1989; Gonzalez, 1990). Upon the gradual appearance of atmospheric oxygen, its main role is believed to have become that of a line of defence (Wickramasinghe and Ville, 1975),

and only later evolving its manifold reactions of oxidation in (a) the anabolism and/or catabolism of endogenous compounds (e.g. steroids, fatty acids, leukotrienes, prostaglandins, biogenic amines, pheromones), and (b) in the detoxication of xenobiotics (in particular in the so-called animal-plant warfare). The long evolution of cytochrome P450 is certainly compatible with its present-day ubiquity, versatility and multiplicity (Estabrook *et al.*, 1982; Nebert and Gonzalez, 1985; Nebert and Gonzalez, 1987; Nebert and Negishi, 1982; Nelson *et al.*, 1993). The present section is centered on cytochrome P450 and examines first its structure and multiplicity. The functioning of cytochrome P450 is next considered by breaking down its catalytic cycle into individual steps discussed successively.

1.1.2. The Chemical Structure of Cytochrome P450

Cytochrome P450, being a hemoprotein, consists of a **protein** (the apoprotein or apoenzyme) and a **heme** moiety, namely iron-protoporphyrin IX. This porphyrin is common not only to all cytochrome P450 enzymes, but also to other hemoproteins and enzymes such as hemoglobin, myoglobin, catalase and most peroxidases. In contrast to the constant porphyrin moiety, the protein part of the enzyme varies markedly from one enzyme/isozyme to the other. This accounts for the differences in the P450 molecular size (e.g. molecular weight approximately ranges from 45 to 60 kDa), substrate and product specificities, and sensitivity to inhibitors.

The iron cation (which exists in the formal ferric and ferrous states) is liganded to the four pyrrole nitrogens. Two additional non-porphyrin ligands in axial positions, the fifth ligand being the **thiolate** ligand and the sixth ligand Y are represented in Figure 1-1 (Ortiz de Montellano, 1986; Ortiz de Montellano, 1995a).

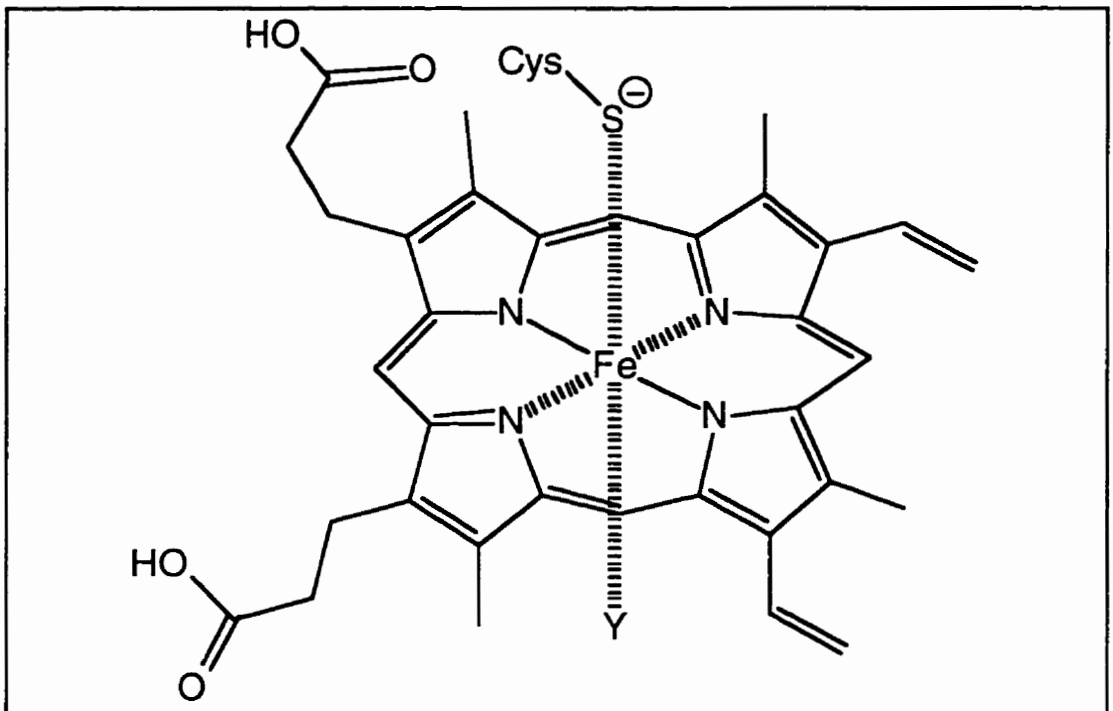


Figure 1-1: Structure of Iron-Protoporphyrin IX in the Prosthetic Heme of Cytochrome P450. Adopted from Ortiz de Montellano (1986)

The detailed structure of protoporphyrin IX has been revealed by X-ray crystallographic studies (Caughey and Ibers, 1977). The fifth ligand to the iron cation is a thiolate group from a conserved cysteine near the carboxyl end of the protein ($R\text{-}S^-$; note the anionic form). The iron-sulfur bond is an

unusually strong one, presumably due to other bonding forces, and it transfers considerable electron density to the iron (Black and Coon, 1985; Poulos and Raag, 1992). These electronic features are indispensable for the catalytic activity of cytochrome P450. The formation of the inactive cytochrome P420, as induced for example by denaturation of the protein, involves displacement of the thiolate ligand (Fe-S rupture) or perhaps simply its protonation (thiol ligation). The sixth ligand (Y in Figure 1-1) has been the subject of lengthy debate. It became clear some years ago that this group, rather than being a stronger nitrogen-containing ligand, must be a weaker, oxygenated one (Dawson *et al.*, 1982; White and Coon, 1982). A hydroxyl group, either from an adjacent amino acid residue or a water molecule, was postulated (Kumaki and Nebert, 1978). In some cases, the sixth ligand has now been shown to be a hydroxyl group belonging to a tyrosinyl residue located near the heme, while in cytochrome P450cam it appears to be a water molecule (Janig *et al.*, 1984; Poulos and Raag, 1992).

The liganded heme can exist in a number of discrete electronic states which are responsible for many of the properties of cytochrome P450, most significantly for the binding of ligands and the activation of molecular oxygen. The iron atom formally exists in either the ferric or the ferrous oxidation state, although higher oxidation states also play a role in oxygen activation in peroxidase/peroxygenase type of reaction as discussed in section 1.1.5.2. Two catalytically relevant spin states exist for both Fe(III) and Fe(II), i.e. low spin

(abbreviated *"ls"*) and high spin (abbreviated *"hs"*). The low spin states of Fe(III) (S = 1/2) and Fe(II) (S = 0) are hexacoordinated in hemoproteins; the iron atom is located in the plane of the four pyrrole nitrogens, and the six ligands occupy the vertices of an octahedron. In contrast, the high spin states of Fe(III) (S = 5/2) and Fe(II) (S = 2) are too large in diameter to be coplanar with the four nitrogen ligands. The iron atom will therefore lie outside the plane of the porphyrin ring [0.3 A for Fe(III) and 0.7 A for Fe(II)] and as a result will be pentacoordinated (no sixth ligand Y) (Hahn *et al.*, 1982).

1.1.3. A Modern Nomenclature System for the Cytochrome P450 Enzymes

Over the years, the number of cytochromes P450 that have been isolated and characterized by a variety of criteria has risen considerably. In particular, human cytochromes P450 have recently received increasing interest and a wealth of significant data can be found in the literature (Beaune *et al.*, 1986; Boobis and Davies, 1984). The result of this extraordinary international effort has been on the one hand the publication of innumerable data, and on the other hand much confusion about the identity, and about similarities or dissimilarities between them. Without the essential contributions of molecular biology, such a state of confusion would now have reached absurd proportions. Unravelling the sequence of P450 genes and isozymes has progressively led to a meaningful and robust classification, and a milestone paper was published in 1987 by Nebert and colleagues (Nebert *et*

al., 1987) who proposed a nomenclature for the *P450* gene superfamily and gene products based on evolution (Coon and Porter, 1988; Nebert *et al.*, 1989b; Puga and Nebert, 1990). In this nomenclature, genes and gene products are classified in families and subfamilies. The protein sequences within one family are defined as usually having >40% amino acid identity, but there are exceptions. For example, two mitochondrial P450 proteins scc and 11β, are included in the CYP11 family although the enzyme sequences are only 34% to 39% identical. Within the same subfamily, mammalian sequences are always >55% identical, but inclusion of non-mammalian vertebrates within the same subfamily drops this value to >46% (Nelson *et al.*, 1993).

The listing of P450 genes, new protein sequences and recommended nomenclature is now updated regularly (Nebert *et al.*, 1989a; Nebert *et al.*, 1991; Nelson *et al.*, 1993; Nelson *et al.*, 1996). As of October 1995, 481 P450 genes and 22 putative pseudogenes had been described in 85 eukaryotes and in 20 prokaryotes (Nelson *et al.*, 1996). Of 74 gene families so far described, 14 families exist in all mammals examined. These 14 families comprise 26 mammalian subfamilies, of which 20 and 15 have been mapped in the human and mouse genome, respectively. Most of the xenobiotic metabolizing P450 enzymes, however, are clustered in the first 3 families, i.e. CYP1-CYP3 families.

The CYP1 family contains the enzymes that metabolize, and are induced by, polycyclic aromatic hydrocarbons such as 3-methylcholanthrene

whereas the CYP2 family enzymes are inducible by phenobarbital and metabolize steroids as well as a large variety of xenobiotics (Anari *et al.*, 1995a; Waxman *et al.*, 1985). The CYP3 family enzymes are inducible by macrolide antibiotics and dexamethasone and metabolize steroids and many different drugs. Essential physiological functions are fulfilled by members of the CYP4 family (which oxidize fatty acids) and of the CYP7 and CYP11 families (which metabolize cholesterol). The biosynthesis of steroids involves members of the CYP11, CYP17, CYP19, CYP21 and CYP27 families (Gonzalez, 1992). In humans, a number of cytochromes P450 are of particular significance in the metabolism of drugs and other xenobiotics, i.e. lA1, 1A2, 1B1, 2B6, 2C9, 2C10, 2C18, 2C19, 2D6, 2E1, and 3A. The substrate specificity of most of them is illustrated in the next section.

1.1.4. Substrate Specificity of Cytochromes P450

As far as xenobiotic metabolism is concerned, a major consequence of cytochrome P450 multiplicity is the substrate specificity of these enzymes. Several isozymes can metabolize a given substrate by the same reaction, but one or a few isozymes are always much more active than the others. In practical terms, many xenobiotic substrates are metabolized mainly or exclusively by a single cytochrome P450. The important human cytochromes P450 and representative xenobiotic substrates (Cholerton *et al.*, 1992; Guengerich, 1990; Guengerich, 1992a; Guengerich, 1992b; Wringhton and

Stevens, 1992) are shown in Table 1-2. Such a list is obviously far from exhaustive, but it is sufficient to convey the concept of isozyme specificity.

Table 1-2: Important Human Cytochromes P450 and Representative Xenobiotic Substrates

CYP1A1	Benzo[a]pyrene and other polycyclic aromatic hydrocarbons
CYP1A2	Caffeine, 2-naphthylamine and other arylamines, phenacetin, theophylline
CYP2C9/10	Hexobarbital, tolbutamide
CYP2C18	Cyclophosphamide, diazepam, hexobarbital, omeprazol, proguanil, propranolol
CYP2D6	Ajmaline, clomipramine, codeine, debrisoquine, dextromethorphan, encainide, haloperidol, imipramine, methadone, metoprolol, minaprine, perhexiline, phenformine, propafenone, propranolol, sparteine, thioridazine, timolol, tropisetrone
CYP2E1	Acetaminophen, acetone, aniline, benzene, carbon tetrachloride, chloroform, chlorzoxazone, diethyl ether, enflurane, ethanol and other alcohols, ethyl carbamate, ethylene dichloride, N-nitrosodimethylamine and other nitrosoamines, styrene, vinyl chloride and bromide
CYP3A	Aflatoxins, bromocryptine, cyclosporine, diazepam, ergotamine, erythromycine, ethynylestradiol, lidocaine, methadone, midozolam, nifedipine and other dihydropyridines, quinidine, terfenadine, triazolam.

1.1.5. Reactions Catalyzed by Cytochrome P450

The mechanisms by which P450 activates oxygen and inserts it into the substrates have long been studied using microsomal fractions or reconstituted purified P450 preparations. A number of functions of cytochrome P450 and their global reactions and stoichiometry are summarized in Table 1-3. The P450 **monooxygenase** activity (Eq. 1-1) which is generally considered as an *in vivo* physiological pathway of substrate oxidation is dependent on the molecular oxygen and two electrons from the NADPH-cytochrome P450 reductase (Guengerich, 1991; White and Coon, 1980). P450s can also catalyze

Table 1-3: Various Functions of Cytochrome P450, Their Global Reactions and Stoichiometry

Monooxygenase function:

$$XH + O_2 + 2e^- + 2H^+ \longrightarrow XOH + H_2O \qquad \text{(Eq. 1-1)}$$

Two-electron oxidase function

$$O_2 + 2e^- + 2H^+ \longrightarrow H_2O \qquad \text{(Eq. 1-2)}$$

Four-electron oxidase function

$$O_2 + 4e^- + 4H^+ \longrightarrow 2H_2O \qquad \text{(Eq. 1-3)}$$

Peroxidase/Peroxygenase function

$$XH + ROOH \longrightarrow XOH + ROH \qquad \text{(Eq. 1-4)}$$

Oxene transferase function

$$XH + RO \longrightarrow XOH + R \qquad \text{(Eq. 1-5)}$$

One-electron reductase function

$$ROOH + H^+ + e^- \longrightarrow RO^{\bullet} + H_2O \qquad \text{(Eq. 1-6)}$$

or

$$XCl + H^+ + e^- \longrightarrow X^{\bullet} + HCl \qquad \text{(Eq. 1-7)}$$

the oxidation of various substrates independent of molecular oxygen, NADPH and cytochrome P450 reductase if organic or lipid hydroperoxides are used as the oxidant source (Hrycay *et al.*, 1976; Hrycay and O'Brien, 1971a; Hrycay and O'Brien, 1971b; O'Brien, 1978; Rahimtula and O'Brien, 1974; Rahimtula and O'Brien, 1975; Rahimtula and O'Brien, 1977a). The splitting of peroxides with transfer of one oxygen atom to a substrate constitutes the **peroxidase/peroxygenase** function of cytochrome P450 (Eq. 1-4). Both monooxygenase and the peroxygenase functions imply peroxide cleavage, followed by transfer of the retained atom of oxygen to the substrate without exchange with an oxygen atom from H_2O, as has repeatedly been shown using oxygen isotopes. However, there exists a further source of oxygen atom, namely compounds able to act as oxygen atom donors, e.g. iodosobenzene (Gustafsson *et al.*, 1979; Lichtenberger *et al.*, 1976). Here, the oxygen atom is transferred directly to the ferricytochrome P450-substrate complex, and substrate oxygenation occurs without NADPH and O_2 (Eq. 1-5). In this reaction, cytochrome P450 acts as an **oxene transferase.**

1.1.5.1. Cytochrome P450 Monooxygenase Activity

The catalytic cycle of cytochrome P450 in its monooxygenase function involves a number of steps (Estabrook *et al.*, 1982; Gander and Mannering, 1980; Guengerich, 1991; Koymans *et al.*, 1993; Mesnil and Testa, 1984; Ortiz de

Montellano, 1986; Ortiz de Montellano, 1995b; White and Coon, 1980) as illustrated in Figure 1-2 and can be summarized as follows :

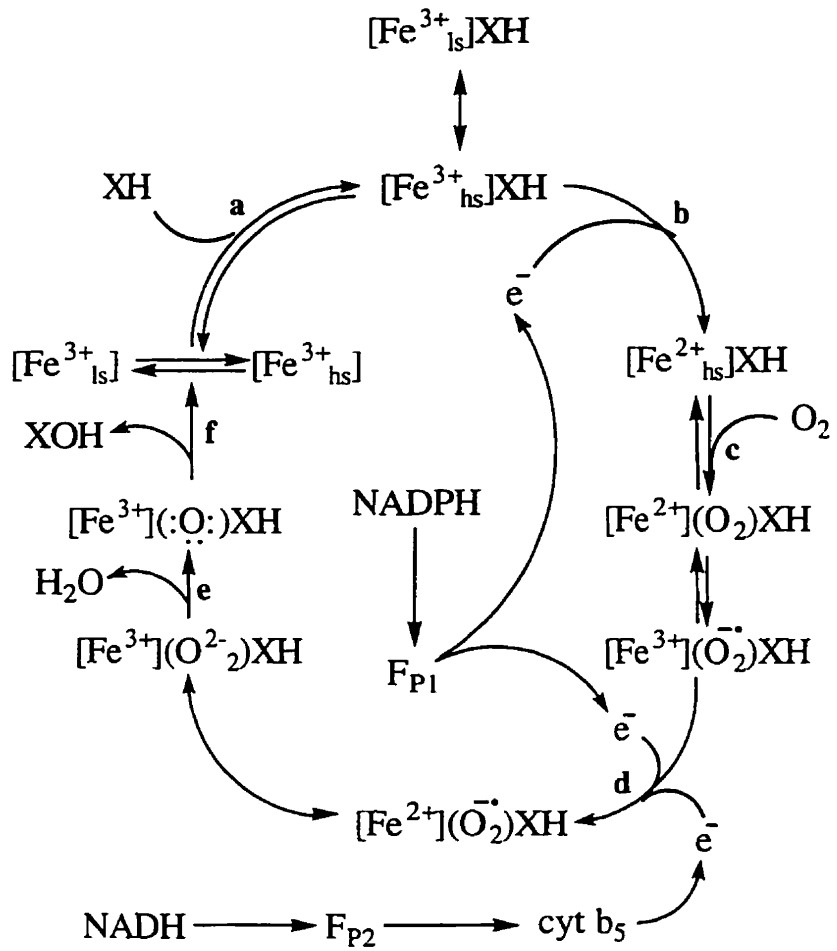


Figure 1-2: Catalytic Cycle of Cytochrome P450 Associated with Monooxygenation Reactions. [Fe^{3+}]=ferricytochrome P450; hs=high spin; ls=low spin; [Fe^{2+}]=ferrocytochrome P450; F_{P1}=NADPH-cytochrome P450 reductase; F_{P2}=NADH-cytochrome b_5 reductase; cyt b_5=cytochrome b_5; XH=substrate. Adopted from Mesnil *et al.* (1984).

(a) Binding of the substrate to the ferric form of the enzyme, followed by a shift to the high-spin form (reaction **a**)

(b) First reduction step to the ferrous form (reaction **b**)

(c) Binding of molecular oxygen (reaction **c**)

(d) Second reduction step (reaction **d**)

(e) Splitting of the dioxygen molecule, i.e. oxygen activation (reaction **e**)

(f) Substrate oxidation and product release (reaction **f**).

1.1.5.2. Cytochrome P450 Peroxygenase/Peroxidase Activities

It was discovered in the early 1970s that hydroperoxides or hydrogen peroxide can support the cytochrome P450-catalyzed hydroxylation of various substrates in a reaction that requires neither O_2, NADPH nor NADPH-cytochrome P450 reductase, and which is not inhibited by carbon monoxide (Hrycay *et al.*, 1976; Hrycay and O'Brien, 1971a; Hrycay and O'Brien, 1971b; O'Brien, 1978; Rahimtula and O'Brien, 1974; Rahimtula and O'Brien, 1975; Rahimtula and O'Brien, 1977a). This reaction that was first viewed as a laboratory curiosity has yielded many valuable insights into the catalytic mechanism of cytochrome P450. The cytochrome P450 peroxygenase /peroxidase reactions show analogies and differences with those of monooxygenases (Cstro, 1980; O'Brien, 1978). A particularly significant difference concerns the origin of the active oxygen, which is not derived from molecular oxygen but from reduced forms thereof (i.e. hydrogen peroxide [H_2O_2] or organic hydroperoxides [ROOH]). The various enzymes that function solely or occasionally as peroxidases include cytochrome P450,

prostaglandin-endoperoxide synthase and catalase, as well as other peroxidases such as chloroperoxidase and myeloperoxidase.

1.1.5.2.1. Catalytic Mechanisms

In simplified form, both peroxygenase and peroxidase activities of cytochrome P450 can be described by Eq. 1-5 as described in Table 1-3, where XH is the substrate and ROOH the hydroperoxide. By the heterolytic reduction

Table 1-4: Hydroperoxide-Supported P450-Catalyzed Oxidation Reactions

P450 Peroxygenase:

						Compound I
ROOH	+	Fe^{3+}	------(1)----->	ROH	+	$[Fe^{5+}=O]^{3+}$
$[Fe^{5+}=O]^{3+}$	+	X-H	------(2)----->	X-OH	+	Fe^{3+}
ROOH	+	X-H	-----(1,2)---->	ROH	+	X-OH

P450 Peroxidase:

						Compound II
ROOH	+	Fe^{3+}	------(1)----->	$RO^{\cdot}$	+	$[Fe^{4+}\text{-}OH]^{3+}$
$RO^{\cdot}$	+	X-H	------(2)----->	ROH	+	$X^{\cdot}$
$[Fe^{4+}\text{-}OH]^{3+}$	+	X-H	------(3)----->	Fe^{3+}	+	$X^{\cdot}$ + H_2O
$[Fe^{4+}\text{-}OH]^{3+}$	+	$X^{\cdot}$	------(4)----->	Fe^{3+}	+	X-OH
ROOH	+	2X-H	---(1,2,3)--->	ROH	+	$2X^{\cdot}$ + H_2O

or

ROOH	+	X-H	---(1,2,4)--->	ROH	+	X-OH

of O-O hydroperoxide bond as shown in Table 1-4, the enzyme functions as a **"peroxygenase"**, a term sometimes used to draw a parallel with its monooxygenase activity and meaning that the oxygen atom transferred to the substrate comes from a hydroperoxide. The cytochrome P450 peroxygenase activity begins with the formation of a ternary complex having the two substrates (i.e. the hydroperoxide and the substrate undergoing hydroxylation) bound to the oxidized (ferric) form of cytochrome P450 (Koop and Hollenberg, 1980). Such a complex has been designated as Complex C by some authors; its formation can be monitored spectrophotometrically, as can the formation of binary hydroperoxide-cytochrome P450 complexes (Blake and Coon, 1980; Blake and Coon, 1981; Blake and Coon, 1981b; Rahimtula and O'Brien, 1977b). The hypervalance intermediate iron-oxygen species, i.e. $P450^{\cdot+}(Fe^{IV}=O)$, is identical to the iron-oxene complex in P450 monooxygenase pathway and analogous to the peroxidases Compound I (Groves *et al.*, 1978; O'Brien, 1978; Ortiz de Montellano, 1995a; Shimizu *et al.*, 1994; Wand and Thompson, 1986) as shown in Figure 1-3. By contrast a homolytic one electron reduction of the hydroperoxide O-O bond by P450s, i.e. "**peroxidase** reaction", results in the formation of alkoxyl radicals and an iron-oxo species analogous to peroxidase Compound II (Wand and Thompson, 1986) (Table 1-4).

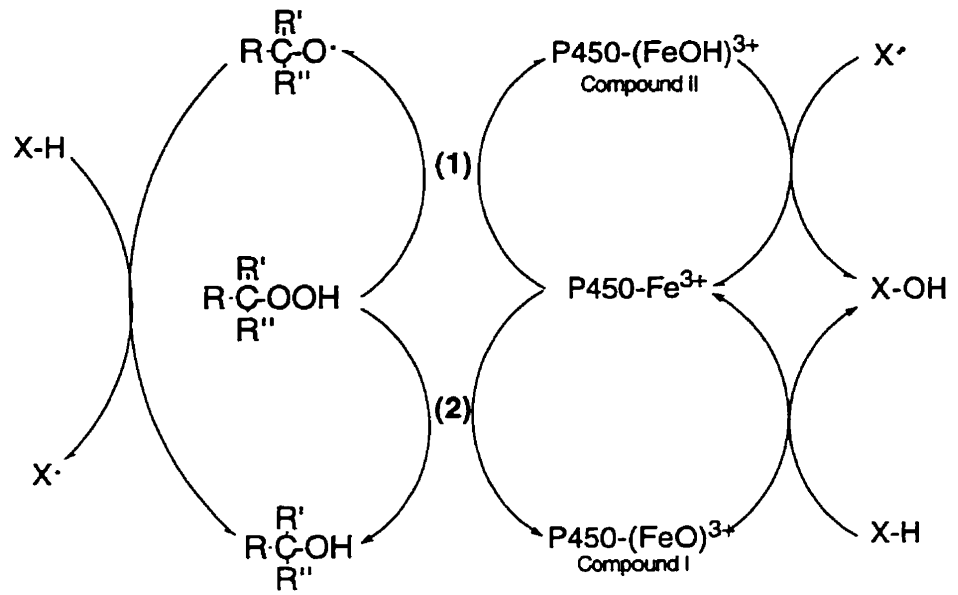
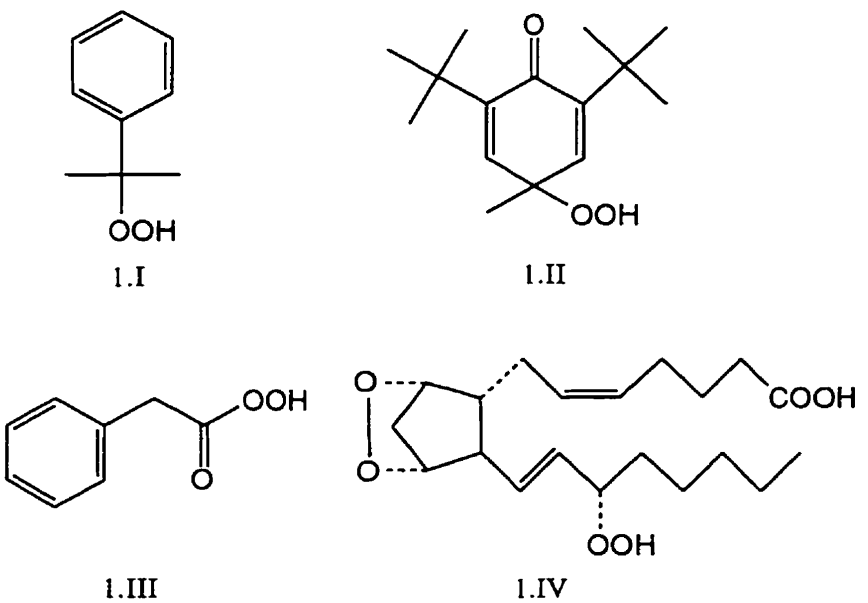


Figure 1-3: Schematic Diagram for the Catalytic Cycles of Microsomal Cytochrome P450 Peroxidase (1) and Peroxygenase (2)

The relative contributions of the homolytic and/or heterolytic pathways was found to depend on the chemical nature of the hydroperoxide used (Thompson and Yumibe, 1989). Traylor and colleagues (1989) have stressed the mechanistic unity of the hemin-catalyzed heterolytic cleavage of alkyl hydroperoxides, acyl hydroperoxides, hydrogen peroxide, and iodosobenzenes (Traylor *et al.*, 1989). This is the common mechanism of peroxidases and catalase, and cytochrome P450 appears to be unique among these enzyme systems in that it can act both heterolytically and homolytically (McCarthy and White, 1983a; McCarthy and White, 1983b). But only in the heterolytic cleavage mechanism can peroxides, iodosobenzenes and other oxidizing agents be considered to act as oxene donors. Among **alkyl**

hydroperoxides, the frequently used cumene hydroperoxide (Figure 1-4, 1.I) appears to be cleaved with slight predominance by the heterolytic pathway. In contrast 2,6-di-*tert*-butyl-4-hydroperoxy-4-methyl-2,5-cyclohexadienone (1.II) appears to react homolytically and heterolytically with comparable ease.



1.I 1.II 1.III 1.IV

Figure 1-4: Chemical Structural of Some of the Widely Studied Organic Hydroperoxides

Acyl hydroperoxides (peracids) such as phenylperacetic acid (1.III) act like alkyl hydroperoxides in supporting cytochrome P450-catalyzed hydroxylations, with the oxygen-oxygen cleavage being predominantly heterolytic (Bruice *et al.*, 1988; Traylor and Xu, 1990). **Hydrogen peroxide** can support cytochrome P450 N-demethylation reactions and aniline hydroxylation but not estradiol 2- and 4-hydroxylation (Bui and Weisz, 1988) at much higher concentrations than alkyl hydroperoxides (Estabrook *et al.*,

1984). The O-O cleavage is known to be predominantly heterolytic (Shimizu *et al.*, 1994). The unsaturated **fatty acid hydroperoxides**, e.g. prostaglandin endoperoxides like PGG_2 (1.IV), can also be metabolized by cytochrome P450 to allene oxides as precursors of prostaglandin-like products. The reaction is believed to involve a homolytic cleavage followed by hydrogen abstraction from the β-carbon and radical recombination (Song and Brash, 1991). Cytochromes P450 can catalyze a variety of novel transformations of fatty acid hydroperoxides presumably via radical reaction (Song *et al.*, 1993; Ullrich and Graf, 1984). Estrogens and retinoic acid can also be cooxidized by fatty acid hydroperoxides through the P450 peroxidase reaction (Muindi and Young, 1993; Roy *et al.*, 1992).

1.5.2.2. Fate of the Hydroperoxide Substrate

While the heterolytic pathway transforms a hydroperoxide solely to the corresponding alcohol, the alkoxyl radical generated by homolytic cleavage can react intramolecularly to give various rearrangement products. Thus, cumene hydroperoxide is transformed in part to acetophenone by β-scission (in this case loss of a CH3 radical) as shown in Figure 1-5, while the hydroperoxide 1.II and homologous 4-alkyl-1,4-peroxyquinols yield a variety of products resulting from a ß-scission, ring expansion and ring contraction (Thompson and Yumibe, 1989; Wand and Thompson, 1986; Yumibe and Thompson, 1988). In the heterolytic pathway, some hydroperoxides can serve

as both **oxygen donor and acceptor.** This is seen for example with 1.II, which following heterolytic cleavage is hydroxylated on the tert-butyl group, or in the metabolism of 20-hydroperoxycholesterol with CYP11A1 (Yumibe and Thompson, 1988).

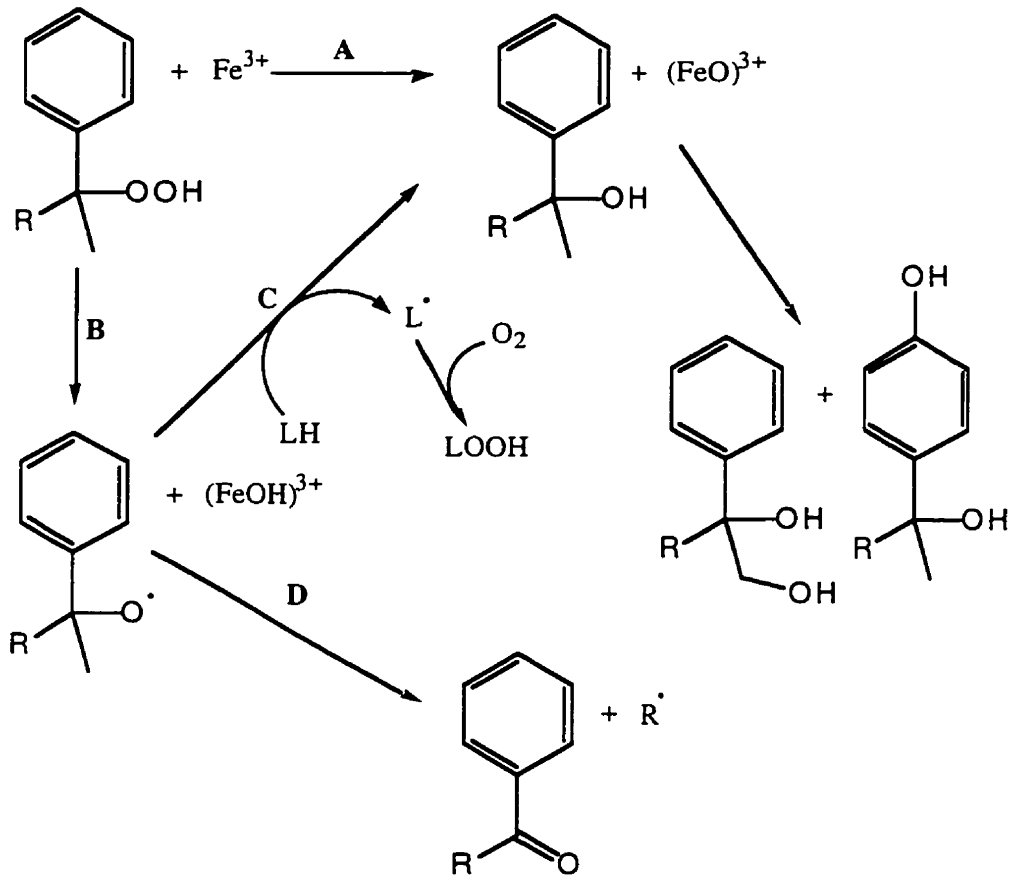


Figure 1-5: Summary of Reaction Pathways for the P450-Catalyzed Reduction of CumOOH (R=CH_3) and BzOOH (R=$C_6H_5CH_2$). Mechanisms **A-D** correspond to the heterolytic, homolytic, lipid hydrogen abstraction, and β-scission cleavage pathways of alkyl hydroperoxide metabolism respectively. Adopted from Thompson and Yumbi, 1989.

The interaction between cytochrome P450 and hydroperoxides, however, is not limited to mechanisms outlined in Table 1-4. In the presence of linoleate hydroperoxide, rat liver microsomes exhibited a **consumption of molecular oxygen** approximately 100 fold greater than that for usual monooxygenase reactions (Wheeler, 1983) which is associated with significant level of microsomal lipid peroxidation (Figure 1-5). Another reaction of probable physiological significance involves the **reductive cleavage** of lipid hydroperoxides and other hydroperoxides by cytochrome P450 in presence of NAD(P)H. This reaction, which was initially identified as the "cytochrome P450 NAD(P)H peroxidase" activity (Hrycay and O'Brien, 1973; Hrycay and O'Brien, 1974), is in fact a one electron reduction of hydroperoxide's dioxygen bound by cytochromes P450 as shown in Table 1-3 (Eq. 1-6).

1.5.2.3. Hydroperoxide-Supported P450-Catalyzed Xenobiotic Oxidations

Over the years, a large number of xenobiotics have been shown to be oxidized *in vitro* by peroxygenase activity of cytochrome P450. Representative examples covering a broad range of reactions, xenobiotic substrates and peroxides are assembled in Table 1-5, some of which warrant a brief discussion.

The **oxidation of aromatic substrates** by the hydroperoxide- and NADPH-dependent reactions proceed by similar mechanisms. Indeed,

Table 1-5: Representative Hydroperoxide-Dependent Oxidations Catalyzed by Cytochrome P450

Reactions/Substrate	ROOH	Enzyme	Reference
Aromatic Oxidation			
Acetanilide	CumOOH	Rat LM	Rahimtula *et al.*, 1978
		Rabbit LM	
		Pur. P450s	
Phenanthrene		Rat LM	
Aniline	H_2O_2	Rabbit LM	Mohr *et al.*, 1979
Benzo[a]pyrene	CumOOH	Rat LM	Capdevila *et al.*, 1980
Propranolol		Human LM	Otton *et al.*, 1990
Aliphatic Hydroxylation			
Cyclohexane		Pur. P450	Nordblom *et al.*, 1976
N-Dealkylation			
Benzphetamine	H_2O_2	Rabbit LM	Mohr *et al.*, 1979
		Pur. P450	Nordblom *et al.*, 1976
	Sodium chlorite		
	Peracids		
	Alkyl hydroperoxides		
Aminopyrine	CumOOH	Rat LM	Estabrook *et al.*, 1984
N-Methylaniline			
N,N-Dimethylaniline			
Diphenhydramine	H_2O_2		
Ethylmorphine			
Imipramine			
Ketamine			
Methamphetamine			
Mephentermine			
Morphine			
Phendimetrazine			
Pargyline			Weli & Lindeke, 1986

A blank means "same as above".
CumOOH: cumene hydroperoxide.
LM: liver microsomes
Pur.: Purified

Table 1-5: Continued.

O-Dealkylation			
para-Nitroanisole	Peracids Alkyl hydroperoxides Peroxyesters	Rabbit LM	Koop & Hollenberg, 1980
7-Isopropoxy-coumarin	H_2O_2 CumOOH	P450 cam	Shinohara *et al.*, 1987
C-Denitrification			
2-Nitropropane		Mouse LM	Marker & Kulkarni, 1986
N- Oxidation			
N,N-Dimethylaniline		Rabbit LM	Hlavica *et al.*, 1993
	H_2O_2	CYP2B4	
Pargyline		Rat LM	Weli & Lindeke, 1986
Felodipine	CumOOH		Baarnhielm *et al.*, 1986
Phenylalkylamines	H_2O_2		Jonsson & Lindeke, 1990
O-Oxidation			
Alkanols	CumOOH		Rahimtula & O'Brien, 1977b
Acetaminophen			Potter & Hinson, 1987

A blank means "same as above". LM: liver microsomes
CumOOH: cumene hydroperoxide. Pur.: Purified

comparable NIH shifts were seen in the hydroxylation of acetanilide, while the primary metabolite of phenanthrene was the 9,10-oxide (Rahimtula *et al.*, 1978). However, some differences in regioselectivity must occur since the major metabolites formed from benzo[a]pyrene were not the same; while the monooxygenase reaction yielded mainly phenols (3-, 6-and 9-hydroxy-BP) and less dihydrodiols and quinones, the hydroperoxide-dependent reaction produced mostly quinones (1,6-, 3,6- and 6,12-quinone) (Capdevila *et al.*, 1980). These quinones are known to be formed readily by enzymatic (peroxygenase

activity of cytochrome P450) or non-enzymatic oxidation of 6-hydroxy-BP; formation of the latter was demonstrated to occur via a radical cation mechanism (Cavalieri *et al.*, 1988).

The **N-dealkylations** listed in Table 1-5 are all N-demethylations apart from some reaction of pargyline. Indeed, this MAO inhibitor was N-demethylated, N-debenzylated, N-depropargylated, and N-oxygenated with comparable ease in the presence of microsomes and H_2O_2 (or cumene hydroperoxide) (Weli and Lindeke, 1986). Thus there are similarities in the types of N-C cleavage supported by hydroperoxides and NADPH. A further analogy is seen in the denitrification of 2-nitropropane to acetone and nitrite (Marker and Kulkarni, 1986). The fact that this reaction is supported by cumene hydroperoxide confirms a mechanism of C(α)- hydroxylation for the monooxygenase denitrification.

The reactions of **N-oxidation** reported in Table 1-5 include two tertiary amine (dimethylaniline and pargyline), a secondary amine of the 1,4-dihydropyridine type (felodipine) and primary phenylalkylamines. The peroxidatic N-oxidation of felodipine yields a pyridine derivative as does cytochrome P450 acting as a monooxygenase. As for the phenylalkylamines, different substrate selectivities were seen between the monooxygenase in peroxygenase N-oxygenations and the formation of cytochrome P450-nitroso complexes (Jonsson and Lindeke, 1990). This suggests that the presence of the

hydroperoxide can affect the three-dimensional structure of the substrate binding site.

The alkanols mentioned in Table 1-5 are ethanol and a few higher homologues; their hydroperoxide-dependent **O-oxidation** yielded the corresponding aldehyde (Rahimtula and O'Brien, 1977c). In contrast to its NADPH-dependent oxidation, the hydroperoxide-supported oxidation of acetaminophen was shown to be mainly a one-electron process yielding N-acetyl-p-benzosemiquinone imine as the metabolic intermediate; the latter then formed acetaminophen polymers (Potter and Hinson, 1987). Homolytic oxygen-oxygen cleavage presumably accounts for this one-electron oxidation.

1.2. NATURALLY OCCURRING HYDROPEROXIDES

1.2.1. Sources

Naturally occurring hydroperoxides are generated physiologically in many tissues through the action of prostaglandin H synthase (Boyd, 1990) or cellular lipoxygenase (Veldink and Vliegenthart, 1984) (Figure 1-6). Recent investigations indicate that reactive oxygen species and lipid hydroperoxides are continuously generated in healthy individuals, because 8-hydroxydeoxyguanosine and malondialdehyde:DNA adducts were identified as a major liver DNA metabolite/adducts *in vivo* (Chaudhary *et al.*, 1994). However, the level of cellular hydroperoxides can be markedly enhanced during the course of **oxidative stress**, the

phenomenon defined as a disturbance in the cellular prooxidant-antioxidant balance in favor of the former (Sies, 1985). Beside the formation of lipid and hydrogen peroxides during the course of oxidative stress, various cellular structural and biochemical elements, e.g. phospholipids, amino acids and nucleic acid bases may also be oxidized to form a wide range of endogenous hydroperoxides (Sies, 1985).

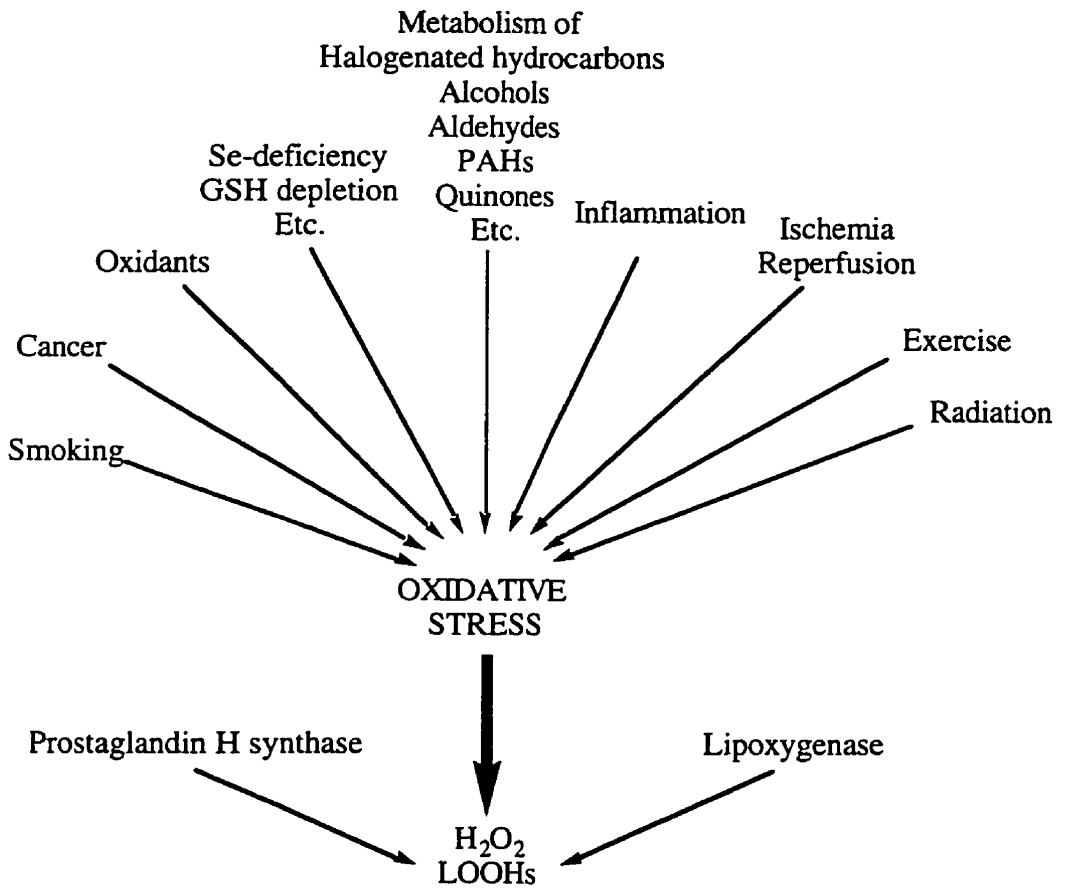


Figure 1-6: Mechanisms of Hydroperoxide Generation *In vivo*.

Among the fatty acid hydroperoxides formed during oxidative stress *in vivo*, arachidonic acid, linoleic acid and linolenic acid hydroperoxides were detected as the predominant products.

1.2.2. Oxidative Stress-Mediated Formation of Hydroperoxides

The molecular mechanism of cell injuries mediated by oxidative stress and its role in the pathogenesis of various diseases has been the focus of many recent investigations, and oxidative stress has been implicated in several diseases such as atherosclerosis, cancer, and rheumatoid arthritis, as well as in drug-associated toxicity, postischemic reoxygenation injury, and aging (Biasi *et al.*, 1994; Gotz *et al.*, 1994; Loughrey *et al.*, 1994; Sipe *et al.*, 1994; Toyokuni *et al.*, 1995).

Oxidative stress can be triggered under various pathological conditions (Figure 1-6) including radiation therapy, inflammation and activation of host immune response (Dargel, 1992), reperfusion injury (Zimmerman and Granger, 1994), malignant disease (Toyokuni *et al.*, 1995), deficiency of antioxidant defensive enzymes/cofactors and smoking (Sies, 1991). The metabolism of many drugs and chemicals such as halogenated hydrocarbons, aldehydes, alcohols, paraquat, nitrofurantoins, quinones, estrogens and adriamycin can also lead to extensive oxidative stress and hence markedly raise hydroperoxide levels in tissues (Dargel, 1992; Nordmann, 1994; Savard and Josephy, 1986; Sies, 1985; Sies, 1991). The metabolism of these chemicals is

generally carried out by the monooxygenase for mixed function oxidase system and form either radical species initiating extensive lipid peroxidation and oxygen activation, through a free radical chain reaction (Yamamoto *et al.*, 1990), or intermediate metabolites capable of activating oxygen by continued oxidation reduction (redox cycling) (Smith *et al.*, 1985).

1.2.3. Stability and Distribution

The oxidation of arachidonic acid through the physiological cyclooxygenase pathway generates potent prostaglandin endoperoxides, i.e. prostaglandin G_2 and prostaglandin H_2, which are rearranged to form various prostaglandins and thromboxanes. These cyclic endoperoxides are relatively stable in aqueous solution (half-life about 5 min) (Hamor, 1989) and can serve as cofactors for prostaglandin H synthase to cooxidize various substrates (Vuillaume, 1987). The hydrophobic nature of lipid hydroperoxides enable them to cross easily the cellular membrane barriers and diffuse to target sites far from the site of generation.

H_2O_2, a byproduct of aerobic respiration that is found at high concentrations in many tissues (20-55 μM), is also stable and could cross cell membranes and react directly or generate other oxidants close to cellular target site (Sies, 1991).

1.3. HYPOTHESIS AND OBJECTIVES

The fact that naturally occurring hydroperoxides can support the *in vitro* P450-catalyzed oxidation of xenobiotics suggests the functional significance of the hydroperoxide-supported P450-catalyzed xenobiotic metabolic activation in humans.

Since endogenous hydroperoxides, generated in many physiological and pathological situations in human tissues, are capable of supporting the microsomal P450-catalyzed oxidation reactions *in vitro*, we hypothesize that such reactions could also occur in intact cells or *in vivo* in tissues with substantial levels of P450 enzymes. The objective of this thesis was primarily to elucidate the functional significance of cytochrome P450 peroxygenase in the metabolic activation of xenobiotics by hydroperoxides, including physiological hydroperoxides, which will be addressed in chapters 3 and 4.

The biological significance of P450 peroxidase reactions that can potentially metabolize hydroperoxides to cytotoxic radical species has also not been studied in intact cells. The second objective of this thesis was to elucidate the role of P450 enzymes in hydroperoxide-induced oxidative stress in intact cells, which will be addressed in chapter 5.

Chapter 2

MATERIALS AND METHODS

2.1. Chemicals

Ethylene glycol-bis(β-aminoethyl ether) N,N,N'N'-tetraacetic acid, 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl, hydrogen peroxide, linolenic acid, linoleic acid, resorufin, methoxyresorufin, soybean lipoxygenase, 4-nitroanisole, 4-hydroxyanisole, 4-nitrophenol, hydroquinone, 4-ethoxyphenol, 4-propoxyphenol, phenanthrene, phenol, naphthalene, 1-naphthol, bromobenzene, reduced glutathione, salicylamide, Trypan Blue, chlorzoxazone, α-naphthoflavone, antimycin A, thiobarbituric acid, digitonin, fluoro-2,4-dinitrobenzene, iodoacetic acid, sodium azide, and cumene hydroperoxide were obtained from Sigma Chemical Co. (St. Louis, MO).

1-Bromoheptane, N,N'-diphenyl-1,4-phenylenediamine (DPPD), bathophenanthroline disulfonate, oxidized glutathione, tert-butylhydroperoxide, 3-methylcholanthrene, *p*-nitrophenol, SKF-525A (2-dimethylaminoethyl-2,2-diphenyl-n-pentanoate), and metyrapone were obtained from Aldrich Chemical Company Inc. (Milwaukee, WI). Collagenase (from *Clostridium histolyticum*), 4-(2-hydroxyethyl)-1-piperazine

ethanesulfonic acid (Hepes), and bovine serum albumin were obtained from Boehringer-Mannheim (Montreal, PQ).

Goat anti-rat P450 2E1 serum (catalog number 219221) was purchased from Gentest Corporation (Woburn, MA). 2-amino-3-methylimidazo[4,5f]quinoline (IQ) [67730-10-3] and 2-nitro-3-methylimidazo[4,5-f]quinoline (nitro-IQ) were obtained from Toronto Research Chemicals (Downsview, ON). Desferoxamine was a gift from Ciba Geigy Canada Ltd. (Toronto, ON). HPLC grade solvents were obtained from Caledon (Georgetown, ON).

2.2. Animal Treatment and Hepatocyte Preparation

Adult male Sprague-Dawley rats, 250-300g, were obtained from Charles River Canada Laboratories (Montreal, PQ), fed *ad lib.* and were allowed to acclimatize for one week on clay chip bedding. Freshly isolated hepatocytes were chosen as the intact cell model in this study as their high P450 levels make them suitable for both toxicology and drug metabolism studies (Berry et al., 1992, Moldeus, 1978). 3-Methylcholanthrene (3MC)-treated hepatocytes were prepared from rats pretreated daily by 3MC (25 mg/kg body wt in oil, ip) for three days.

The hepatocytes were prepared by collagenase perfusion of the liver as described by Moldeus (Moldeus *et al.*, 1978). Damaged cells, debris, and Kupffer cells were removed by centrifugation with Percoll (45% Percoll, 10

min centrifugation at 50 X g)(Kreamer *et al.*, 1986). The cells were preincubated in Krebs-Henseleit bicarbonate buffer [NaCl (13.9 g), KCl (0.71 g), KH_2PO_4 (0.32 g), $MgSO_4.7H_2O$ (0.59 g), and $CaCl_2.2H_2O$ (0.76 g), $NaHCO_3$ (4.2 g) and Hepes (6.0 g) dissolved in 1 L of deionized water and adjusted to pH 7.4] supplemented with 12.5 mM Hepes for 30 min in an atmosphere of 1% O_2/5% CO_2/94% N_2 in continuously rotating 50 mL round bottom flasks at 37° C before the addition of chemicals. Stock solutions of chemicals were made either in incubation buffer or in methanol (maximum 0.1% which had no significant effect on cell viability or the assays) and added to hepatocyte suspensions 10 min before CumOOH addition unless indicated. GSH-depleted hepatocytes were obtained by 20 min preincubation of normal hepatocytes with 200 μM of 1-bromoheptane, which resulted in depletion of 95% of total hepatocyte GSH, as described previously (Khan and O'Brien, 1991). With this method hepatocyte cytosolic and mitochondrial GSH are depleted as a result of GSH conjugation without causing cytotoxicity even at concentrations as high as 4 mM bromoheptane. The tert-butylhydroperoxide (tBHP) concentrations used as a non-toxic peroxidant cofactor in Chapter 3 did not affect hepatocyte viability. Nevertheless, desferoxamine, a ferric chelator was added (500 μM, -30 min) to all tBHP-treated groups to prevent possible hydroperoxide-induced iron mediated cytotoxicity (Minotti and Aust, 1992) although similar results were obtained in the absence of desferoxamine.

2.3. Cell Viability

The viability of the hepatocytes was assessed by plasma membrane disruption as determined by the Trypan Blue (0.2% w/v) exclusion test (Moldeus *et al.*, 1978). Dead cells were indicated with the appearance of dark blue stained nuclei after Trypan Blue addition. The blue staining of the nuclei is the result of damage to the plasma membrane, a prime indicator of cell death. Viability was examined at different time points during the incubation up to 4 hours and the cells were at least 90% viable before use.

2.4. Microsomal Preparations

Adult male Sprague-Dawley rats, 250-300g, were obtained from Charles River Canada Laboratories (Montreal, PQ), fed *ad lib.*, and were allowed to acclimatize for one week on clay chip bedding. Animals were pretreated by daily injection of 3-MC (25 mg/kg body wt in oil, ip) for three days. The animals were anesthetized by sodium pentobarbital (60 mg/kg body wt) on day four and livers were removed under sterile conditions and perfused with KCl solution (1.18% w/v, 4° C). Hepatic microsomes were prepared under sterile conditions by differential centrifugation as previously described (Dallner, 1978). The microsomal pellet was suspended and homogenized in sterile potassium phosphate buffer:KCl solution [50 mM KH_2PO_4 and 0.23% (w/v) KCl, pH 7.4] and was stored at -80° C. Microsomes containing human P450 1A2 (hP4501A2) were obtained from Gentest Corporation (Woburn, MA)

and had been prepared from the human AHH-1 TK+/- cell line expressing human P450 1A2 cDNA. hP4501A2 was sterilized by gamma radiation (680 Gy) for 2 h which had no effect on P450 1A2 enzymatic activity. The total cytochrome P450 content was 880 and 41 pmol P450/mg protein for the rat 3-MC-induced liver microsomes (rP4501A) and hP4501A2, respectively. The ethoxyresorufin O-deethylation activities of rP4501A and hP4501A2 were 12,500 and 155 pmol resorufin/min/mg microsomal protein respectively.

2.5. Enzymatic Assays

Microsomal protein was determined by the method of Lowry *et al.*, (1951) and total cytochrome P450 content was assayed as described by Omura and Sato (1964).

Methoxyresorufin O-demethylation (MROD), an enzymatic probe for the P450 1A2 activity (Yang *et al.*, 1988), was determined as follows: Briefly, microsomes were diluted with 0.1 M potassium phosphate buffer (pH 7.4), to a concentration of 50 µg/mL which provided a linear rate of reaction for at least 10 minutes. Methoxyresorufin in DMSO was then added (0.5 µM final concentration in the cuvette) and the mixture was incubated at 37° C for 3 minutes. A stable baseline was established and the reaction was initiated by the addition of various concentrations of hydroperoxides. Due to the high microsomal associated catalase activity, the catalase inhibitor, sodium azide (1 mM) was added to the incubation mixture just before hydrogen peroxide

addition. The progressive increase of resorufin fluorescence was recorded using a Shimadzu RF5000U spectrofluorophotometer with excitation and emission wavelengths of 530 and 586 nm, respectively. The fluorometer was calibrated by 100 pmol resorufin in each experiment and the Km for each hydroperoxide was obtained by using Lineweaver-Burk plot of the reciprocal rate of reaction (1/V) versus reciprocal hydroperoxide concentration (Cornish-Bowden, 1979).

The NADPH-supported ***p*-nitrophenol hydroxylase** (PNPH) assay was used as an enzyme probe of P450 2E1 activity and determined as described by Koop (Koop, 1986).

2.6. Mutagenicity Assays

The Ames mutagenicity assay has been widely used for the screening of mutagenic metabolites of xenobiotics and was chosen in this study to elucidate the potential formation of IQ mutagenic metabolites through the P450 peroxygenase pathway. The *Salmonella typhimurium* YG1012 strain (TA1538 1,8-DNP pYG213) was graciously provided by Dr. M. Watanabe, National Institute of Hygienic Sciences, Tokyo, Japan. This strain expresses an elevated level of acetyl-CoA: arylamine N-acetyltransferase (NAT) and has higher sensitivity for detection of mutagenic nitroarenes and aromatic amines (Morrison *et al.*, 1993; Watanabe *et al.*, 1990). The P450-catalyzed mutagenicity assay was performed according to the modified Ames

mutagenicity assays as described previously for the prostaglandin-H synthase bioactivation system (Josephy *et al.*, 1989; Petry *et al.*, 1988; Petry *et al.*, 1989) except that arachidonic acid and ram seminal vesicle microsomes were replaced by hydroperoxides and rP4501A or hP4501A2 respectively. In brief, IQ, microsomes, and bacteria were preincubated in 0.5 ml of KH_2PO_4 buffer (100 mM, pH 7.4) at 37° C for 2 min and then incubated for additional 30 min following hydroperoxide addition. The mixture was plated on minimal glucose medium and incubated at 37° C for 72 h prior to scoring of His^+ revertant colonies with "Optimas" Microsoft Windows-based video image analysis system.

2.7. Fatty Acid Hydroperoxides Preparation

Linoleic and linolenic acid hydroperoxide were prepared by the enzymatic peroxygenation of the corresponding fatty acid by soybean lipoxidase and were further purified as described (Teng and Smith, 1985). The peroxidase activity for each fatty acid hydroperoxide was determined by measuring the rate of oxidation of tetra-methylphenylenediamine as described previously (Hrycay and O'Brien, 1971a). A rate of 8.3 and 2.54 μmol oxidized tetra-methylphenylenediamine/min/mg protein for linoleic and linolenic acid hydroperoxides was found respectively.

2.8. HPLC Analysis of Hepatocyte and Microsomal 4-HA Metabolites

The microsomal reaction mixture contained 1 mg microsomal protein/mL, desferoxamine (500 μM), 4-HA (1 mM), glutathione (5 mM) and NADPH (1 mM) or tBHP (2 mM) in a total volume of 2 mL. Incubations were carried out at 37 °C for various lengths of time and stopped by the addition of hydrochloric acid (3 M final concentration for characterizing GSH conjugates) or metaphosphoric acid (12% w/v final concentration for determining GSH levels). Samples were centrifuged at 15,000g for 5 min to pellet the proteins, and the supernatants were then directly assayed for metabolite formation by LC/MS or HPLC.

Hepatocytes (5×10^6 cells/mL) were preincubated for 30 min in Krebs-Henseleit bicarbonate-Hepes buffer at 37° C in the presence of desferoxamine (500 μM) as described earlier. Phenylimidazole (300 μM) or equal volume of vehicle (methanol 0.1%) was added 10 min before the addition of 4-HA (1 mM) and tBHP (500 μM). 2 mL of the incubation mixture was taken at 30 min and mixed with 3 mL trichloroacetic acid (20% w/v aqueous) on ice. The supernatants were separated by centrifugation and 4-HA metabolites were analyzed by a reverse phase HPLC system (Shimadzu SCL-6B system and LC-6A pump) equipped with Suppelco 250 mm × 4.6 mm Suppelcosil LC-18 column. The samples (5 μL) were eluated by a mobile phase (flow rate 1.0 mL/min) consisting of 85/15 (v/v) acetate buffer (100 mM, pH 4.8)/methanol and detected by a ESA 5200A Coulochem® II electrochemical detector with a

model 5010 analytical cell. On the detector, the guard cell potentiostat was set at +550 mV and both oxidative and reductive currents were monitored. The first cell potentiostat was set at +500 mV with the output of +1.0 V, a gain range of 5 μA and a filter of 2 s and the second potentiostat was set at -300 mV with the output of -1.0 V. The current setting enabled the selective detection of the hydroquinone and benzoquinone metabolites with the t_R of 3.1 and 5.4 min, respectively.

2.9. LC/MS Analysis of 4-HA Metabolite:GSH Conjugates

For the LC/MS detection of the GSH conjugates, the aqueous supernatants of the microsomal incubations were extracted with acetonitrile and concentrated under a stream of nitrogen at 55 °C. The sample was redissolved in water and analyzed by LC/MS using selective ion monitoring (SIM) or LC/MS/MS at a constant flow rate of 0.2 mL/min, of which 20 μL/min entered the mass spectrometer. Narrow-bore C18 Phenomenex columns (Torrance, CA) packed with 5 μm i.d. Ultracarb ODS 30 were used because this packing does not require triethylamine to prevent tailing of amines and triethylamine severely inhibits ionization in the mass spectral analysis. The dimensions of the columns used were 2 × 100 mm with a 2 × 30 mm guard column. A mobile phase of water:acetonitrile:acetic acid (15:85:1, v/v) including 1 mM ammonium acetate was used.

Mass spectrometry and combined liquid chromatography-tandem mass spectrometry (LC/MS and LC-MS/MS) were carried out on a Sciex API III triple quadrupole mass spectrometer (Perkin-Elmer Sciex, Thornhill, Ontario) equipped with an IonSpray interface. Analyses were performed with an ionizing voltage of 5 kV, and high-purity air was used as the nebulizing gas at operating pressure of 40 psi. The orifice voltage was 70 V. Collisional-induced dissociation (CID) of selected precursor ions was performed in the rf-only quadrupole region and employed argon as target gas.

2.10. LC/MS Analysis of IQ Metabolite:2'-Deoxyguanosine Conjugates

For identification of the deoxyguanosine:IQ metabolite adducts, a reaction mixture containing 3-MC-induced microsomes (0.2 mg/mL), 2'-deoxyguanosine (5 mM), and IQ (1 mM) in potassium phosphate buffer (pH 7.4) was preincubated for 2 min at 37 °C. Hydroperoxides (5 mM) were added in four equal portions during the 30 min incubation and the reaction stopped by the addition of equal volume of acetonitrile. Samples were centrifuged at 15,000g for 10 min to pellet the proteins. The supernatants were concentrated by an organic solvent extraction under a stream of nitrogen at room temperature. The residuals were redissolved in 100 µL of 50% (v/v) water:methanol and analyzed by LC/MS using selective ion monitoring (SIM) as previously described in section 2.9. (Anari *et al.*, 1995b). Briefly, narrow-bore C18 Phenomenex columns (Torrance, CA) packed with 5 µm i.d.

Ultracarb ODS 30 were used because this packing does not require triethylamine to prevent tailing of amines and triethylamine severely inhibits ionization in the mass spectrometer. The dimensions of the columns used were 2 × 100 mm with a 2 × 30 mm guard column. A mobile phase of acetonitrile:water:acetic acid (30:70:1, v/v) including 1 mM ammonium acetate was used.

2.11. Antimycin A-Resistant Respiration and Lipid Peroxidation

Hepatocyte mitochondrial respiration was inhibited by the addition of antimycin A (25 μM) and the rate of oxygen uptake was determined with a Clark-type oxygen electrode (Model 5300; Yellow-spring Instrument Co., Inc., Yellow Springs, OH, USA) following the addition of CumOOH to hepatocytes (10^6 cells/mL) maintained in a 2-mL incubation chamber at 37° C. Inhibitors were added after the baseline of the antimycin A resistant respiration had stabilized. Hepatocyte lipid peroxidation was determined by measuring the amount of thiobarbituric acid reactive substances (TBARS), i.e. the lipid hydroperoxide decomposition products, spectrophotometrically (Beckman DU®-7 spectrophotometer) at 532 nm after treating aliquots of hepatocyte suspension with trichloroacetic acid (7.5% w/v) and boiling the supernatant with thiobarbituric acid (0.27% w/v) for 20 min (Smith *et al.*, 1982).

The hepatic microsomal CumOOH-induced lipid peroxidation was determined in a reaction mixture containing 0.2 mg of microsomal

protein/mL, 150 μM CumOOH, 10 μl of 100-fold stock solutions of P450 inhibitors in water or methanol and 0.1 mM potassium phosphate buffer (pH 7.4) in a total volume of 1 mL. The P450 inhibitors were first preincubated with microsomal suspensions for 2 min at 37 °C before the addition of CumOOH or *p*-nitrophenol.

2.12. Determination of Hydroperoxide Metabolism

The rate of hepatocyte CumOOH or tBHP decomposition was measured by the spectrophotometric analysis of the ferrithiocyanate complex at 490 nm formed by the oxidation of ferrous thiocyanate as previously reported (Thurman *et al.*, 1972). The linear regression analyses were indicative of linearity of metabolism over 5 min incubation as the correlation coefficients (r) of all curves were above 0.995.

2.13. Determination of Hepatocyte Iron Release

The intracellular free ferrous ion concentration in hepatocytes was determined by bathophenanthroline as described previously (Brumby and Massey, 1967). Aliquots of hepatocytes were taken at different time points and were filtered through 0.45 μm membrane filters after addition of 0.3% digitonin to permeabilize the plasma membrane. Bathophenanthroline disulfonate (1 mM) was added to 1 mL of filtrate and the absorbance of the pink complex was measured at 537 nm.

2.14. HPLC Analysis of Hepatocyte GSH/GSSG

The total amounts of GSH and GSSG in isolated hepatocytes were measured by the HPLC analysis of deproteinized samples (5% meta phosphoric acid) after derivatization with iodoacetic acid and fluoro-2,4-dinitrobenzene (Reed *et. al.*, 1980). Briefly, 800 µl of hepatocyte suspensions were mixed with 200 µl of 25% (w/v) metaphosphoric acid and the mixture was centrifuged at 50g for 5 min. A tipful quantity of solid $NaHCO_3$ (approximately 50 mg) was placed in a test tube and 500 µl of the supernatant and 50 µl of iodoacetic acid (15 mg/ml H_2O) were simultaneously added to solid sodium bicarbonate. After vortexing, the mixture was kept in the dark for 1 hour. To the mixture, 500 µl of ethanolic (100%) fluoro-2,4-dinitrobenzene (1.5% v/v) was added and the mixture was kept for 4 hours at room temperature in the dark. After centrifugation (50g for 4 min), 200 µl of supernatant was used for GSH/GSSG analysis in a Waters HPLC system (model 510 pumps, WISP 710B auto injector, and model 410 UV/vis detector) equipped with a Waters µ Bondapak® NH_2 (10 µm) 3.9 × 300 mm column. The mobile phase consisted of two eluants: Buffer A (80% v/v methanol in water, and Buffer B (80% v/v Buffer A in Stock acetate buffer). Stock acetate buffer consisted of 122 ml H_2O, 378 ml acetic acid, and 272 g sodium acetate (trihydrate). The flow rate was adjusted to 1.5 ml/min with the following linear gradient programming: initial condition at 90% buffer A, 20 min at up to 90% buffer B, 2 min at up to 90% buffer A, and hold for 5 min. The

detection was performed at 365 nm and under the condition described, the GSH and GSSG eluated at 12.5 and 15 min, respectively.

2.15. HPLC Analysis of Hepatocyte ATP

Intracellular ATP in hepatocytes was extracted using an alkaline extraction procedure and was analyzed by a reverse phase HPLC (Stocchi *et al.*, 1984), using Shimadzu SCL-6B, LC-6A pump, SPD-6AV detector system equipped with a Waters μ Bondapak® C18 (10μm) 3.9 × 300 mm column. The mobile phase consisted of two eluants: Buffer A (0.1 M KH_2PO_4 solution, pH 6), and Buffer B (10% v/v of methanol in Buffer A). The flow rate was adjusted to 1.3 ml/min with the following linear gradient programming: 9 min at 100% buffer A, 6 min at up to 25% of buffer B, 2 min at up to 100% of buffer B, and hold for 6 min. The detection was performed at 254 nm and under the condition described, the ATP peak eluated at 6.5 min.

2.16. P450 2E1 Inhibition Studies

In the inhibition study with anti-rat P450 2E1 serum, 50 μL of antiserum (115.6 mg protein/mL) were added instead of P450 inhibitors and the microsomal suspensions were preincubated at room temperature for 30 min before the addition of CumOOH or *p*-nitrophenol. Incubations were carried out at 37° C for 30 min and stopped by the addition of trichloroacetic acid (7.5% w/v). The addition of thiobarbituric acid and analysis of

colorimetric TBARS was carried out as described for hepatocytes (Smith *et al.*, 1982).

2.17. Spectral Analyses of Hepatocyte Quinone-GSH Conjugates

Quinone- GSH conjugates formed during the hydroperoxide supported 4-hydroxyanisole metabolism in hepatocytes were analyzed by monitoring the UV/vis absorption in the range of 310-360 nm (differential absorbance of 350 nm vs 500 nm) recorded at timed intervals as previously described (Eckert *et al.*, 1990; Lau *et al.*, 1989). The SLM DW-2000™ UV/vis spectrophotometer equipped with a controlled temperature stirring device was used for spectral analysis of hepatocyte 4-HA metabolites.

2.18. Hepatocyte 4-Nitroanisole O-Demethylation Assay

Non conjugated 4-nitrophenol formed by the hepatocyte metabolism of 4-NA (0.5 mM) was assayed as described by Brown and Bidlack (1991). Hepatocytes were preincubated with salicylamide (2 mM, -10 min) to prevent any sulfation and glucuronidation of the 4-nitrophenol metabolite (Burke and Orrenius, 1978). Briefly, hepatocytes (1 ml) taken at different time points were mixed with 0.5 ml of perchloric acid (0.6 N) and kept on ice for 15 min to terminate the reaction and allow the complete precipitation of proteins. The mixture was centrifuged and the clear supernatants (1 ml) were mixed with NaOH (100 µl of 10 N). The nitrophenolate yellow color developed

immediately which was assayed spectrophotometrically at 400 nm within 5 min.

2.19. Statistical Analysis

One- and two-way ANOVA followed by the Scheffe's test were used for comparison amongst the multiple-treated groups and the relevant controls. Results represent the mean ± standard error of the mean of triplicate samples.

Chapter 3

CYTOCHROME P450 PEROXIDASE/PEROXYGENASE MEDIATED XENOBIOTIC METABOLIC ACTIVATION AND CYTOTOXICITY IN ISOLATED HEPATOCYTES*

3.1. ABSTRACT

Cytochrome P450 (P450) can utilize organic hydroperoxides and peracids to support hydroxylation and dealkylation of various P450 substrates. However the biological significance of this P450 peroxygenase/peroxidase activity in the bioactivation of xenobiotics in intact cells has not been demonstrated. We have shown that t-butylhydroperoxide (tBHP) markedly enhances 3-20-fold the cytotoxicity of various aromatic hydrocarbons and their phenolic metabolites. The tBHP-enhanced hepatocyte cytotoxicity of 4-nitroanisole (4-NA) and 4-hydroxyanisole (4-HA) was also accompanied by an increase in the hepatocyte O-demethylation of 4-NA and 4-HA up to 7.5- and 21-fold, respectively. Hepatocyte GSH conjugation by 4-HA was also markedly increased by tBHP. A LC/MS analysis of the GSH conjugates identified hydroquinone:GSH and 4-methoxycatechol:GSH conjugates as the

* Reprinted with permission from M. Reza Anari, S. Khan, Zhao Chao Liu and P. J. O'Brien (1995) Cytochrome P450 peroxidase/peroxygenase mediated xenobiotic metabolic activation and cytotoxicity in isolated hepatocytes. *Chemical Research in Toxicology* **8**, 997-1004. Copyright 1995 American Chemical Society.

predominant adducts. Pretreatment of hepatocytes with P450 inhibitors, e.g. phenylimidazole, prevented tBHP-enhanced 4-HA metabolism, GSH depletion and cytotoxicity. In conclusion, hydroperoxides can therefore be used by intact cells to support the bioactivation of xenobiotics through the P450 peroxidase/peroxygenase system.

3.2. INTRODUCTION

Cytochrome P450 catalyzes the oxidation of various xenobiotics to cytotoxic, mutagenic, teratogenic and carcinogenic reactive metabolites (Gonzalez and Gelboin, 1994; Guengerich, 1988). By introducing one atom of molecular oxygen to substrates and reducing the other oxygen atom to water, P450 isoforms catalyze the "monooxygenase" hydroxylation and dealkylation of various exogenous and endogenous substrates (Guengerich and Shimada, 1991). The monooxygenase function requires molecular oxygen and a reductant (NADPH through NADPH:cytochrome P450 reductase) to catalyze oxidation reactions efficiently (White and Coon, 1980). However, P450s can catalyze the oxidation of various substrates independent of molecular oxygen, NADPH and cytochrome P450 reductase if organic or lipid hydroperoxides are used as the oxidant source (O'Brien, 1978). The hydroperoxide specificity for the peroxidase activity of cytochrome P450 is broader than that of the peroxidase enzymes and includes lipid hydroperoxides (Hrycay and O'Brien, 1971a), tertiary organic hydroperoxides (Hrycay and O'Brien, 1972), steroid hydroperoxides (Hrycay and O'Brien, 1972), and to a lesser extent hydrogen

peroxide (Hrycay and O'Brien, 1971b). The P450 peroxygenase/peroxidase activity supports aromatic and aliphatic hydroxylations (Hrycay *et al.*, 1976; Rahimtula and O'Brien, 1974), N- and O-dealkylation (Kadlubar *et al.*, 1973; Kedderis *et al.*, 1983; Rahimtula and O'Brien, 1975), N-hydroxylation (Hlavica *et al.*, 1983), alcohol oxidation (Rahimtula and O'Brien, 1977a) and arene epoxidation (Rahimtula *et al.*, 1978). The xenobiotic metabolites formed are usually similar to those formed by the NADPH/O_2-supported mixed function oxidase (Burke and Mayer, 1975; Capdevila *et al.*, 1980; O'Brien, 1978).

Studies of hydroperoxide-supported oxidation reactions have provided useful information regarding the mechanisms by which P450 activates oxygen and forms dioxygen P450 species (Guengerich and MacDonald, 1990). Recent findings regarding the physiological function of P450 peroxidase/peroxygenase in the oxidative metabolism of endobiotics in plants (Song *et al.*, 1993), platelets (Haurand and Ullrich, 1985) and endothelial cells (Pereira *et al.*, 1993; Ullrich and Graf, 1984) suggest that P450 may also contribute in the metabolism of xenobiotics in the presence of endogenously generated hydroperoxides. In spite of the numerous studies characterizing the P450 peroxygenase activity in isolated hepatic microsomes (O'Brien, 1978), the ability of P450 to catalyze hydroperoxide-dependent xenobiotic metabolism in intact cells, and thereby modulate xenobiotic cytotoxicity, has yet to be investigated. The present study was conducted to determine if P450 in intact cells can exert enough peroxygenase activity to modulate the metabolism and

cytotoxicity of P450 substrates. It was found that non-toxic concentrations of tBHP enhanced the P450-dependent bioactivation of xenobiotics to such an extent that concentrations of hepatotoxins normally not toxic to isolated hepatocytes became toxic. This enabled cytotoxic mechanisms and the reactive intermediates or cytochrome P450 isoforms involved to be more easily identified.

3.3. RESULTS

3.3.1. tBHP-Enhanced Xenobiotic Metabolism in Hepatocytes

The 4-NA O-demethylation to 4-nitrophenol in the presence and absence of tBHP (150 μM) was assayed in isolated hepatocytes (Figure 3-1). The O-demethylation rate in the presence of tBHP (21 μmol/10 min/10^6 cells) was 22-fold faster than in the absence of tBHP. The hydroperoxide-supported 4-NA O-demethylation reached a plateau after 60 min and the total metabolite formed (13.5 μmol/10^6 cells) was 7-fold more than the cellular monooxygenase rate which reached a plateau in 30 min. The tBHP/4-NA cotreated hepatocytes were still more than 70% viable after 60 min of incubation which indicates the 4-nitrophenol's plateau at 60 min was not due to cell death. As shown in Figure 3-1, phenylimidazole (300 μM, -10 min), a potent type II P450 inhibitor (Testa and Jenner, 1981), prevented the tBHP-enhanced metabolism of 4-NA to 4-nitrophenol (84% inhibition of total metabolite at 60 min, $P<0.01$).

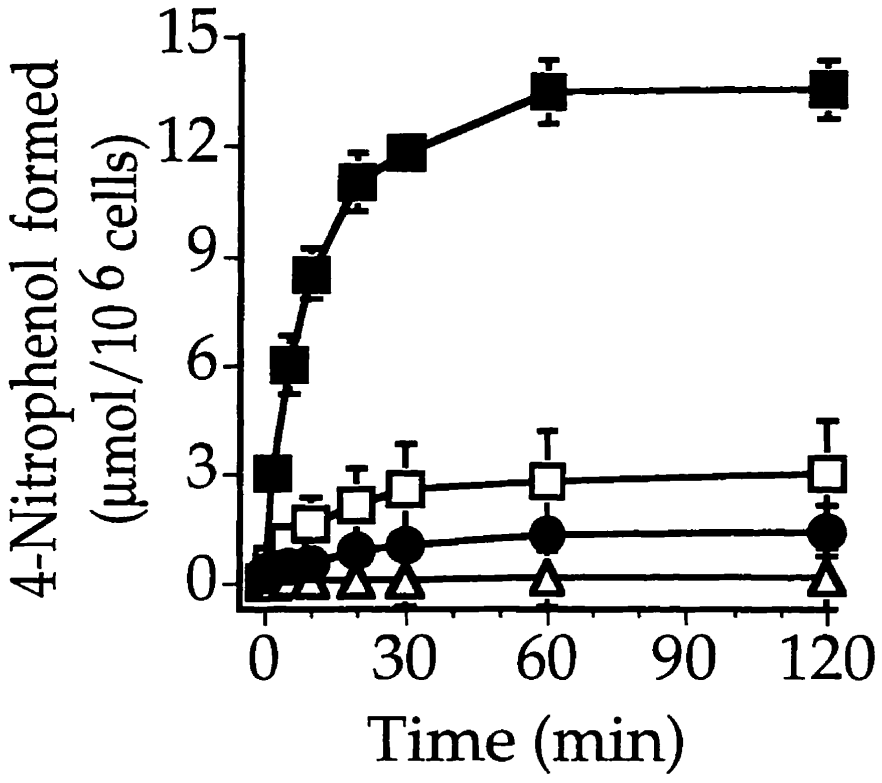


Figure 3-1: Hydroperoxide-Enhanced 4-Nitroanisole O-Demethylation in Isolated Rat Hepatocytes. Hepatocytes (3×10^6 cells/mL) were incubated with tBHP (150 µM) (Δ), 4-nitroanisole (500 µM) (●), 4-nitroanisole + tBHP (■), and with phenylimidazole (300 µM) + 4-nitroanisole + tBHP (❑). Non conjugated 4-nitrophenol was determined by the spectrophotometric analysis as described in section 2.18. Values represent mean ± SE of three separate experiments.

Table 3-1: Hydroperoxide-Enhanced O-Demethylation of 4-Hydroxyanisole in Isolated Rat Hepatocytes[a]

Treatment	O-demethylated metabolites (nanomol/10^6 cells/30 min)
Control	N.D.[b]
4-HA	2.2 ± 0.9[c]
" + t-BHP	38.3 ± 4.2[d]
" + " + Phenylimidazole	3.7 ± 0.8[e]

[a] Hepatocytes (5×10^6 cells/mL) were preincubated for 30 min in Krebs-Henseleit bicarbonate-Hepes buffer at 37 °C in the presence of desferoxamine (500 μM). Phenylimidazole (300 μM) or equal volume of vehicle (methanol 0.1%) was added 10 min before the addition of 4-HA (1 mM) and tBHP (500 μM). The deproteinized samples were used for the HPLC analysis of hydroquinone and benzoquinone metabolites as described in section 2.8. Data represent mean ± SE of three separate experiments. [b] Not detected. [c] Hydroquinone was the major metabolite.

[d] Benzoquinone was the major metabolite. $P<0.01$ compared with 4-HA.

[e] $P<0.01$ compared with 4-HA+tBHP treated group and $P>0.05$ compared with 4-HA treated group.

The 4-HA O-demethylation to hydroquinone and/or its oxidized form benzoquinone in the presence and absence of tBHP was assayed in isolated hepatocytes (Table 3-1). The total amount of O-demethylated metabolites formed in 30 min was 21 times more in the presence of tBHP. Benzoquinone was the main metabolite formed in the presence of tBHP whereas hydroquinone was the main metabolite formed in the absence of tBHP.

The rate of metabolism of tBHP by hepatocytes was assayed in the presence and absence of 4-NA (Figure 3-2) to determine if the hydroperoxide-supported metabolism of 4-NA correlated with tBHP metabolism. tBHP was rapidly metabolized by hepatocytes and 40 and 20 percent of tBHP remained after 10 min incubation in the presence and absence of 4-NA respectively. The slower rate of tBHP metabolism in the presence of 4-NA suggests that part of the hydroperoxide decomposition was P450 dependent (O'Brien, 1978). In addition the rate of tBHP disappearance appears to correlate with the rate of hepatocyte tBHP-supported 4-NA O-demethylation to 4-nitrophenol (Figure 3-1).

3.3.2. tBHP-Enhanced Xenobiotic Cytotoxicity

The cytotoxicity of 4-NA towards isolated hepatocytes was assayed in the presence and absence of tBHP (Figure 3-3). 4-NA alone at 0.5 mM was slightly cytotoxic towards isolated hepatocytes (15% loss of cell viability after 2 h, $P>0.05$ compared to control), however addition of a non-cytotoxic concentration of tBHP (150 μM) markedly increased 4-NA mediated hepatocyte toxicity (100% loss of cell viability after 2 h). Furthermore, cytochrome P450 inhibitors, e.g. phenylimidazole (300 μM), prevented the tBHP-enhanced 4-NA cytotoxicity (25% loss of viability at 2 h, $P<0.05$ compared to tBHP/4-HA cotreated group).

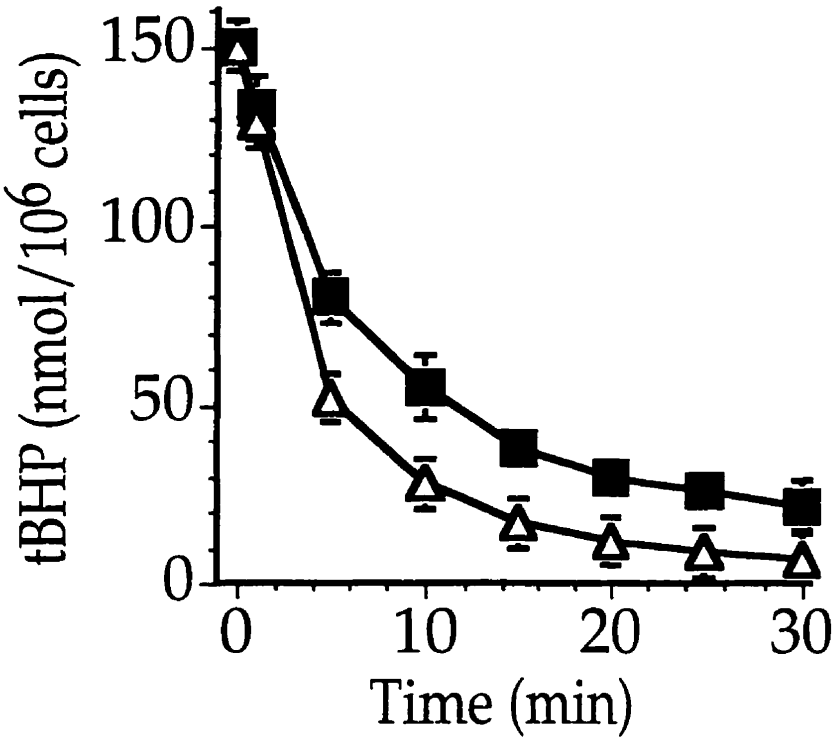


Figure 3-2: Hepatocyte Metabolism of tBHP alone and in the Presence of 4-NA. Hepatocytes (3×10^6 cells/mL) were incubated with tBHP (150 μM) (Δ) or 4-nitroanisole + tBHP (■) and the time-dependent hydroperoxide consumption was determined by the spectrophotometric analysis of ferrithiocyanate complex at 490 nm as described in section 2.12. Values represent mean ± SE of three separate experiments.

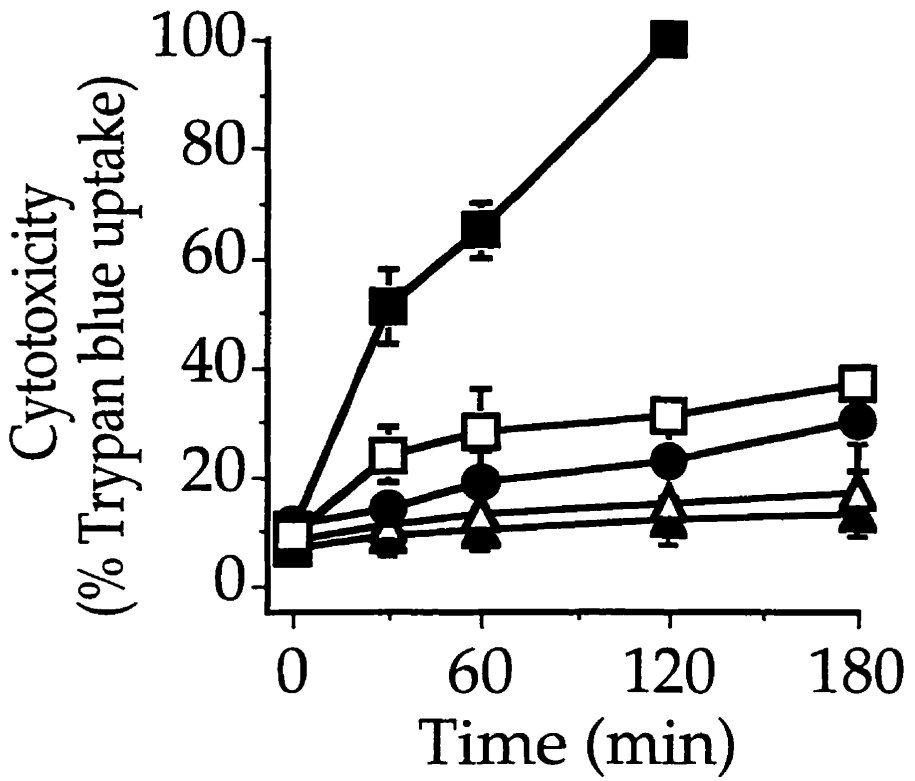


Figure 3-3: Hydroperoxide-Enhanced 4-Nitroanisole Toxicity and Cytoprotection by a P450 Inhibitor. Hepatocytes (10^6 cells/mL) were incubated alone (▲), or with tBHP (150 μM) (Δ), 4-nitroanisole (0.5 mM) (●), 4-nitroanisole + tBHP (■), and with phenylimidazole (300 μM) + 4-nitroanisole + tBHP (❑). Cytotoxicity was monitored by using Trypan Blue uptake as described in section 2.3. Values represent mean ± SE of three separate experiments.

Table 3-2: P450 Peroxygenase Catalyzed Xenobiotic Activation in Isolated Hepatocytes[a]

XENOBIOTICS	LC_{50} (mM)[b] No addition	LC_{50} (mM)[b] Hydroperoxide
Benzene	5.0	0.8
Phenol	>10.0	1.5
Hydroquinone	2.0	0.1
Bromobenzene	3.0	0.5
4-Hydroxyanisole	>10.0	1.5
4-Ethoxyphenol	4.0	1.1
4-Propoxyphenol	3.0	0.7
Naphthalene	1.0	0.3
1-Naphthol	0.5	0.1
Phenanthrene	4.0	0.5

[a] Hepatocytes (10^6 cells/mL) were preincubated for 30 min in Krebs-Henseleit bicarbonate buffer at 37 °C in the presence of desferoxamine (500 μM). Xenobiotics were dissolved in incubation buffer, methanol or dimethyl sulfoxide (0.1%) and added at various concentrations in the presence or absence of tBHP (150 μM and the viability was assessed by determining the percentage of cells excluding Trypan Blue as described in the section 2.3.

[b] LC_{50}, i.e. the concentration of the xenobiotic that kills 50% of the cells at 2 h, was estimated from the linear regression graph of the percentage of dead cells against the logarithm of xenobiotic concentration. The variation of estimated LC_{50} values was within the 15% of the averaged values and the viability of tBHP treated cells at 2 h was 85 ± 3 percent.

The cytotoxicity of 4-HA and various aromatic xenobiotics, which could be oxidized to phenolic and quinoid metabolites, was also evaluated in the presence or absence of tBHP and the results are summarized in Table 3-2.

Most of the xenobiotics tested were only cytotoxic at high millimolar concentrations.

However, xenobiotic cytotoxicity was markedly increased by tBHP and a 3- to 20-fold lower xenobiotic concentration was required to cause a similar cytotoxic response. The magnitude of the tBHP-enhanced cytotoxicity was greatest with the benzene metabolite, hydroquinone.

3.3.3. tBHP-Enhanced GSH Depletion and 4-HA Metabolite:GSH Conjugation

Hepatocyte GSH levels were measured to confirm the formation of reactive metabolites during the tBHP-enhanced 4-HA metabolism. As shown in Figures 3-4 and 3-5, 4-HA alone did not alter hepatocyte GSH or GSSG levels. However, tBHP alone transiently oxidized 90% of the hepatocyte GSH to GSSG which was reduced back to GSH within 10 min. When both 4-HA and tBHP were added, GSSG was transiently formed but the GSSG initially formed disappeared without the appearance of GSH. This suggests that the GSH regenerated from the GSSG reduction was trapped by the reactive metabolites produced from 4-HA in the presence of tBHP. Hepatocyte GSH was regenerated if the hepatocyte P450 was inhibited with phenylimidazole before addition of 4-HA and tBHP. The formation of GSH:4-HA metabolite conjugates were also markedly enhanced in the presence of tBHP as measured by the spectral monitoring of the hepatocyte quinone-

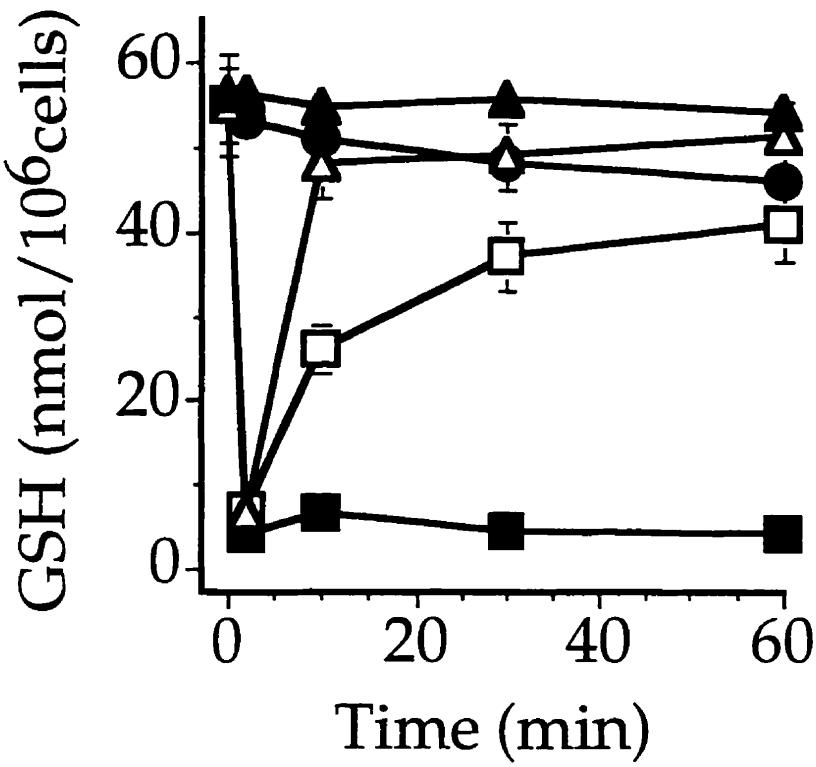


Figure 3-4: tBHP-Enhanced 4-Hydroxyanisole Mediated Modulation of Hepatocyte GSH. Hepatocytes (10^6 cells/mL) were incubated alone (▲), or with tBHP (150 μM) (Δ), 4-hydroxyanisole (1 mM) (●), 4-hydroxyanisole + tBHP (■), and with phenylimidazole (300 μM) + 4-hydroxyanisole + tBHP (❑). GSH was determined by HPLC after derivatization as described in section 2.14. Values represent mean ± SE of three separate experiments.

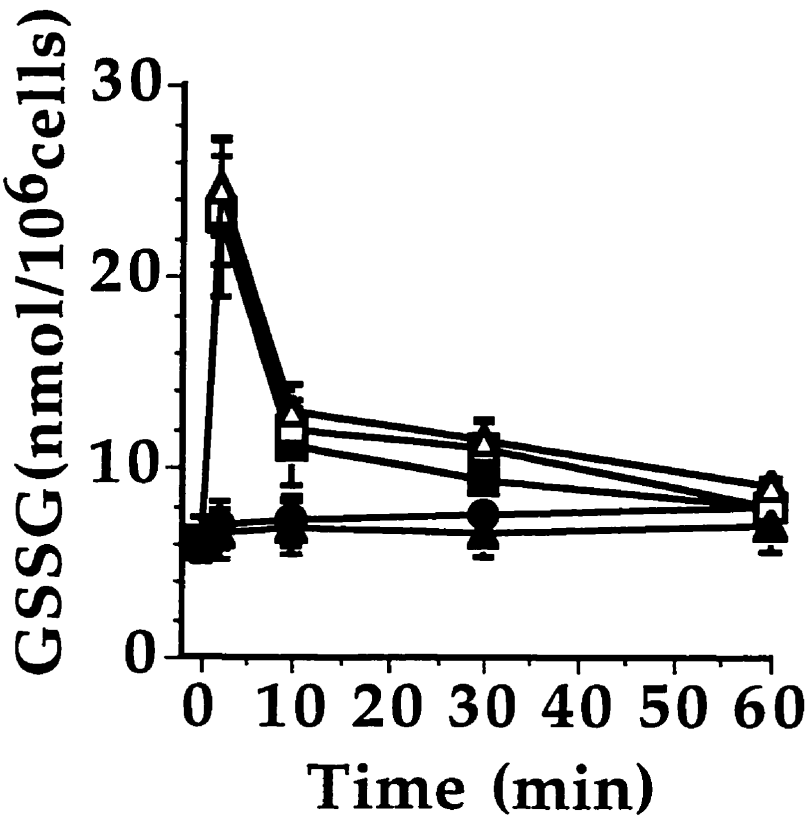


Figure 3-5: tBHP-Enhanced 4-Hydroxyanisole Mediated Modulation of Hepatocyte GSSG. Hepatocytes (10^6 cells/mL) were incubated alone (▲), or with tBHP (150 μM) (Δ), 4-hydroxyanisole (1 mM) (●), 4-hydroxyanisole + tBHP (■), and with phenylimidazole (300 μM) + 4-hydroxyanisole + tBHP (❑). GSSG was determined by HPLC after derivatization as described in section 2.14. Values represent mean ± SE of three separate experiments.

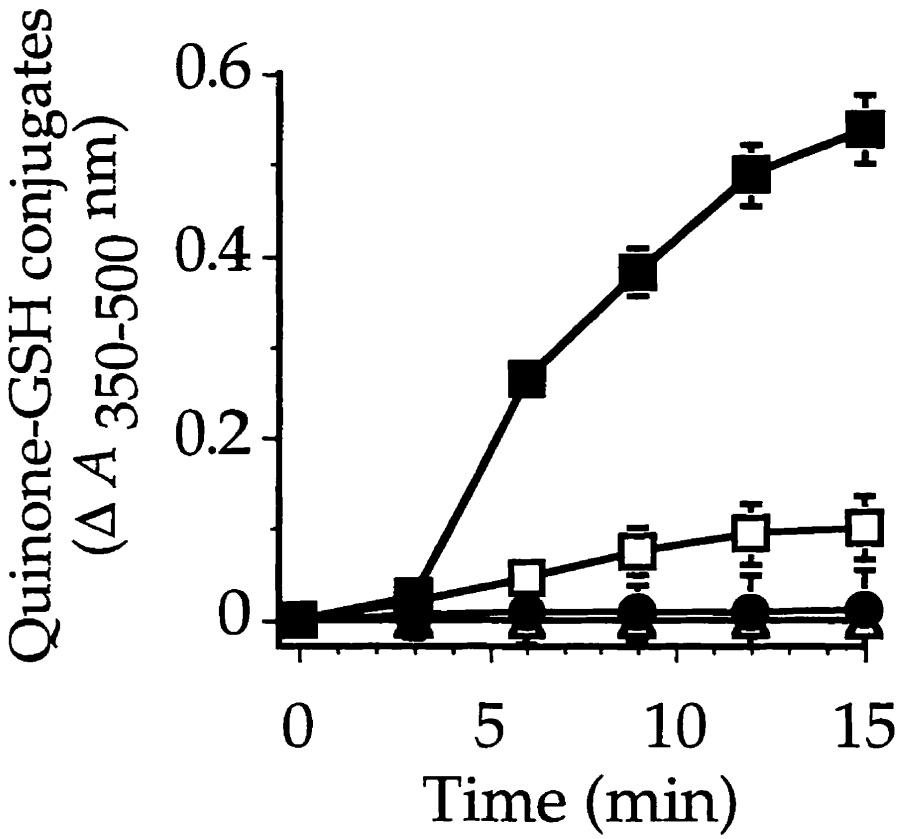


Figure 3-6: Hydroperoxide-Enhanced 4-Hydroxyanisole Metabolism to Benzoquinone:GSH Conjugates in Isolated Rat Hepatocytes. Hepatocytes (3×10^6 cells/mL) were incubated with tBHP (150 μM) (Δ), 4-hydroxyanisole (1 mM) (●), 4-hydroxyanisole + tBHP (■), and phenylimidazole (300 μM) + 4-hydroxyanisole + tBHP (❑). A time-dependent increase in the A_{350} which corresponds to the formation of benzoquinone:GSH conjugates was monitored spectrophotometrically as described in section 2.17. Values represent mean ± SE of three separate experiments.

glutathione conjugates (Figure 3-6). After a short lag period of about 3 min, quinone-GSH conjugates were formed and within 15 min were 55-fold higher than those formed in the absence of tBHP ($P<0.01$). Phenylimidazole pretreatment prevented quinone-GSH conjugate formation ($P<0.01$ compared to tBHP/4-HA cotreated group).

3.3.4. Identification of GSH Conjugates of 4-HA

The formation of reactive metabolite:GSH conjugates through the P450 peroxygenase or monooxygenase pathway was further investigated by studying the microsomal metabolism of 4-HA in the presence of GSH (Table 3-3). As shown in Figure 3-7, tBHP was much more effective than NADPH in supporting microsomal catalyzed 4-HA-mediated GSH depletion, suggesting that the hydroperoxide supported metabolism of 4-HA to reactive metabolites is more efficiently catalyzed through the P450 peroxygenase pathway. The LC/MS analysis of the GSH conjugates formed revealed that the hydroquinone-GSH conjugates were the predominant GSH adducts in both tBHP- and NADPH-supported systems (Table 3-3). Candidate GSH conjugates were detected on the basis of the protonated molecular ions at m/z 416 (Figure 3-8) and 446 (Figure 3-9). LC/MS analysis demonstrated that during the first 15 min incubation in the presence of tBHP, a single peak of hydroquinone:GSH conjugate was detected with a t_R of 2.80 min as a major GSH conjugate metabolite. By 60 min incubation, two peaks corresponding to

4-methoxycatechol-GSH conjugates with the t_R of 2.83 and 3.20 min were also detected, the latter being the major peak of 4-methoxycatechol:GSH conjugate (Figure 3-9).

Following the detection of the GSH conjugates, the MH^+ ion of each conjugate was subjected to CID in order to obtain a spectrum of product ions. LC/MS/MS spectra of both hydroquinone-GSH (Figure 3-10) and catechol

Table 3-3: tBHP- and NADPH-Dependent Formation of 4-HA Reactive Metabolites-GSH Conjugates in Isolated Microsomes[a]

Treatment	Hydroquinone:GSH conjugate	4-methoxycatechol:GSH conjugate
Microsomes+4-HA (15 min)	170[b]	71
" + " + tBHP (15 min)	52,000	988
" + " + NADPH (15 min)	198	2,153
Microsomes+4-HA (60 min)	178	65
" + " + tBHP (60 min)	121,900	3,000
" + " + NADPH (60 min)	30,250	7,550

[a] Noninduced rat liver microsomes (1 mg/mL) were incubated with GSH (5 mM), 4-HA (1 mM) and tBHP (2 mM) or NADPH (1 mM). The formation of 4-HA reactive metabolite:GSH conjugates was studied by LC/MS analysis of products as described in section 2.8.

[b] Data represent the average of two experimental arbitrary units obtained from the AUC of each conjugate peak and should be used only to compare the values of a similar conjugate.

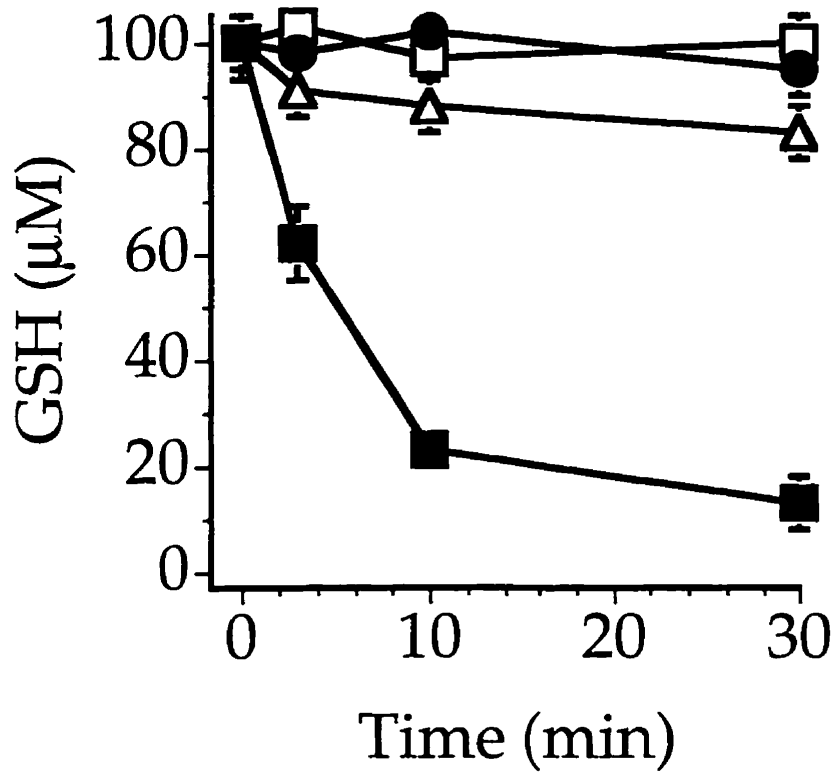


Figure 3-7: Microsomal tBHP-Enhanced 4-Hydroxyanisole Mediated GSH Depletion. GSH (5 mM) was incubated with control rat liver microsomes (1 mg/mL protein) in the presence of tBHP (150 μM) (Δ), or 4-hydroxyanisole (1 mM) + tBHP (■), or 4-hydroxyanisole + NADPH (1 mM) (●), or 4-hydroxyanisole + tBHP while microsomes were inactivated by heating for 5 min at 95 °C (❑). GSH was determined by HPLC after derivatization as described in section 2.14. Values represent mean ± SE of three separate experiments.

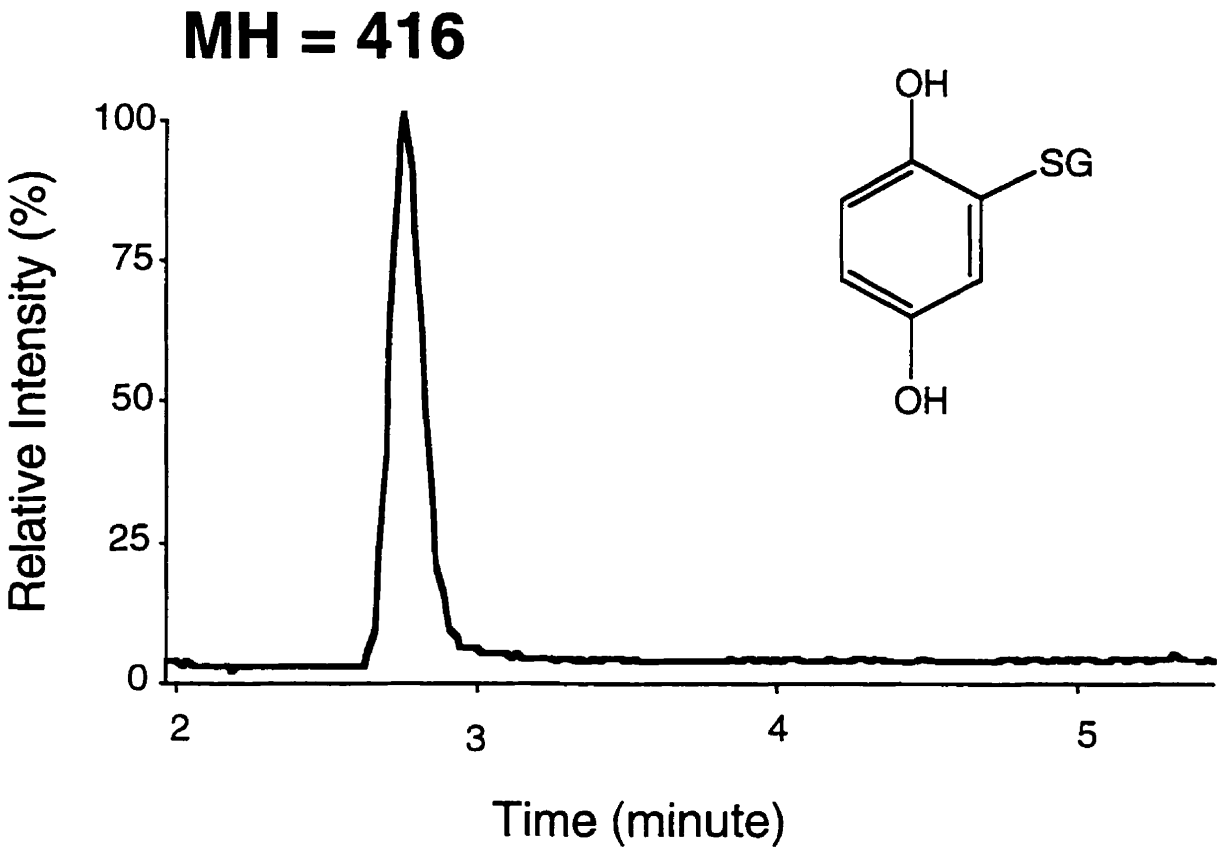


Figure 3-8: Detection of Hydroquinone-GSH Conjugates with Selective Ion Monitoring. Conjugates were isolated from the tBHP-supported microsomal incubation with 4-HA in the presence of GSH at 15 min as described in the section 2.9. The major product was identified as the hydroquinone-GSH conjugate at m/z 416.

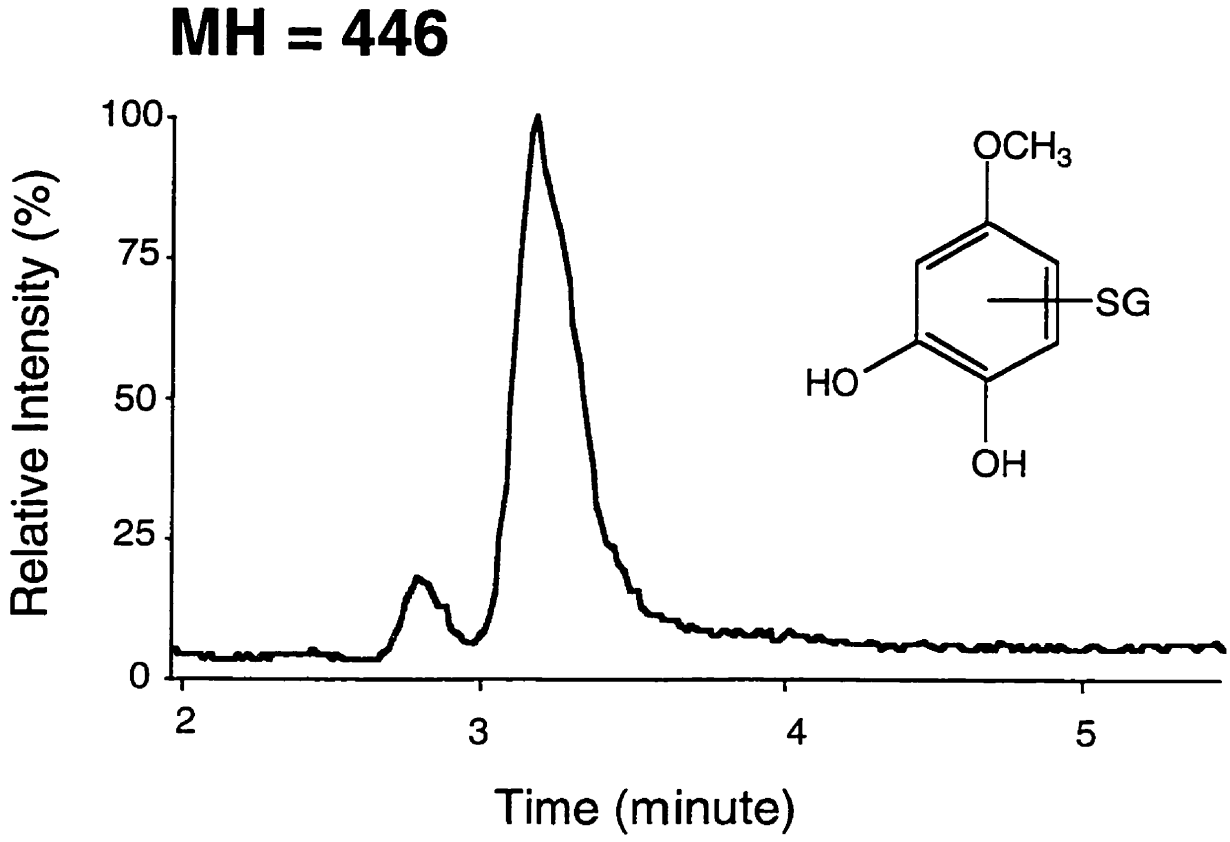


Figure 3-9: Detection of 4-Methoxycatechol-GSH Conjugates with Selective Ion Monitoring. Conjugates were isolated from the NADPH- or tBHP-supported microsomal incubation with 4-HA in the presence of GSH at 60 min as described in the section 2.9. Two peaks corresponding to 4-methoxycatechol-GSH conjugates (m/z 446) with the tR of 2.83 and 3.20 min were detected, the latter being the major peak of 4-methoxycatechol:GSH conjugate.

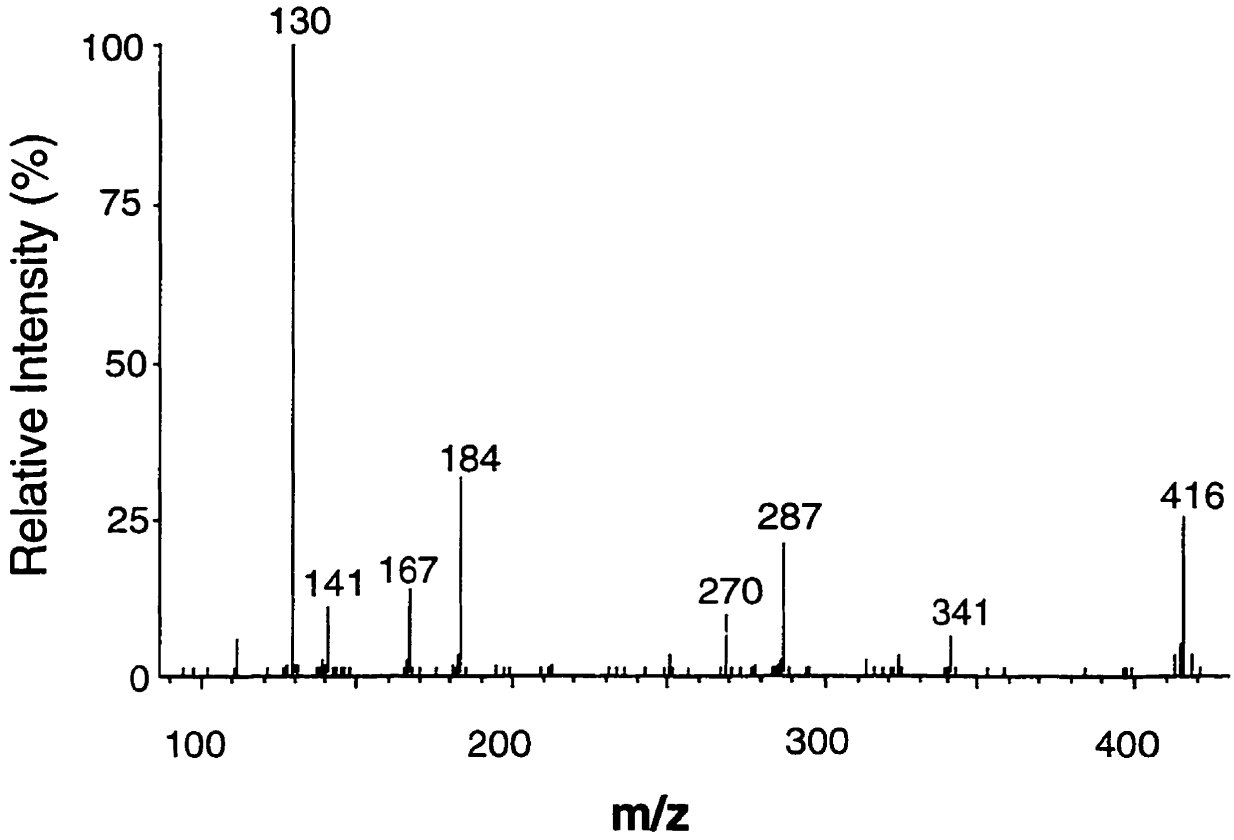


Figure 3-10: Collisional activation Spectra of the Hydroquinone-GSH Conjugate. Spectra were Acquired Using LC/MS/MS at an Energy of 27 eV as described in section 2.9.

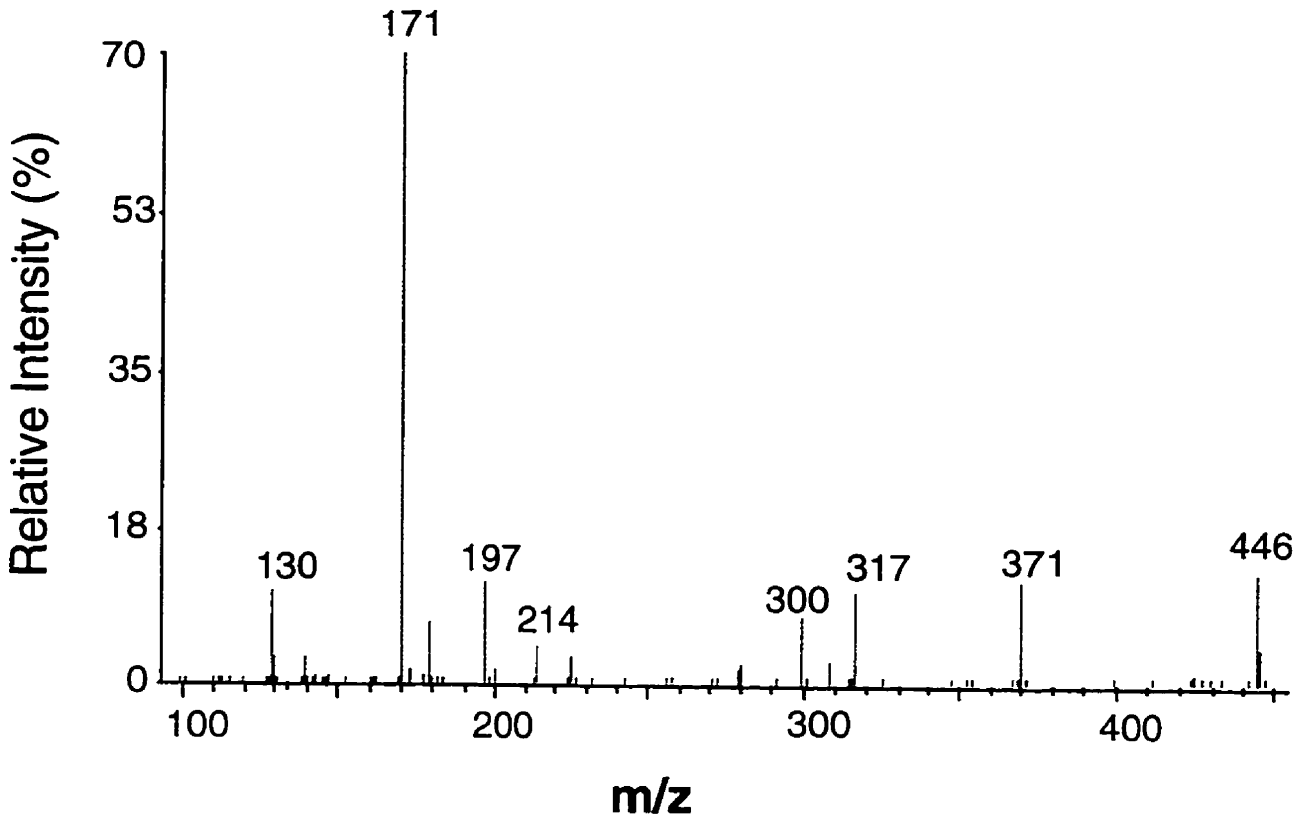


Figure 3-11: Collisional Activation Spectra of the 4-Methoxycatechol-GSH Conjugate. Spectra were acquired using LC/MS/MS at an energy of 27 eV as described in section 2.9.

(Figure 3-11) conjugates exhibited a number of structurally informative fragmented ions. For hydroquinone:GSH (MH^+ = 416), these characteristic ions included: m/z 341 (MH - glycine)$^+$, 287 (MH - pyroglutamic acid)$^+$, 270 (MH - pyroglutamic acid - OH)$^+$, 184 (MH - pyroglutamic acid - glycine - CO)$^+$, 167 (MH - pyroglutamic acid - glycine - COOH)$^+$, 141 (hydroquinone + S - H)$^+$ and 130 (pyroglutamic acid + H)$^+$. For methoxycatechol:GSH conjugates (MH^+ = 446), CID spectrum had the following characteristic ions: m/z 371 (MH - glycine)$^+$, 317 (MH - pyroglutamic acid)$^+$, 300 (MH - pyroglutamic acid - OH)$^+$, 214 (MH - pyroglutamic acid - glycine - CO)$^+$, 197 (MH - pyroglutamic acid - glycine - COOH)$^+$, 171 (4-methoxycatechol + S - H)$^+$ and 130 (pyroglutamic acid + H)$^+$.

3.4. DISCUSSION

In the present study, using freshly isolated rat hepatocytes, we provide for the first time evidence that P450 can function as a peroxidase/peroxygenase in the metabolic bioactivation of xenobiotics in intact cells. Organic hydroperoxides, i.e. tBHP, cumene hydroperoxide, and peracetic acid, were previously shown to be much more efficient than NADPH in supporting the microsomal O-demethylation of 4-NA (Koop and Hollenberg, 1980). A higher rate of tBHP-supported metabolism of these xenobiotics would suggest that P450 peroxygenase can be a much more efficient oxidative pathway for the bioactivation of xenobiotic P450 substrates

in intact cells than that of the monooxygenase system. The hydroperoxide is believed to bypass the rate limiting step of the monooxygenase system, ie. ferric P450 reduction by NADPH: P450 reductase (White and Coon, 1980). The hydroperoxide reaction rate is therefore independent of the reductant cofactor (NADPH) and molecular oxygen (O'Brien, 1978; White and Coon, 1980).

The hepatocyte cytotoxicity of 4-NA was markedly increased by tBHP as was the hepatocyte O-demethylation of 4-NA to the 4-nitrophenol. The increased 4-NA cytotoxicity could be attributed to the 4-nitrophenol metabolite. Nitrophenol causes cytotoxicity at 1 mM which may result from cellular ATP depletion caused by the uncoupling of mitochondrial oxidative phosphorylation (Weinbach and Garbus, 1965). The rate of tBHP metabolism by hepatocytes correlated with the rate and extent of tBHP supported 4-NA O-demethylation to 4-nitrophenol. Hepatotoxicity was reported in recent clinical trials of the antimelanoma agent 4-hydroxyanisole (Belcher *et al.*, 1992). However the metabolic pathways and hepatic toxic mechanisms involved have not been elucidated (Bray *et al.*, 1955). The cytotoxicity of 4-HA towards rat hepatocytes however was so low that an estimation of the dose-dependent cytotoxic response was not feasible and molecular cytotoxic mechanisms were difficult to investigate. However the addition of tBHP established a dose-dependent cytotoxic response for 4-HA and enabled us to perform more detailed metabolic and toxicological mechanistic studies.

Hepatocyte GSH levels were measured as an index of reactive quinoid or epoxide metabolites generated intracellularly during the hydroperoxide-supported metabolism of phenolic xenobiotics. 4-HA metabolism through the monooxygenase pathway did not produce enough reactive metabolites to deplete hepatocyte GSH. However, the addition of nontoxic concentrations of tBHP to hepatocytes resulted in a transient oxidation of GSH via GSH peroxidase and its subsequent regeneration by GSSG reductase (41). Such a transient rapid oxidation of GSH explains the lag period for quinone:GSH conjugate formation observed during the hydroperoxide-supported 4-HA metabolism. The lack of GSH re-generation in tBHP/4-HA co-treated hepatocytes suggests that the GSH regenerated was immediately alkylated by the reactive metabolites of 4-HA produced through the P450 peroxygenase pathway. This was further proved by studying the tBHP versus NADPH supported microsomal metabolism of 4-HA in the presence of GSH. tBHP readily supported the microsomal catalyzed 4-HA-mediated GSH depletion indicating that the 4-HA reactive metabolites formed via the P450 peroxygenase were responsible for the loss of GSH.

The hydroperoxide-enhanced xenobiotic bioactivation in hepatocytes was catalyzed by P450, as phenylimidazole, which forms a ferricytochrome type II P450 complex (Eklow *et al.*, 1981), prevented the tBHP-enhanced 4-NA and 4-HA metabolism as well as 4-HA mediated GSH depletion and cytotoxicity. The prevention of microsomal-catalyzed GSH conjugate

formation by phenylimidazole suggests that the cytoprotection observed with phenylimidazole results from the inhibition of P450 peroxygenase activity.

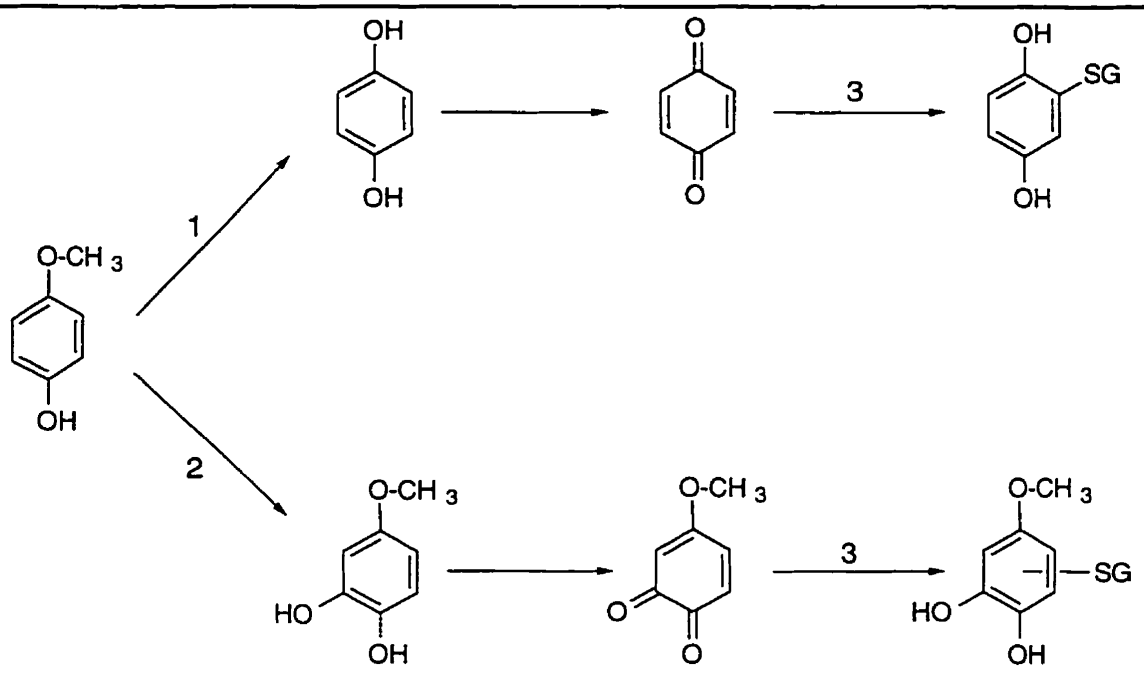


Figure 3-12: Proposed Metabolic Pathways of 4-HA Biotransformation in Isolated Rat Hepatocytes.
(1) O-Demethylation, (2) Ring Hydroxylation, (3) GSH Conjugation.

The LC/MS studies of the GSH conjugates of 4-HA reactive metabolites revealed that benzoquinone-GSH conjugates were formed initially as the predominant adduct. The Michael addition of GSH to benzoquinone most likely led to the formation of hydroquinone:GSH conjugates. The MS/MS spectra of the hydroquinone-GSH conjugate had a fragment ion at m/z 141 that is characteristic of aromatic thioether conjugates which display a

fragment resulting from cleavage of the cysteinyl C-S bond and proton transfer from the aromatic moiety to yield the stabilized (hydroquinone + S - H)$^+$ (Baillie and Davis, 1993; Haroldsen *et al.*, 1988). This fragment ion increased to become the major product ion when the pressure of the collisional gas increased (data not shown). This supports the proposed structure in which the GSH is bound to the aromatic ring of hydroquinone. The major isomer of 4-methoxycatechol-GSH conjugate formed had the most abundant fragment ion at m/z 171 indicating the formation of (4-methoxycatechol + S - H)$^+$. These results suggest that P450 metabolizes 4-HA to hydroquinone and 4-methoxycatechol by O-demethylation and ring hydroxylation respectively (Scheme 1). Cytotoxicity can probably be attributed to the benzoquinone and orthoquinone reactive metabolites, which could potentially alkylate cellular vital macromolecules (O'Brien, 1991).

The marked increase by tBHP of hepatocyte cytotoxicity and GSH depletion induced by benzene, naphthalene, bromobenzene, or naphthol was also prevented by cytochrome P450 inhibitors. This suggests that these xenobiotics were also activated by P450 peroxygenase to epoxide (Rahimtula *et al.*, 1978) or quinone metabolites (Capdevila *et al.*, 1980) which form hepatocyte GSH conjugates (Eckert *et al.*, 1990; Snyder *et al.*, 1993).

Recently, naturally occurring lipid hydroperoxides at low concentrations were shown to markedly increase the microsomal metabolic oxidation of retinoic acid (Muindi and Young, 1993). More recent work has

also demonstrated that a wide variety of physiologically generated lipid hydroperoxides at nanomolar concentrations also efficiently support diethylstilbestrol oxidation to its quinone metabolite catalyzed by microsomes (Wang and Liehr, 1995). Lipid hydroperoxides were also shown to be approximately two orders of magnitude more potent than cumene hydroperoxide in the oxidative activation of diethylstilbestrol by CYP1A1 (Roy *et al.*, 1992; Wang and Liehr, 1995). The increased level of lipid hydroperoxides in the kidney of estradiol-treated hamsters also correlated with the extent of metabolic oxidation of diethylstilbestrol to quinone metabolites in the Syrian hamster kidney (Roy and Liehr, 1992), the target organ of estrogen-induced carcinogenesis (Weisz *et al.*, 1992).

Lipid hydroperoxides are either generated physiologically from phospholipase-released fatty acids through the actions of prostaglandin H synthase or cellular lipoxygenase, or pathologically from the oxidative metabolism of fatty acids during the oxidative stress triggered by inflammation (Dargel, 1992), reperfusion injury (Zimmerman and Granger, 1994), or various xenobiotics, e.g. ethanol (Nordmann, 1994) and estrogens (Dargel, 1992). The effectiveness of physiological hydroperoxides in catalyzing P450 peroxygenase/peroxidase dependent xenobiotic oxidation *in vivo* needs to be assessed. This alternative pathway for reactive metabolite generation could be important in tissues with substantial prostaglandin synthetase and/or low P450 reductase activity. This is shown here to be of a toxicological

importance as xenobiotics not toxic to isolated hepatocytes became so as a result of a P450 peroxygenase/peroxidase catalyzed bioactivation to reactive cytotoxic metabolites which caused GSH depletion. The results presented here could form the basis for a novel *in vitro* toxicology technique for determining the nature of reactive metabolites formed and for identifying the P450 isoforms that modulate the cytotoxicity of a xenobiotic.

3.5. SIGNIFICANCE

In conclusion, we have shown that using an organic hydroperoxide as a P450 peroxygenase/peroxidase cofactor can markedly enhance the metabolism and cytotoxicity of various xenobiotic P450 substrates by intact cells. Using hydroperoxides to markedly enhance P450-dependent oxidative metabolism of xenobiotics, one can now study the toxicokinetics and toxicodynamics of xenobiotics in cells with low monooxygenase bioactivation rates.

Chapter 4

HYDROGEN PEROXIDE SUPPORTS HUMAN AND RAT CYTOCHROME P450 1A2-CATALYZED 2-AMINO-3-METHYLIMIDAZO[4,5f]QUINOLINE BIOACTIVATION TO MUTAGENIC METABOLITES:

Significance of cytochrome P450 peroxygenase*

4.1 ABSTRACT

In this paper, we show that the naturally occurring hydroperoxide, hydrogen peroxide, is highly effective in supporting the cytochrome P450 1A2 peroxygenase-catalyzed metabolic activation of the heterocyclic aromatic amine, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), to genotoxic metabolites. Mutagenicity was assessed by the Ames assay with *S. typhimurium* strain YG1012, and an activation system consisting of hydroperoxides plus either 3-methylcholanthrene-induced rat liver microsomes (rP4501A) or human P450 1A2-containing microsomes (hP4501A2). The mutagenic response was dependent on the concentration of microsomal protein, IQ and hydroperoxides. The addition of hydrogen

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* M. Reza Anari, P. David Josephy, Tracey Henry, Peter J. O'Brien (1996) Hydrogen peroxide supports human cytochrome P450 1A2 catalyzed 2-amino-3-methylimidazo[4,5f]quinoline bioactivation to mutagenic metabolites: significance of cytochrome P450 peroxygenase. *Chemical Research in Toxicology,* submitted.

peroxide or *tert*-butylhydroperoxide (tBHP) to rP4501A greatly enhanced the yield of histidine prototrophic (His^+) revertants. This increase was inhibited, in a concentration-dependent manner, by α-naphthoflavone, a P450 1A inhibitor. Hydrogen peroxide was the most effective peroxygenase cofactor, particularly with hP4501A2 (Km= 0.1 mM). The hydroperoxide-supported activation of IQ produced reactive intermediates which bound to 2'-deoxyguanosine; LC/MS analysis of the adducts revealed the same major (protonated) adduct at m/z=464.4 as previously reported for the DNA adduct formed (*in vivo* or *in vitro*) by the mixed function monooxygenase-catalyzed bioactivation system. None of the peroxidase-catalyzed IQ metabolites (nitro-, azo-, or azoxy-IQ) were detected. In conclusion, hydrogen peroxide in the physiological/pathological concentration range may be able to support the metabolic activation of arylamines to genotoxic products through the cytochrome P450 peroxygenase pathway.

4.2. INTRODUCTION

Heterocyclic aromatic amines (HAAs) are formed in proteinaceous foods as a result of pyrolysis during cooking (Layton *et al.*, 1995). Seventeen HAAs have been identified so far in a variety of foods at parts per billion levels (Layton *et al.*, 1995; Sugimura, 1986). IQ and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) are abundant HAAs, potent bacterial mutagens, and rodent carcinogens (Sugimura, 1986; Sugimura, 1988);

IQ is also a liver carcinogen in monkeys (Adamson *et al.*, 1990). HAAs, like many carcinogens, must be metabolically activated in order to exert their genotoxic effects. Metabolic activation occurs through N-oxidation of the exocyclic amine group catalyzed primarily by cytochrome P450 1A2 (Butler *et al.*, 1989; Guengerich, 1988; Shimada *et al.*, 1989; Yamazoe *et al.*, 1983).

Cytochrome P450 (P450) enzymes are extraordinarily versatile haemoproteins that catalyze the oxidation of physiological substrates and foreign chemicals, such as drugs, pesticides, polycyclic aromatic hydrocarbons and HAAs (Gonzalez and Gelboin, 1994; Guengerich and Shimada, 1991). The versatility also extends to the oxygen donor. Molecular oxygen serves as the natural donor when electrons are supplied to cytochrome P450 by NADPH via NADPH-cytochrome P450 reductase in the P450 monooxygenase pathway (eqs 1 and 2). In the overall reaction, equimolar amounts of substrate, O_2, and NADPH are consumed and equimolar amounts of oxidized substrate, H_2O, and $NADP^+$ are formed (Coon, 1978).

$$P450\text{-}Fe^{III} + O_2 + 2NADPH + 2e^- \longrightarrow P450^{\cdot+}(Fe^{IV}{=}O) + H_2O + 2NADP^+ \quad (1)$$

$$P450^{\cdot+}(Fe^{IV}{=}O) + X\text{-}H \longrightarrow P450\text{-}Fe^{III} + X\text{-}OH \quad (2)$$

Cytochrome P450 can also utilize reduced oxygen equivalents, e.g., hydroperoxides, to support the oxidation of various substrates, in a reaction that is independent of molecular oxygen, NADPH, and NADPH-cytochrome P450 reductase (Hrycay *et al.*, 1976; Hrycay and O'Brien, 1971a; Hrycay and O'Brien, 1971b; Hrycay and O'Brien, 1972; Kadlubar *et al.*, 1973; O'Brien, 1978;

Rahimtula and O'Brien, 1974). Cytochrome P450 functions as a "peroxygenase" in this reaction and forms iron-oxygen species identical to the iron-oxene complex in P450 monooxygenase pathway and analogous to peroxidase Compound I, as shown in eqs 3 and 4 (O'Brien, 1978; Ortiz de Montellano, 1995b; Wand and Thompson, 1986).

$$P450\text{-}Fe^{III} + ROOH \longrightarrow P450^{\cdot+}(Fe^{IV}=O) + ROH \quad (3)$$

$$P450^{\cdot+}(Fe^{IV}=O) + X\text{-}H \longrightarrow P450\text{-}Fe^{III} + X\text{-}OH \quad (4)$$

Analysis of the microsomal hydroperoxide-supported oxidation reaction has provided valuable insights into the mechanism of oxygen activation by P450 (Guengerich and MacDonald, 1990; O'Brien, 1978; Ortiz de Montellano, 1995b), but the significance of this pathway in intact cells remains unresolved. Recently, we reported that cytochrome P450-catalyzed xenobiotic metabolism in, and acute cytotoxicity to, isolated rat hepatocytes could be enhanced markedly by the addition of a nontoxic concentration of the alkyl hydroperoxide, *tert*-butyl hydroperoxide (tBHP) (Anari *et al.*, 1995b). The peroxidase or peroxygenase nature of xenobiotic oxidation was not studied in this preliminary report, although further studies with intact hepatocytes showed that the P450-catalyzed homolytic cleavage of hydroperoxides initiates the formation of cytotoxic radical species (Anari *et al.*, 1996b). The objective of the present study was to determine whether hydrogen peroxide or fatty acid hydroperoxides, which are being produced *in vivo* particularly during the course of oxidative cell stress (Sies, 1991), can support the bioactivation of

promutagens/procarcinogens to genotoxic metabolites through the cytochrome P450 peroxygenase pathway. Previously it was shown that hydrogen peroxide was much less effective that alkyl hydroperoxides in supporting cytochrome P450 peroxygenase activities (Estabrook *et al.*, 1984; Nordblom *et al.*, 1976; Penneberg *et al.*, 1978). However, here we show that low concentrations of hydrogen peroxide effectively support cytochrome P450 1A2-catalyzed metabolic bioactivation of a heterocyclic arylamine to DNA-reactive mutagenic metabolites.

4.3. RESULTS

The bioactivation system used in the Ames assay usually comprises rat liver S9 fraction and an NADPH generating system (Maron and Ames, 1983). We used 3-MC-induced rat liver microsomes or human P450 1A2-containing microsomes (Rodrigues and Prough, 1991), but replaced the NADPH generating system with hydrogen peroxide or an organic hydroperoxide. The complete system of microsomes, IQ, and hydroperoxide gave a strong mutagenic response, which was dependent on the concentrations of microsomal protein (Figure 4-1), IQ (Figure 4-2), and hydroperoxide (Figure 4-3). Using a concentration of microsomal protein within a linear range (25 μg/plate), the effect of various concentrations of naturally occurring hydroperoxides (fatty acid hydroperoxides and H_2O_2) as well as *tert*-butylhydroperoxide on the mutagenicity of IQ (10 pmol/plate, 5 nM) was investigated (Figure 4-3). Hydrogen peroxide showed

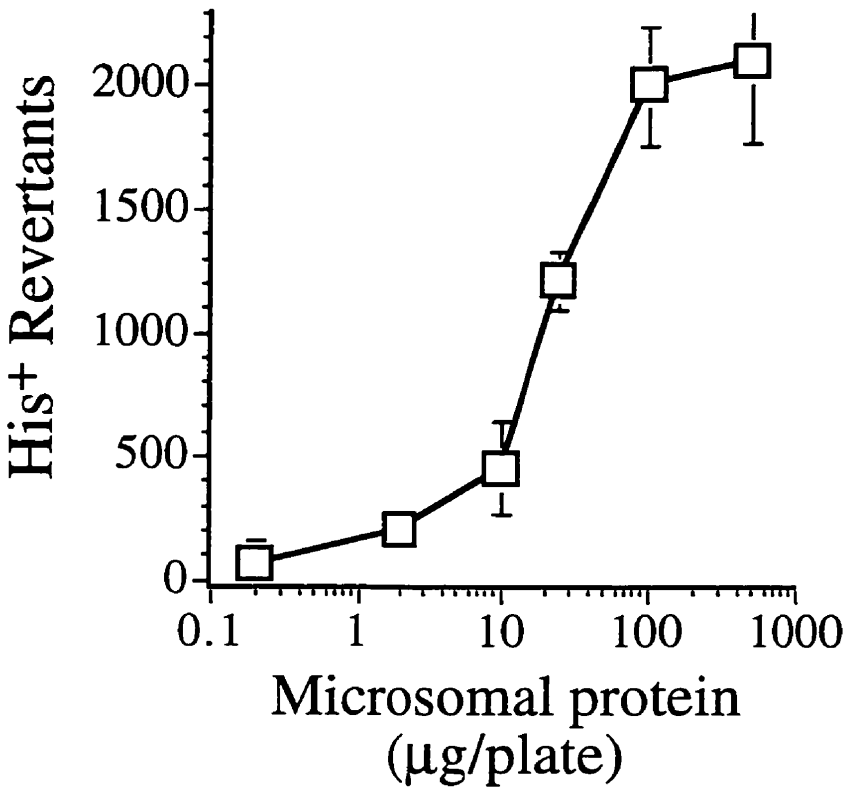

Figure 4-1: Hydrogen Peroxide-Supported P450-Catalyzed Mutagenic Activation of IQ: Enzyme concentration-response curve. The frequency of His+ revertants was determined using various concentrations of rP4501A in the presence of hydrogen peroxide (50 nmol/plate) and IQ (10 pmol/plate) as described in section 2.6.

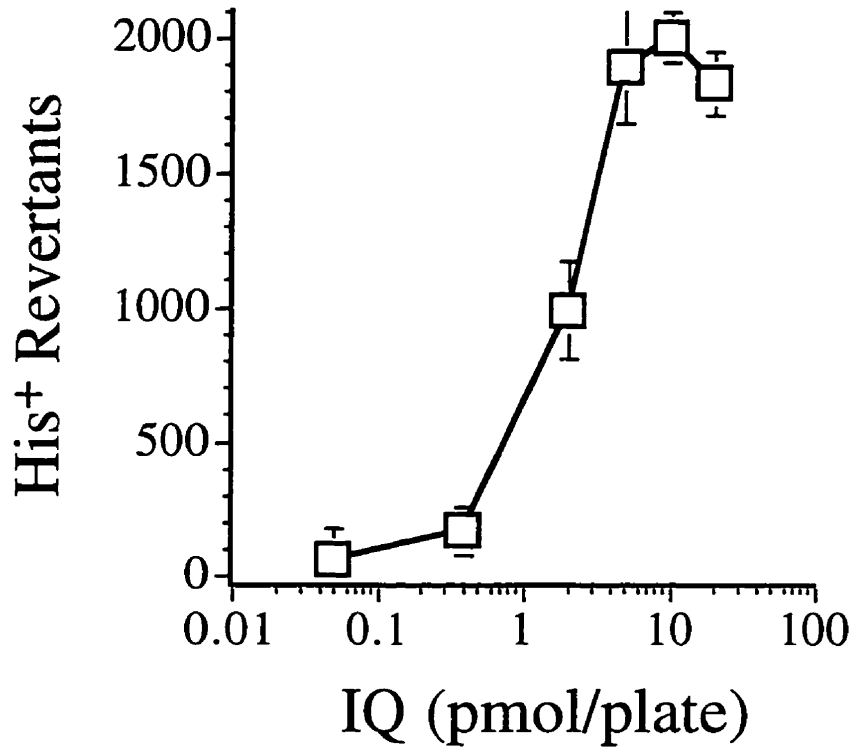

Figure 4-2: Hydrogen Peroxide-Supported P450-Catalyzed Mutagenic Activation of IQ: Mutagen concentration-response curve. The frequency of His^+ revertants was determined using rP4501A (25 μg/plate) in the presence of hydrogen peroxide (50 nmol/plate) and various concentrations of the IQ as described in section 2.6. Values represent means ± SE of three separate experiments.

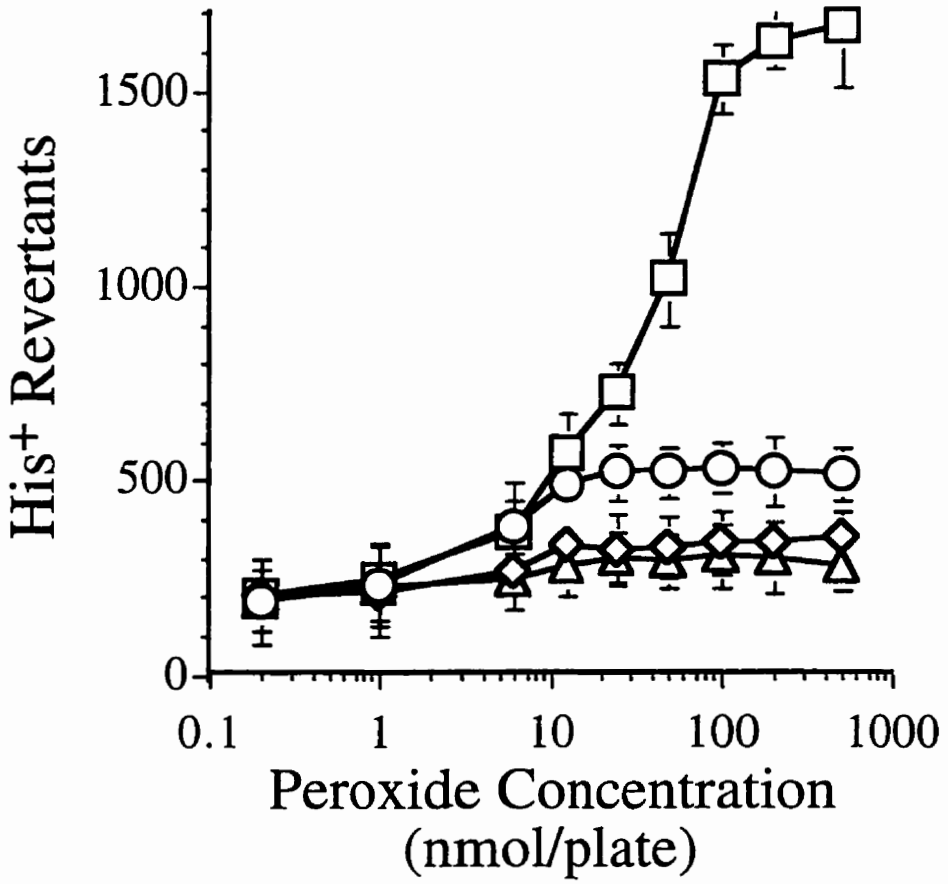

Figure 4-3: The Effectiveness of Various Hydroperoxides at Supporting the Mutagenic Activation of IQ. The frequency of His^+ revertants was determined using rP4501A (25 µg/plate) in the presence of IQ (10 pmol/plate) and various concentrations of hydrogen peroxide (❑), tBHP (○), Linoleic acid hydroperoxide (◇), or linolenic acid hydroperoxide (Δ) as described in section 2.6. Values represent means ± SE of three separate experiments.

the highest efficacy in this system with a maximum mutagenic response of 1650 His^+ revertants/plate whereas *tert*-butylhydroperoxide showed a maximum mutagenic response of 500 His^+ revertants/plate. Linoleic and linolenic acid hydroperoxides were not effective in supporting the 3MC-induced microsomal-catalyzed mutagenic bioactivation of IQ ($P>0.05$ compare to control group without hydroperoxide). α–NF, an inhibitor of P450 1A isoforms (Rodrigues and Prough, 1991), inhibited the H_2O_2-dependent mutagenicity of IQ, in a concentration-dependent manner (Figure 4-4). Human P450 1A2-containing microsomes were also effective in the H_2O_2-dependent system (Table 4-1). Again, all three components of the bioactivation mixture (P450 1A2, IQ, and hydrogen peroxide) were required for the induction of the mutagenic response and the mutagenic response was almost double of that obtained by the NADPH-supported monooxygenase system under similar conditions.

We also evaluated hydroperoxide-supported P450 activity by direct enzyme assay (methoxyresorufin O-demethylation; MROD). In the human P450 1A2-enriched microsomal system (Figure 4-5), MROD activity was efficiently supported by hydrogen peroxide (Vmax= 1000 pmol resorufin/min/mg protein, Km= 103 μM H_2O_2). With a rat liver microsomal system, MROD was also supported by hydrogen peroxide (Vmax = 500 pmol resorufin/min/mg protein, Km= 1208 μM)(Figure 4-6) or tBHP (Vmax = 500 pmol resorufin/min/mg protein, Km= 158 μM)(Figure 4-7). MROD activity

Table 1. Hydrogen Peroxide- and NADPH-Supported Human P4501A2-Catalyzed Metabolic Activation of IQ in *S. Typhimurium* Strain YG1012[a]

Treatment			His^+ Revertants
hP4501A2	IQ	Cofactor	
+	+	H_2O_2	2200 ± 83[b]
-	+	-	56 ± 6
+	-	-	23 ± 4
+	+	-	225 ± 37
+	-	H_2O_2	33 ± 3
+	+	NADPH	1186 ± 75[b]

[a] The Ames assay was performed with IQ (10 pmol/plate, 5 nM), human P4501A2-containing microsomes (2.05 pmol P450/plate, 1.025 nM), and hydrogen peroxide (50 nmol/plate, 25 μM) or NADPH (1 mM) as described in section 2.6. In the absence of an activation system, nitro-IQ, 0.2 pmol/plate, gave 1570 ± 22 revertants per plate.

[b] Data represent the means of three separate experiments ± SE.

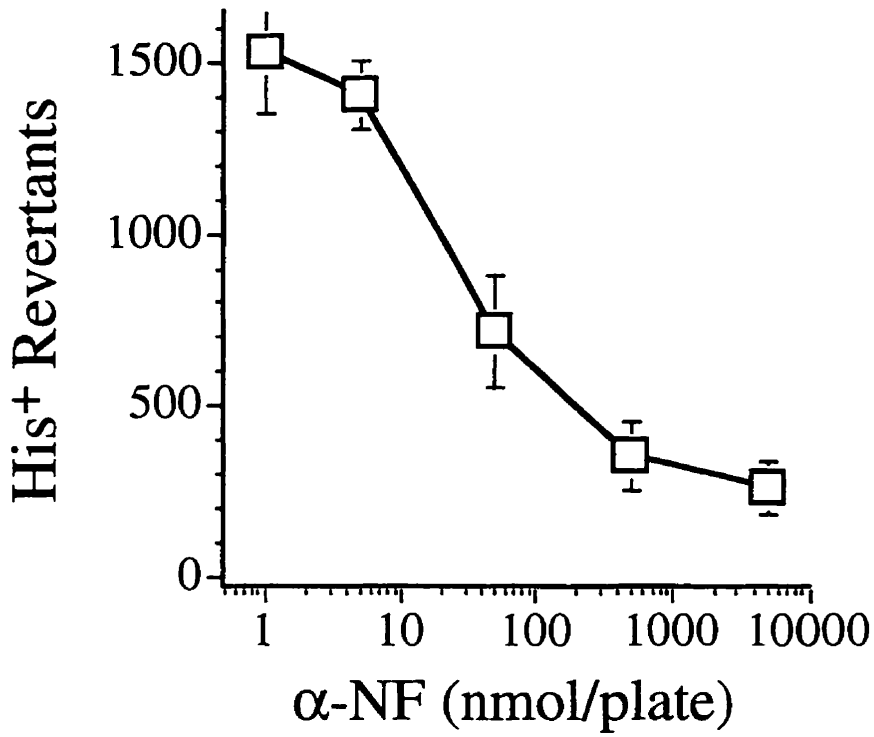

Figure 4-4: Hydrogen Peroxide-Supported P450-Catalyzed Mutagenic Activation of IQ: Effect of α-NF. The frequency of His^+ revertants was determined using rP4501A (25 μg/plate) in the presence of IQ (10 pmol/plate), hydrogen peroxide (50 nmol/plate), and various concentrations of α-NF as described in section 2.6. Values represent means ± SE of three separate experiments.

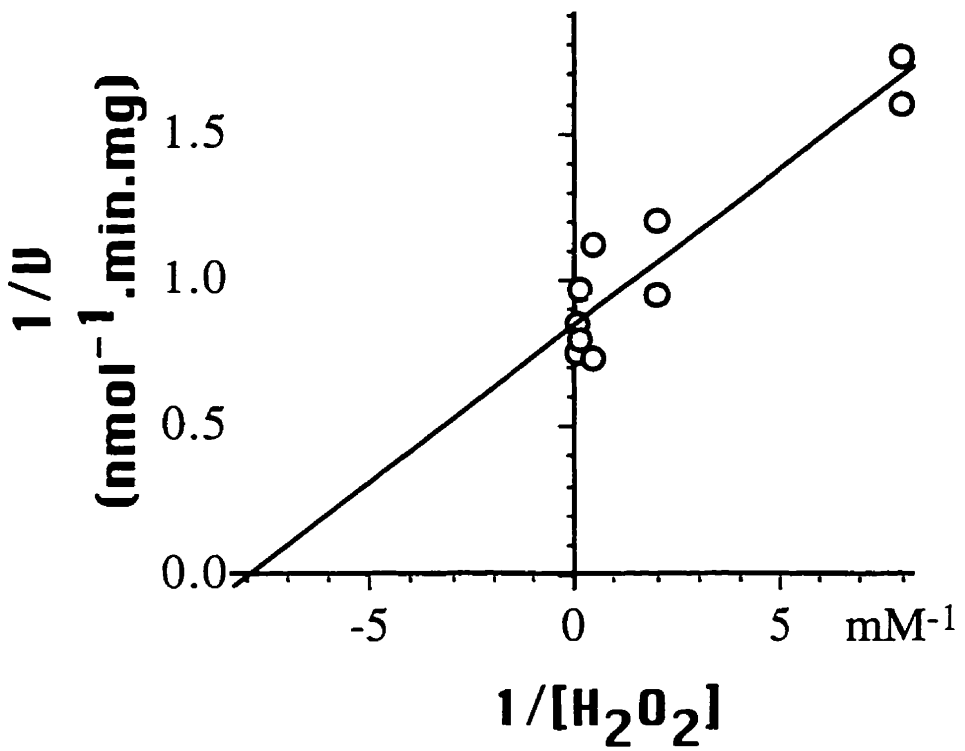

Figure 4-5: Kinetic Analysis of Hydrogen Peroxide-Supported Methoxyresorufin O-Demethylation by Human Microsomal P450 1A2. Incubation mixtures contained methoxyresorufin (0.5 mM), hP4501A2 (0.1 mg/mL), and hydrogen peroxide. Reciprocal rate of MROD is plotted against reciprocal hydroperoxide concentration. The linear regression equation was: $Y = 0.103\ X + 0.001$; $r^2 = 0.9821$; Vmax = 1000 pmol/(min.mg); Km = 103 μM. Values represent average of two separate experiments.

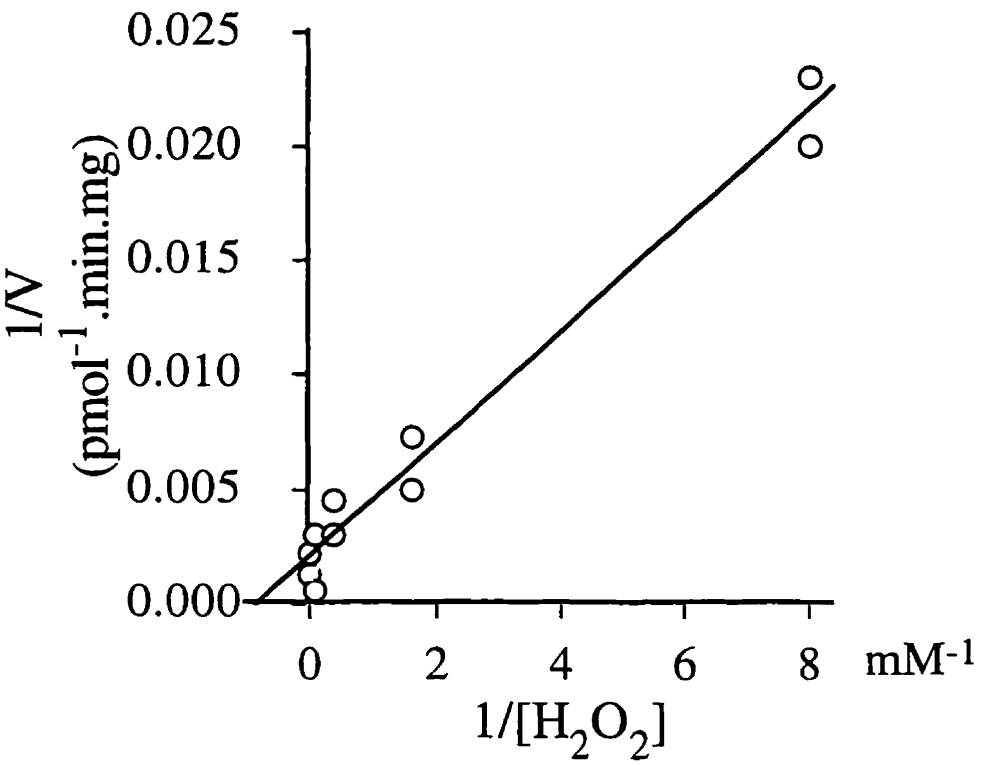

Figure 4-6: Kinetic Analysis of Hydrogen Peroxide-Supported Methoxyresorufin O-Demethylation by Rat Liver Microsomal P450. Incubation mixtures contained methoxyresorufin (0.5 mM), rP4501A (50 mg/mL), and hydrogen peroxide. Reciprocal rate of MROD is plotted against reciprocal hydroperoxide concentration. The linear regression equation was: Y = 2.416 X + 0.002; r^2 = 0.9868; Vmax = 500 pmol/(min.mg); Km = 1208 µM. Values represent average of two separate experiments.

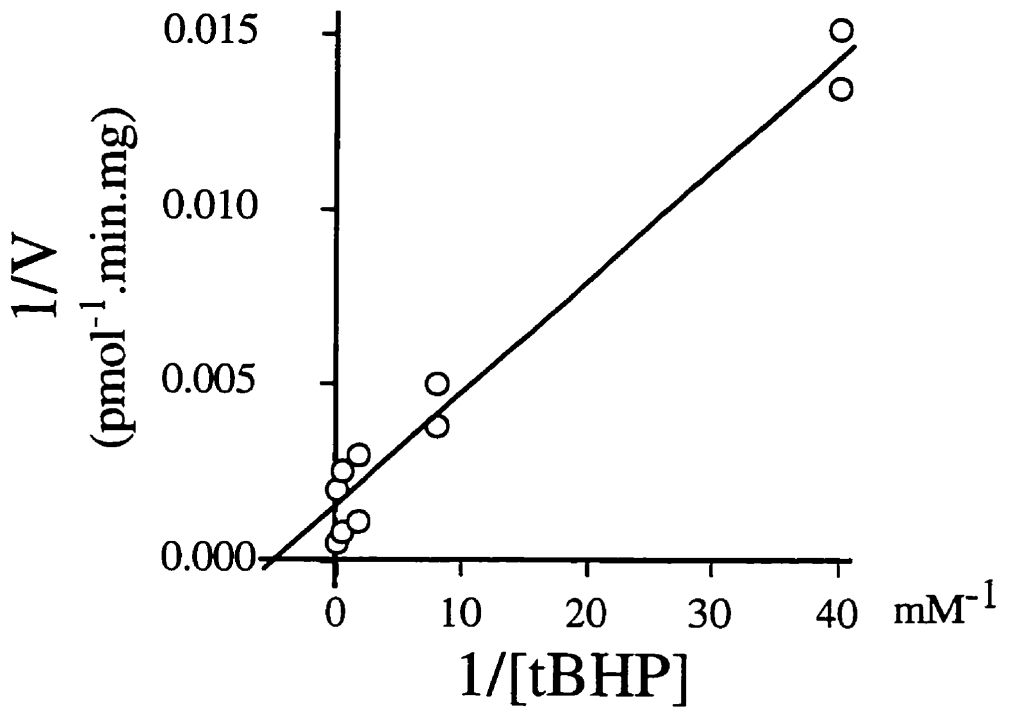

Figure 4-7: Kinetic Analysis of tBHP-Supported Methoxyresorufin O-Demethylation by Rat Liver Microsomal P450. Incubation mixtures contained methoxyresorufin (0.5 mM), rP4501A (50 mg/mL), and tBHP. Reciprocal rate of MROD is plotted against reciprocal hydroperoxide concentration. The linear regression equation was: Y = 0.316 X + 0.002; r^2 = 0.9895; Vmax = 500 pmol/(min.mg); Km = 158 μM. Values represent average of two separate experiments.

however was not supported by linoleic or linolenic acid hydroperoxides (MROD activity of less than 1 pmol resorufin/min/mg protein).

The formation of reactive metabolites from IQ was examined by trapping with the deoxyribonucleoside 2'-deoxyguanosine; adducts were analyzed by LC/MS. The major deoxyguanosine adduct was characterized on the basis of its protonated molecular ions, and is probably either N-(deoxyguanosin-8-yl)-2-amino-3-methylimidazo-[4,5-*f*]quinoline or (deoxyguanosin-N2-yl)-2-amino-3-methylimidazo[4,5-*f*]quinoline (Figure 4-8). Selective ion monitoring (m/z = 464.4) confirmed the formation of a single major adduct (t_R = 2.49 min; Figure 4-9). Distinguishing between the two possible isomeric forms of the adduct will require further investigation. Adduct yield was dependent on hydroperoxide (Table 4-2), in a manner which paralleled the results of the mutagenicity and enzyme assay experiments. Neither the NADPH-supported microsomal P450 1A2 monooxygenase system nor the hydrogen peroxide-supported horseradish peroxidase system produced deoxyguanosine adducts.

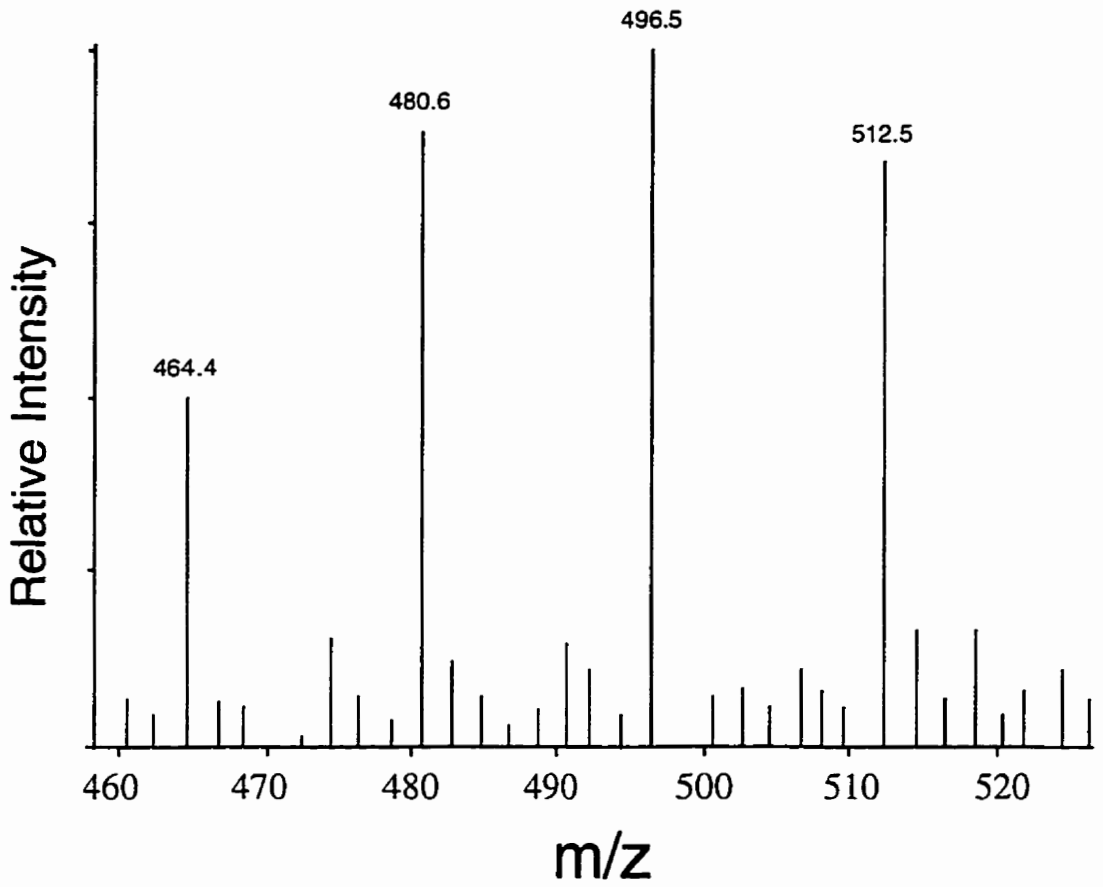

Figure 4-8: LC/MS Analysis of IQ:Deoxyguanosine Adducts. Adducts were formed by the microsomal metabolism (rP4501A, 0.2 mg/mL) of IQ (1 mM) in the presence of deoxyguanosine (5 mM) and hydrogen peroxide (5 mM). Major protonated adduct peaks (MH^+) occurred at m/z = 464.4, 480.6, 496.5, and 512.5, and reached up to 14.8% of the total ion current.

Table 4-2: Hydroperoxide-Dependent Formation of IQ-Deoxyguanosine Adducts[a]

Treatment			IQ:Deoxyguanosine conjugates at m/z			
rP4501A	IQ	Cofactor[b]	464.4	480.4	496.2	512.4
+	+	H_2O_2, 5 mM	1.0×10^5[c]	1.9×10^5	2.2×10^5	1.7×10^5
+	+	–	ND	ND	ND	ND
–	+	H_2O_2, 5 mM	ND	ND	ND	ND
+	+	tBHP, 1 mM	2.4×10^4	4.4×10^4	8.3×10^4	7.5×10^4
+	+	LinoleicHP, 0.5 mM	ND	ND	ND	ND
+	+	LinolenicHP, 0.5 mM	ND	ND	ND	ND
+	+	NADPH, 1 mM	ND	ND	ND	ND

[a] Incubations contained rat liver microsomal preparation (0.2 mg/mL), IQ (1 mM), 2'-deoxyguanosine (5 mM), and hydroperoxides, as indicated. Adducts were measured by LC/MS analysis, as described in section 2.10.

[b] Fatty acid hydroperoxide cofactors used: LinoleicHP = Linoleic acid hydroperoxide; LinolenicHP = Linolenic acid hydroperoxide.

[c] Data represent the average of two measurements (arbitrary units) obtained from the area-under-curve of each adduct peak, and should be used only for comparison of values for a given adduct.

[d] ND = Not detected

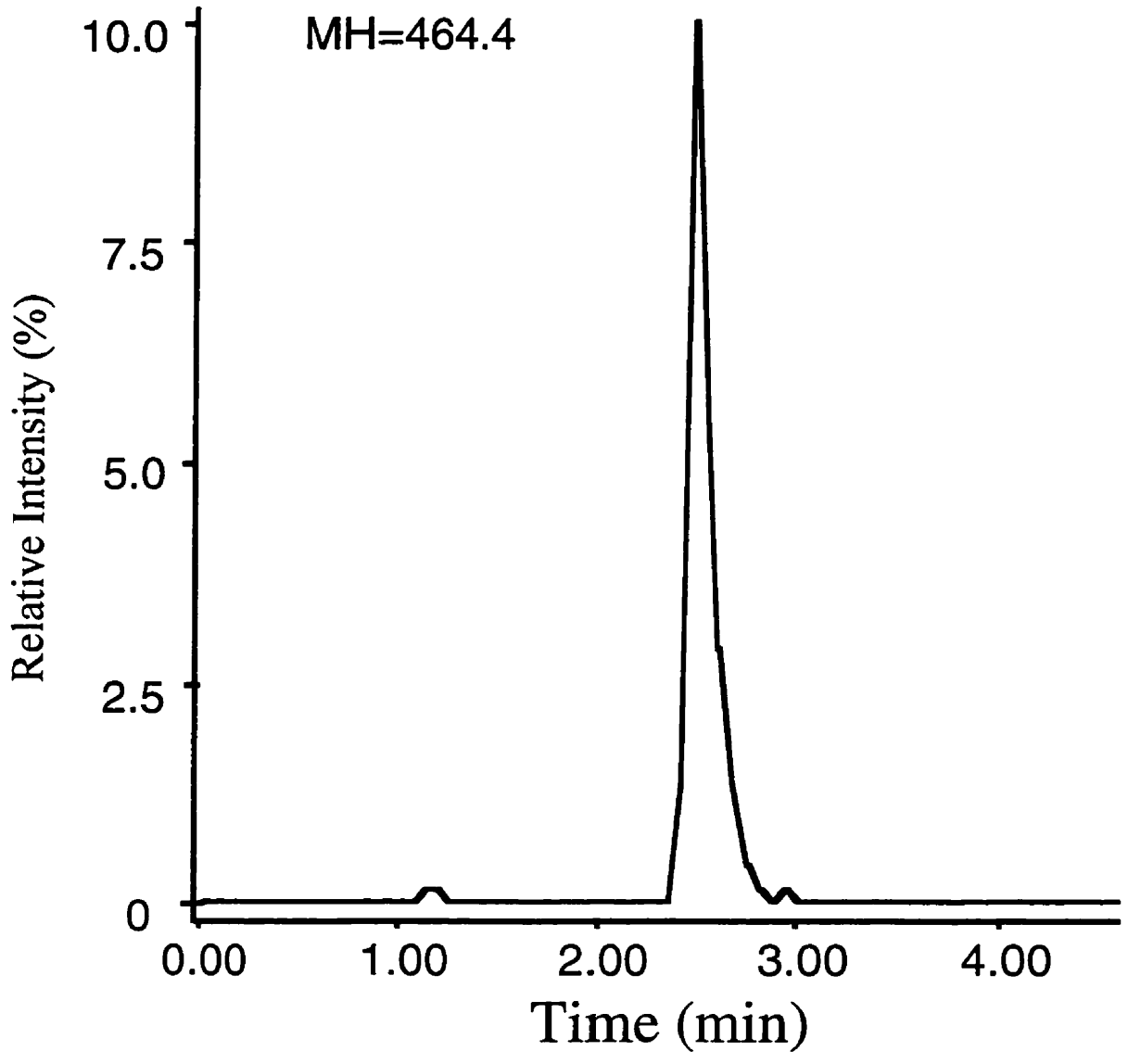

Figure 4-9: Selective ion monitoring at m/z = 464.4 of IQ:deoxyguanosine adducts. Adducts were formed by the microsomal metabolism (rP4501A, 0.2 mg/mL) of IQ (1 mM) in the presence of deoxyguanosine (5 mM) and hydrogen peroxide (5 mM). t_R of major peak = 2.49 min.

4.4. DISCUSSION

Following our finding that P450 peroxidase/peroxygenase can bioactivate xenobiotics in isolated hepatocytes (Anari *et al.*, 1995b), we have investigated whether naturally occurring hydroperoxides can support the P450-catalyzed metabolic activation of promutagens to DNA-reactive and genotoxic metabolites. Both human and rat P450 1A2 can utilize hydroperoxides and bioactivate IQ to mutagenic metabolites. The mutagenic response was dependent on each component of the bioactivation system (enzyme, peroxide, and promutagen). Hydrogen peroxide was much more efficient than *tert*-butylhydroperoxide though fatty acid hydroperoxides were ineffective.

Site-directed mutagenesis studies of rat P450 1A2 have demonstrated that the putative distal amino acid residues, Glu 318 and Thr 319, are essential for high peroxygenase activity (Shimizu *et al.*, 1994). Optical absorption spectroscopy studies indicated that the hydrogen peroxide O-O bond was cleaved heterolytically by the wild type rat P450 1A2, resulting in the formation of an oxo-P450 heme complex analogous to horseradish peroxidase compound I (Shimizu *et al.*, 1994). The amino acid residues Glu 318 and Thr 319, in the oxygen binding pocket as well as neighboring residues are highly conserved between the rat and human 1A2 (Ikeya *et al.*, 1989; Shimizu *et al.*, 1994). Our results show for the first time that the human form of P450 1A2 can effectively catalyze the hydrogen peroxide-supported IQ bioactivation.

P450 isoforms which lack oxygen binding pockets and utilize physiological lipid hydroperoxides as oxygen sources have recently been characterized in plants (Song *et al.*, 1993), platelets (Haurand and Ullrich, 1985), and endothelial cells (Pereira *et al.*, 1993; Ullrich and Graf, 1984). The homolytic cleavage of the hydroperoxide O-O bond is involved in the catalytic cycle of these novel cytochrome P450 enzymes. Naturally occurring lipid hydroperoxides can also support the microsomal-catalyzed oxidation of retinoic acid (Muindi and Young, 1993) and diethylstilbestrol (Roy *et al.*, 1992). However, linoleic and linolenic acid hydroperoxides did not support the mutagenic bioactivation of IQ by rat or human P450 1A2. It is known that the dioxygen bond in unsaturated fatty acid hydroperoxides is primarily metabolized homolytically by certain cytochrome P450 enzymes and P420 to free radicals which catalyze the above oxidations (O'Brien, 1978). Therefore, these hydroperoxides, although excellent peroxidase cofactors, cannot efficiently support the P450 peroxygenase-catalyzed metabolism of P450 substrates.

Hydrogen peroxide was also an efficient cofactor for another P450 1A2-catalyzed activity, i.e. methoxyresorufin O-demethylation (Km= 0.103 mM for human P450 1A2 and Km= 1.2 mM for 3MC-induced rat liver microsomes). This was surprising as other investigators have reported that hydrogen peroxide was several orders of magnitude less effective that alkyl hydroperoxides in supporting microsomal P450-catalyzed benzphetamine N-

demethylation (Km= 250 mM) (Nordblom *et al.*, 1976) or aniline hydroxylation (Km= 18 mM) (Penneberg *et al.*, 1978). The high concentrations of hydrogen peroxide required as a peroxygenase cofactor for P450 found by others may be attributed to catalase associated with the microsomal preparations and the possibility that it is the peroxide anion (HO_2^-) that is the reactive species interacting with other cytochrome P450 isoforms (Estabrook *et al.*, 1984). The unusual high affinity of P450 1A2 for hydrogen peroxide warrants further investigation, particularly as such hydrogen peroxide concentrations may be formed in vivo by various oxidases and hydrogen peroxide formation could be higher during oxidative stress (Sies, 1991).

Covalent modification of DNA by genotoxins may be an initiating event in carcinogenesis (Miller and Miller, 1981). HAAs form DNA adducts primarily at the C-8 and N^2 atoms of guanine and to a lesser extent at the C-8 and N^6 position of adenine (Snyderwine *et al.*, 1988b; Turesky and Markovic, 1995; Turesky *et al.*, 1996b). The activated metabolite (2-(hydroxyamino)-3-methylimidazo[4,5-*f*]quinoline; NHOH-IQ) formed by monooxygenase-dependent metabolism of IQ reacts with DNA primarily to form N-(deoxyguanosine-8-yl)-2-amino-3-methylimidazo[4,5-*f*]quinoline, with 5-(deoxyguanosine-N^2-yl)-IQ as a minor adduct (Snyderwine *et al.*, 1988a; Turesky *et al.*, 1992) (Figure 4-10). ^{32}P-Postlabeling analysis of DNA adducts of IQ formed *in vivo* (rodents, monkey) identified the same two 2'-deoxyguanosine adducts (Turesky *et al.*, 1996a; Turesky and Markovic, 1995;

Turesky *et al.*, 1996b). We identified, by LC/MS, a major adduct which may be identical to these adducts. The formation of the deoxyguanosine adduct and its oxidized derivatives was dependent on the presence of all components of the bioactivation system. Again the order of potency for hydrogen peroxide and tBHP at supporting adduct formation was similar to that obtained in supporting the MROD reaction as well as enhancing IQ mutagenicity. Whether or not the deoxyguanosine adducts formed under these in vitro conditions are responsible for the mutagenic properties of IQ requires further investigation.

The N-hydroxy metabolites of IQ and related compounds require O-acetylation to generate a metabolite sufficiently reactive to form 2'-deoxyguanosine adducts (Frandsen *et al.*, 1994). The bacterial strain used in this study expresses high levels of acetyl-CoA:arylamine N-acetyltransferase, which also catalyzes acetyl CoA-dependent O-acetylation. Prostaglandin H synthase metabolizes IQ to mutagenic products, via the peroxidase reaction; however, this metabolism proceeds by one-electron oxidation, to form, ultimately, nitro metabolites (Josephy *et al.*, 1989; Morrison *et al.*, 1993; Smith *et al.*, 1992). HPLC analysis of the hydrogen peroxide-supported P450 1A2 catalyzed IQ metabolites did not indicate the formation of nitro or nitroso metabolites.

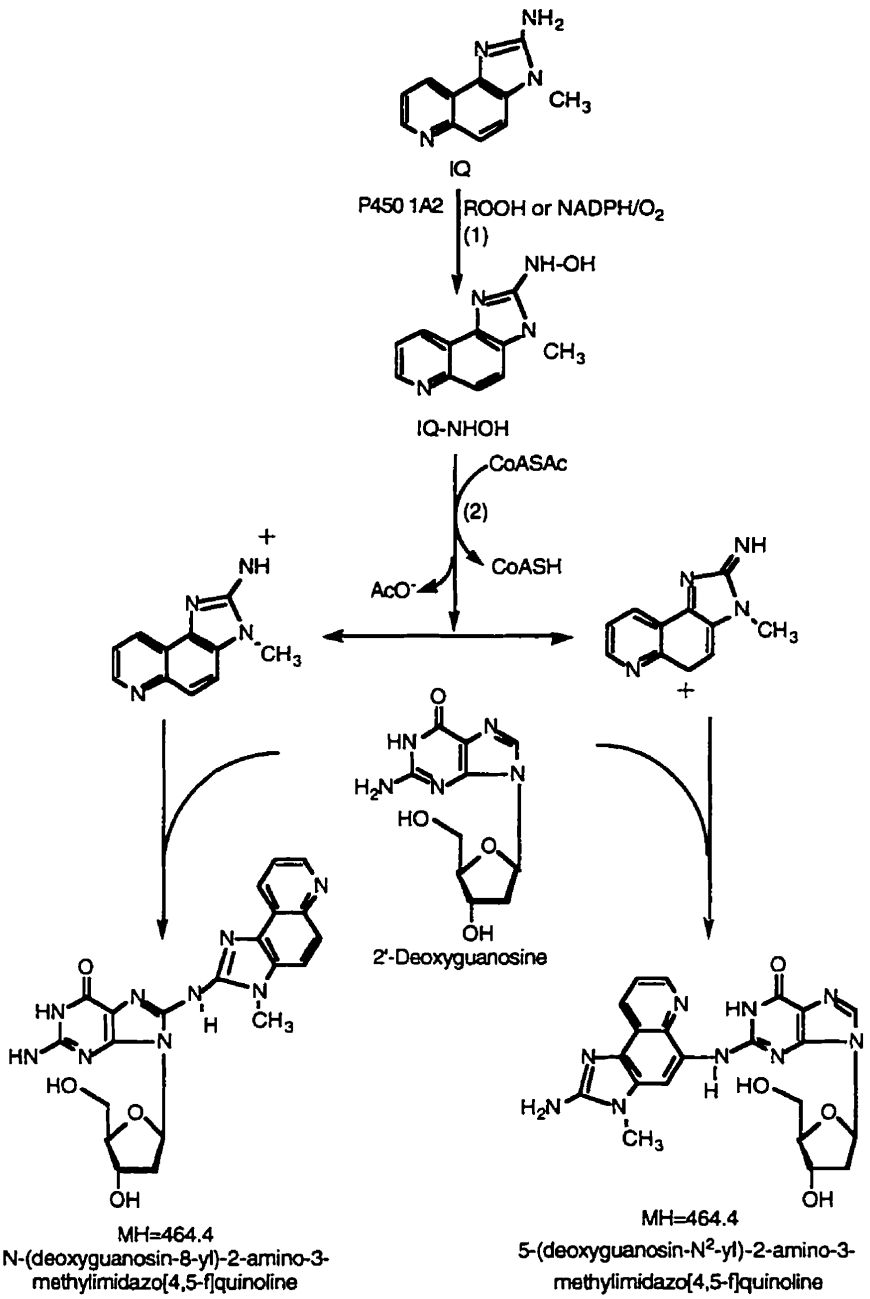

Figure 4-10: Proposed Metabolic Pathway of IQ Bioactivation and Formation of Deoxyguanosine Adducts. (1) P450 monooxygenase/peroxygenase oxidation of IQ to hydroxylamine metabolite (2) Acetyl CoA:arylamine N-acetyltransferase-dependent formation of nitrenium and carbon centered cations.

In conclusion, hydrogen peroxide and alkyl hydroperoxides can support the rat and human P450 1A2-catalyzed metabolic bioactivation of arylamines to reactive genotoxic metabolites. P450 1A2 may be a particularly active peroxygenase with hydrogen peroxide, as a result of its distal active site structure, which favors the heterolytic cleavage of hydroperoxides. The activities of other human P450 enzymes towards hydroperoxide-supported oxidation reactions also require investigation.

4.5. SIGNIFICANCE

In conclusion, hydrogen peroxide and alkyl hydroperoxides can support the rat and human P450 1A2-catalyzed metabolic bioactivation of arylamines to reactive genotoxic metabolites. P450 1A2 may be a particularly active peroxygenase with hydrogen peroxide, as a result of its distal active site structure, which favors the heterolytic cleavage of hydroperoxides.

The enhanced genotoxicity of P450 substrates that may occur at low physiological concentration of hydroperoxides could be of toxicological significance in bioactivation of chemical carcinogens. The xenobiotic reactive metabolites formed through the P450 peroxygenase pathway could potentially contribute to induction of organ selective toxicity and carcinogenesis.

4.6 ACKNOWLEDGMENT

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Chapter 5

THE INVOLVEMENT OF CYTOCHROME P450 PEROXIDASE IN METABOLIC BIOACTIVATION OF CUMENE HYDROPEROXIDE IN ISOLATED RAT HEPATOCYTES*

5.1. ABSTRACT

Organic hydroperoxides are believed to be primarily detoxified in cells by the GSH peroxidase/GSSG reductase system and activated to cytotoxic radical species by non heme iron. However, organic hydroperoxides seem to be bioactivated by cytochrome P450 (P450) in isolated hepatocytes as various P450 (particularly P450 2E1) inhibitors inhibited cumene hydroperoxide (CumOOH) metabolism and attenuated subsequent cytotoxic effects including antimycin-A resistant respiration, lipid peroxidation, iron mobilization, ATP depletion, and cell membrane disruption. CumOOH metabolism was also faster in P450 1A-induced hepatocytes and was inhibited by the P450 1A inhibitor α-naphthoflavone (α-NF). The ferric chelator deferoxamine also prevented cytotoxicity even after CumOOH had been metabolized but had no effect on CumOOH metabolism. This emphasizes the toxicological significance of the iron released following hydroperoxide metabolic activation

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by cytochrome P450. The radical trap, 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO), had no effect on CumOOH metabolism but prevented CumOOH-induced antimycin A-resistant respiration, lipid peroxidation, iron mobilization, and loss of membrane integrity. These results suggest that CumOOH is metabolically activated by some P450 enzymes (eg. P450 2E1) in hepatocytes to form reactive radical metabolites or oxidants that cause lipid peroxidation and cytotoxicity.

5.2. INTRODUCTION

The molecular mechanism of cell injury mediated by oxidative stress and its role in the pathogenesis of various diseases has been the focus of many recent investigations (Biasi *et al.*, 1994; Loughrey *et al.*, 1994; Sies, 1985; Toyokuni *et al.*, 1995). Beside the formation of hydrogen peroxide during the course of oxidative stress, various cellular structural and biochemical elements, e.g. fatty acids, triglycerides, amino acids and nucleic acid bases may also be oxidized to form a wide range of endogenous hydroperoxides (Sies, 1985). However, hydrogen peroxide is rapidly detoxified as a result of decomposition by intracellular catalase and therefore organic hydroperoxides, e.g. tert-butylhydroperoxide or cumene hydroperoxide, which are poor substrates for catalase, are used widely as a model to study oxidative stress mediated cell injury (Bhatnagar, 1994; Glascott *et al.*, 1992; Jones *et al.*, 1981; Masaki *et al.*, 1989).

Studies conducted using hydroperoxides revealed three possible biochemical and molecular cytotoxic mechanisms in isolated hepatocytes. The first mechanism proposed was that organic hydroperoxides were metabolized by intracellular nonheme iron to form free radicals which initiated lipid peroxidation and cell membrane disruption (Caprari *et al.*, 1995; Glascott *et al.*, 1992; Masaki *et al.*, 1989). Malondialdehyde, a lipid peroxidation decomposition product, accumulated before cytotoxicity ensued, and antioxidants or deferoxamine, a ferric ion chelator, prevented cytotoxicity. A second mechanism proposed involves a radical-mediated oxidation of thiols of a mitochondrial permeability transition pore ("megachannel") which opens causing mitochondrial membrane depolarization. Subsequent mitochondrial Ca^{2+} recycling could deplete cellular energy reserves and damage mitochondria (Imberti *et al.*, 1993; Richter and Kass, 1991; Saylor *et al.*, 1995). A third mechanism proposed involved the activation of plasma membrane proteases and phospholipases following an increase in cytosolic Ca^{2+} levels (Bellomo *et al.*, 1982; Richter and Frei, 1988; Rush *et al.*, 1986). By contrast, cytotoxic mechanisms proposed for cultured macrophages, endothelial cells, and fibroblasts involved ATP and NAD^+ depletion resulting from the repair of oxidatively damaged DNA by nuclear poly(ADP-ribose) polymerase (Saylor *et al.*, 1995; Schraufstatter *et al.*, 1988; Thies and Autor, 1991).

In spite of the numerous studies characterizing the mechanism of hydroperoxide-induced lethal injuries (Bellomo *et al.*, 1982; Bhatnagar, 1994; Caprari *et al.*, 1995; Glascott *et al.*, 1992; Imberti *et al.*, 1993; Jones *et al.*, 1981; Richter and Frei, 1988; Richter and Kass, 1991; Rush *et al.*, 1986; Saylor *et al.*, 1995; Schraufstatter *et al.*, 1988; Thies and Autor, 1991; Yamamoto *et al.*, 1993), the cellular biochemical pathways involved in the metabolic activation of hydroperoxides in intact cells have yet to be elucidated.

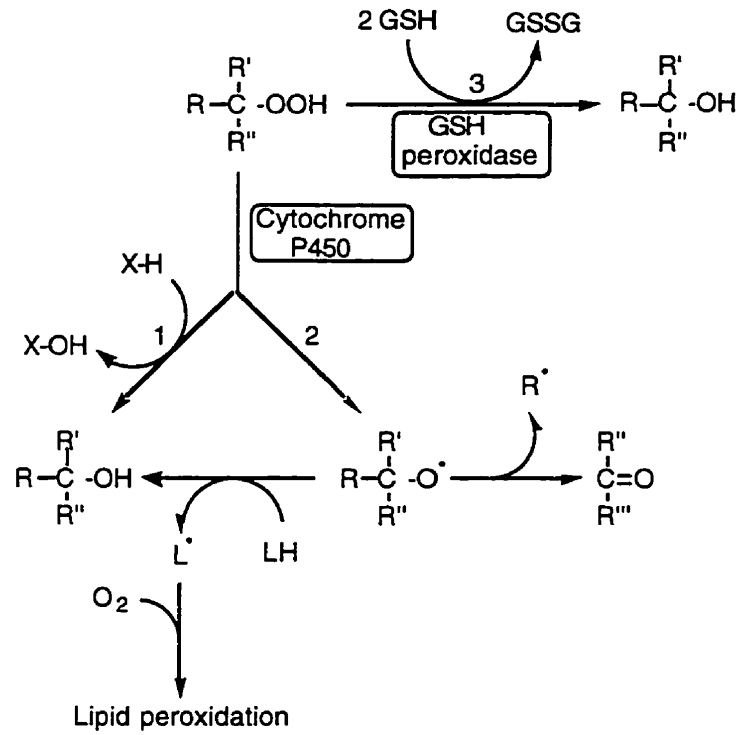

Figure 5-1: Cytochrome P450- and GSH Peroxidase-Dependent Hydroperoxide Metabolism:
(1) Heterolytic cleavage of O-O bond by P450
(2) Homolytic cleavage of O-O bond by P450
(3) Heterolytic cleavage of O-O bond by GSH peroxidase

The metabolism of hydroperoxides and peracids by hepatic microsomal fractions or purified P450 preparations has been extensively studied (Black and Coon, 1987; Blake and Coon, 1981a; Groves *et al.*, 1978; Hrycay and O'Brien, 1971a; Hrycay and O'Brien, 1973; Hrycay and O'Brien, 1974; O'Brien, 1978; O'Brien and Rahimtula, 1975; Vaz and Coon, 1987; Vaz *et al.*, 1990; Wand and Thompson, 1986; White and Coon, 1980) and cytochrome P450s have been shown to carry out both one and two electron reduction of hydroperoxides (Figure 5-1). The homolytic one electron reduction of the hydroperoxide O-O bond (P450 peroxidase activity) results in the formation of alkoxyl radicals and an iron-oxo species analogous to peroxidase Compound II (Wand and Thompson, 1986). The heterolytic two electron reduction of hydroperoxide O-O bond by P450s (P450 peroxygenase activity) results in the formation of an iron-oxo species analogous to peroxidase Compound I, and capable of substrate hydroxylation independent of molecular oxygen and NADPH (Groves *et al.*, 1978; O'Brien, 1978). We have recently shown that hydroperoxides markedly increase the hepatocyte-catalyzed metabolic activation of xenobiotics as a result of cytochrome P450 functioning as a peroxygenase (Anari *et al.*, 1995b). The objective of this study was to determine if cytochrome P450 also contributes to hydroperoxide cytotoxicity in isolated hepatocytes that occurs at higher hydroperoxide concentrations. In the following we present evidence suggesting that hydroperoxide cytotoxicity

and lipid peroxidation are initiated in intact cells by the metabolism of hydroperoxide by certain cytochrome P450 enzymes.

5.3. RESULTS

5.3.1. Effects of P450 Inhibitors and Other Cytoprotective Agents on Hepatocyte CumOOH Metabolism.

CumOOH (150 μM) was rapidly metabolized by isolated hepatocytes with 80% metabolism within 5 min of adding to the hepatocytes (24 nmol CumOOH/min/10^6 cells) as shown in Figure 5-2. The rate of CumOOH metabolism was inhibited up to 61% (10 nmol CumOOH/min/10^6 cells) by the P450 2E1 substrate chlorzoxazone (Bocker *et al.*, 1990) at concentrations that inhibit microsomal P450 2E1 activity. The less selective P450 2E1 substrate, 1-phenylimidazole (Wilkinson *et al.*, 1983) and isoniazid (Zand *et al.*, 1993) also markedly inhibited (54 and 46% inhibition respectively, $p<0.05$) the CumOOH metabolism by untreated hepatocytes. SKF-525A, a non-selective P450 inhibitor, inhibited 33% of the initial rate of hepatocyte CumOOH metabolism ($p<0.05$) whereas α−NF, a P450 1A inhibitor (Rodrigues and Prough, 1991) had no significant effect on CumOOH metabolism by untreated hepatocytes. Moreover, the metabolism of CumOOH was faster in hepatocytes isolated from 3-methylcholanthrene (3MC)-induced rats (29.5 nmol CumOOH/min/10^6 cells, $p<0.05$ compared to untreated hepatocytes) as shown in Figure 5-3. The enhanced rate of

CumOOH metabolism in 3MC-treated hepatocytes was mainly dependent on the activity of P450 1A enzymes as α–NF markedly inhibited CumOOH metabolism (18 nmol CumOOH/min/10^6 cells, $p<0.05$ compared to CumOOH alone). Chlorzoxazone, 1-phenylimidazole, and SKF-525A also inhibited CumOOH metabolism in 3MC-treated hepatocytes (16-25% inhibition, $p<0.05$).

To determine the contribution of GSH peroxidase and cytochrome P450 to the total CumOOH metabolism, the rate of CumOOH metabolism in GSH-depleted hepatocytes was determined in the presence or absence of various P450 substrates or inhibitors. As shown in Figure 5-4, CumOOH was still metabolized in GSH-depleted hepatocytes at 54% of its decomposition rate in control non-treated cells (13 nmol CumOOH/min/10^6 cells). This in turn was inhibited by P450 2E1 substrates up to 45-66% (4-7 nmol CumOOH/min/10^6 cells, $p<0.05$) as well as SKF-525A (36% inhibition, $p<0.05$). On the other hand, α–NF at maximal tolerable concentration had no effect on the rate of CumOOH metabolism in GSH-depleted hepatocytes. The slower rate of CumOOH metabolism in GSH depleted cells enabled us to test the effect of other hydroperoxide cytoprotective agents on CumOOH metabolism. The ferric chelator deferoxamine (Masaki *et al.*, 1989) or the radical trap TEMPO (Voest *et al.*, 1993) did not affect CumOOH metabolism.

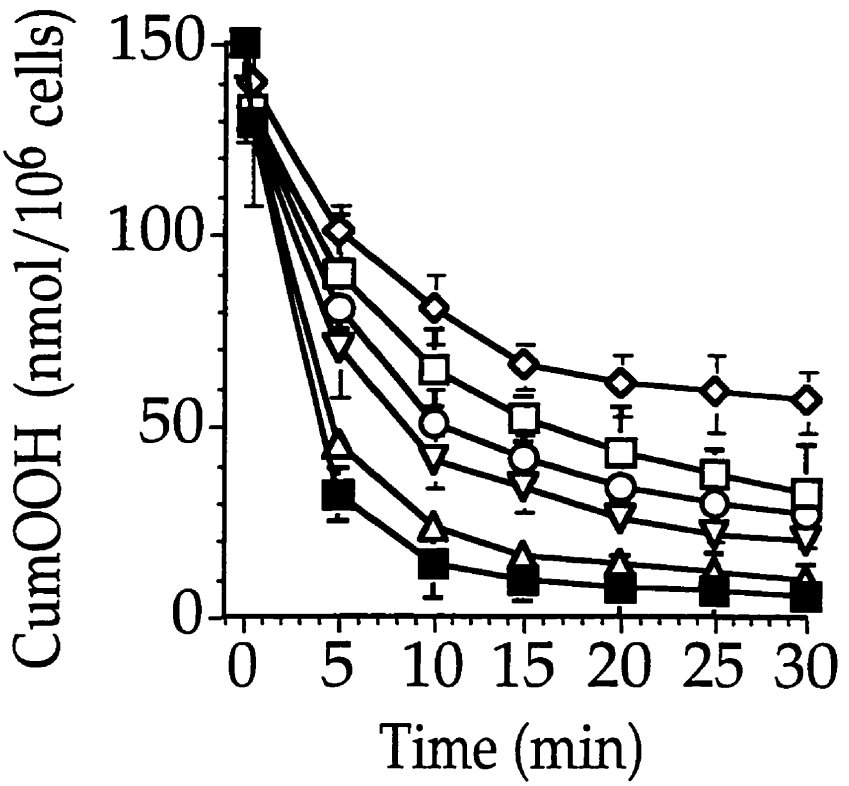

Figure 5-2: Effects of P450 Inhibitors/Substrates and Various Cytoprotective Agents on CumOOH Metabolism in Uninduced Rat Hepatocytes. Hepatocytes (10^6 cells/mL) were incubated with CumOOH (150 μM) (■), chlorzoxazone (400 μM) + CumOOH (◇), phenylimidazole (300 μM) + CumOOH (❑), isoniazid (1 mM) + CumOOH (○), α-naphthoflavone (10 μM) + CumOOH (Δ) or SKF-525A (100 μM) + CumOOH (▽). The analysis of the CumOOH level and the hepatocyte preparation techniques for each group was as described in section 2.12. Values represent mean ± SE of three separate experiments.

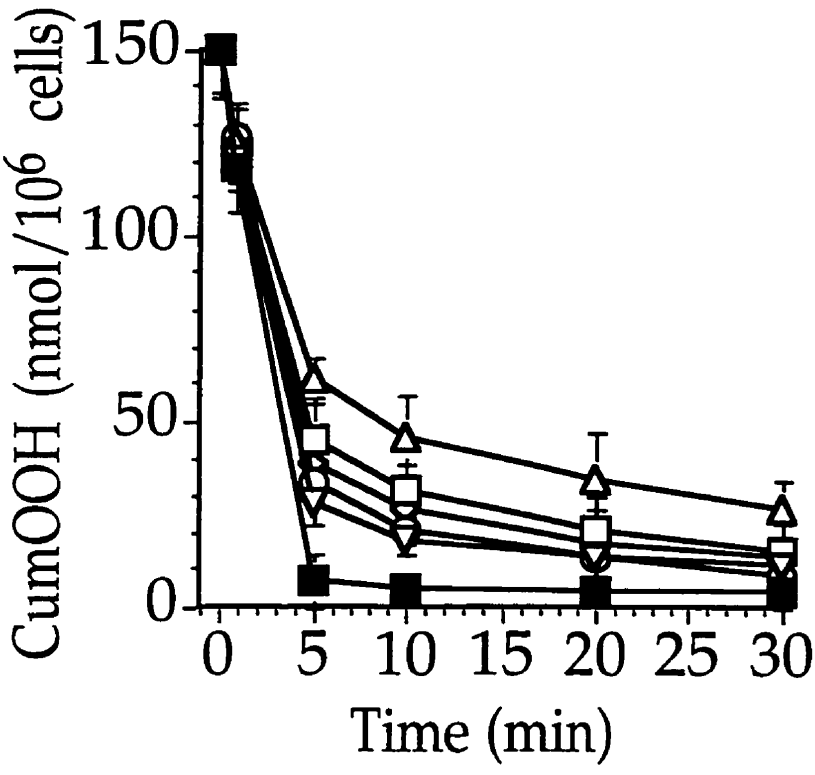

Figure 5-3: Effects of P450 Inhibitors/Substrates and Various Cytoprotective Agents on CumOOH Metabolism in 3-MC-Induced Rat Hepatocytes. Hepatocytes (10^6 cells/mL) were incubated with CumOOH (150 μM) (■), chlorzoxazone (400 μM) + CumOOH (◇), phenylimidazole (300 μM) + CumOOH (❑), isoniazid (1 mM) + CumOOH (○), α- naphthoflavone (10 μM) + CumOOH (Δ) or SKF-525A (100 μM) + CumOOH (▽). The analysis of the CumOOH level and the hepatocyte preparation techniques for each group was as described in section 2.12. Values represent mean ± SE of three separate experiments.

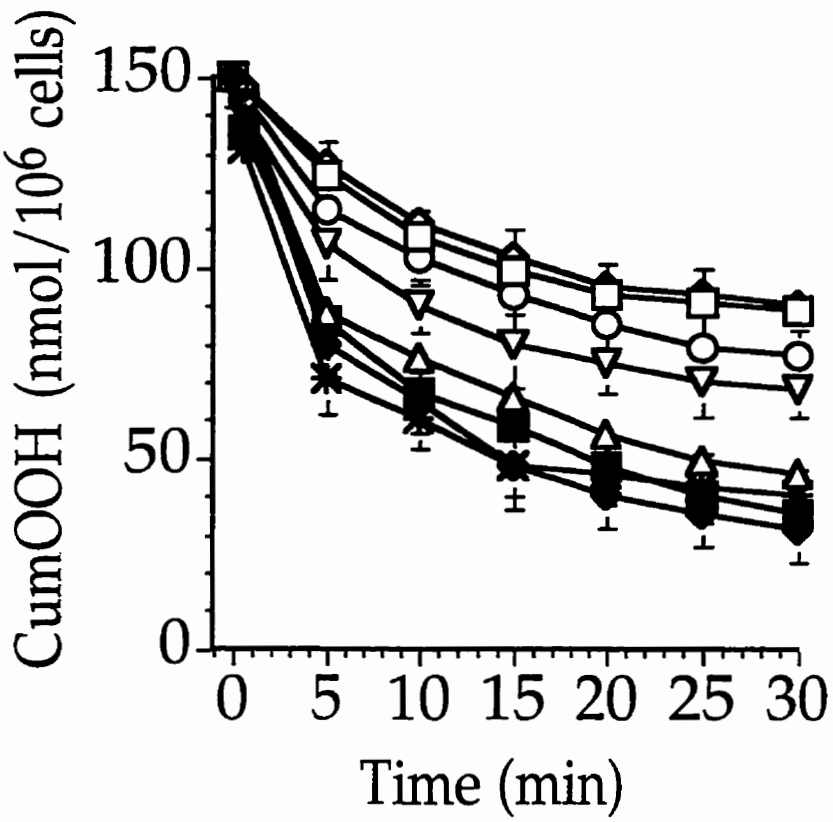

Figure 5-4: Effects of P450 Inhibitors/Substrates and Various Cytoprotective Agents on CumOOH Metabolism in GSH-Depleted Rat Hepatocytes. Hepatocytes (10^6 cells/mL) were incubated with CumOOH (150 μM) (■), chlorzoxazone (400 μM) + CumOOH (◇), phenylimidazole (300 μM) + CumOOH (❑), isoniazid (1 mM) + CumOOH (○), α- naphthoflavone (10 μM) + CumOOH (Δ), SKF-525A (100 μM) + CumOOH (▽), TEMPO (100 μM) + CumOOH (◆) or deferoxamine (500 μM) + CumOOH (*). The analysis of the CumOOH level and the hepatocyte preparation techniques for each group was as described in section 2.12. Values represent mean ± SE of three separate experiments.

5.3.2. Effects of P450 Inhibitors and Other Cytoprotective Agents on CumOOH-Induced Hepatocyte Membrane Disruption

CumOOH at a concentration of 150 μM led to 90% loss of hepatocyte viability at 2 h as determined by the percentage of cells excluding trypan blue (Table 5-1). At maximal non-cytotoxic concentration of P450 substrates/inhibitors, the P450 2E1 substrate, chlorzoxazone, and the non selective P450 2E1 substrates, isoniazid and phenylimidazole, showed high cytoprotection (73-80% inhibition of membrane disruption at 2 h, $p<0.01$) in untreated hepatocytes. Metyrapone, a non-selective P450 inhibitor (Knickle and Bend, 1994), was partially cytoprotective (28% cytoprotection) while α-NF showed no cytoprotection and SKF-525A enhanced CumOOH cytotoxicity (140% increased cell death within 60 min, $p<0.05$).

In the presence of TEMPO or deferoxamine, CumOOH-induced cytotoxicity was prevented up to 79 and 70% respectively ($p<0.01$ compared to control), so that a higher concentration of CumOOH (300 μM) was required to induce a similar cytotoxic response. Cytochrome P450 inhibitors still prevented cytotoxicity at this high CumOOH concentration in the presence of TEMPO indicating that the cytoprotection by P450 inhibitors could not be attributed to any antioxidant activity of these inhibitors (Table 5-1). By contrast, GSH depletion markedly increased the susceptibility of hepatocytes to CumOOH (270% increased cell death at 30 min, $p<0.05$). This suggests that

Table 5-1: Modulation of CumOOH-Induced Hepatocyte Cytotoxicity by Cytochrome P450 Inhibitors/Substrates and Various Cytoprotective Agents[a]

Treatment	Cytotoxicity (per cent trypan blue uptake at time min)			
	30	60	120	180
Control	7 ± 2	7 ± 2	8 ± 3	10 ± 3
CumOOH 0.15 mM	43 ± 4	75 ± 5	91 ± 6	100
" + Chlorzoxazone 400 μM	13 ± 3[b]	17 ± 3[b]	20 ± 2[b]	24 ± 3[b]
" + Phenylimidazole 300 μM	14 ± 3[b]	16 ± 2[b]	18 ± 3[b]	22 ± 4[b]
" + Isoniazid 1mM	17 ± 2[b]	21 ± 3[b]	25 ± 3[b]	27± 4[b]
" + *p*-Nitrophenol 100 μM	27 ± 3[b]	33 ± 3[b]	47 ± 3[b]	58± 5[b]
" + Metyrapone 0.7 mM	31 ± 3	52 ± 4	69 ± 5	88 ± 5
" + SKF-525A 100 μM	76 ± 5	93 ± 7	100	
" + α- NF 10 μM	43 ± 4	71 ± 4	92 ± 5	98 ± 6
" + Bromoheptane 200 μM	100[b]			
" + Deferoxamine 500 μM	18 ± 3	29 ± 3[b]	34 ± 4[b]	39 ± 5[b]
" + Deferoxamine 500 μM (15 min)	25 ± 3	31 ± 3[b]	37 ± 4[b]	41 ± 5[b]
" + TEMPO 100 μM	15 ± 3[b]	23 ± 3[b]	27 ± 2[b]	29 ± 4[b]
CumOOH 0.3 mM	100			
" + TEMPO 100 μM	58 ± 7	78 ± 8	84 ± 8	100
" + " + Chlorzoxazone 400 μM	23 ± 3[b]	28 ± 4[b]	37 ± 4[b]	41 ± 5[b]
" + " + Phenylimidazole 300 μM	21 ± 2[b]	25 ± 3[b]	31 ± 2[b]	34 ± 4[b]
" + " + Isoniazid 1mM	20 ± 3[b]	36± 3[b]	41 ± 4[b]	43 ± 4[b]

[a] Hepatocytes (10^6 cells/mL) were incubated in Krebs-Henseleit buffer (pH 7.4) at 37 °C. Cells were maintained under 1% O_2, 94% N_2 and 5% CO_2 as described under material and methods. Cell viability was determined as the percentage of cells that uptake trypan blue. All cytoprotective agents were added 10 min before CumOOH addition unless indicated. Values are expressed as means of three separate experiment ± SE.

[b] Significantly different from the control CumOOH treated groups. ($p<0.05$).

Table 5-2: Modulation of CumOOH-Induced Antimycin A-Resistant Respiration in Isolated Hepatocytes[a] .

Treatment	Rate of oxygen consumption (nmol oxygen/min/10^6 cells)	%inhibition
CumOOH 150 μM	29.3 ± 3.2	0
" + Chlorzoxazone 400 μM	1.1 ± 0.2[b]	100
" + Phenylimidazole 300 μM	4.3 ± 0.2[b]	89
" + Isoniazid 1mM	2.4 ± 0.2[b]	95
" + in GSH depleted hepatocytes	49.9 ± 4.6[c]	
" + Deferoxamine 500 μM	29.2 ± 2.4	<1
" + TEMPO 100 μM	3.3 ± 0.2[b]	92

[a] Hepatocyte (10^6 cells/mL) mitochondrial respiration was inhibited by the addition of antimycin A (25 μM) and the rate of oxygen uptake was determined with a Clark-type oxygen electrode following the addition of CumOOH to hepatocytes as described in Section 2.11. The control respiration rate before and after antimycin A addition was 32.6 ± 3.8 and 1.1 ± 0.1 nmol oxygen/min/10^6 cells respectively. The P450 inhibitors/substrates, TEMPO, and deferoxamine were added after the baseline of the antimycin A resistant respiration had been stabilized and did not affect the rate of antimycin A resistant respiration.

[b] Significantly different from CumOOH treated group. ($p<0.05$).

[c] 170% increase in the initial rate of oxygen uptake was observed relative to the CumOOH treated group.

GSH peroxidase detoxifies CumOOH whereas cytochrome P450 enzymes bioactivate CumOOH to cytotoxic metabolites.

5.3.3. Effects of P450 Inhibitors and Other Cytoprotective Agents on CumOOH-Induced Oxygen Uptake and Lipid Peroxidation

Cyanide or antimycin-A resistant respiration and lipid peroxidation are generally considered as indices of reactive oxygen radical formation. As shown in Table 5-2, the addition of a cytotoxic CumOOH concentration to hepatocytes caused an immediate increase in antimycin A-resistant respiration of up to 27 fold ($p<0.01$) which was comparable to the normal rate of mitochondrial respiration seen without antimycin A addition (32.2 nmol oxygen/min/10^6 cells). Chlorzoxazone, phenylimidazole and isoniazid markedly prevented (89-100% inhibition, $p<0.01$) CumOOH-induced antimycin A-resistant respiration. TEMPO inhibited the CumOOH-induced antimycin A-resistant respiration (92% inhibition, $p<0.01$) whereas deferoxamine had no effect. The rate of CumOOH-induced oxygen consumption was also 170% greater in GSH-depleted hepatocytes than control ($p<0.01$).

As shown in Figure 5-5, lipid peroxidation accompanied the antimycin A-resistant oxygen uptake and increased up to 32 fold (3.2 nmol TBARS/10^6 cells) after 30 min of CumOOH addition. Lipid peroxidation was also

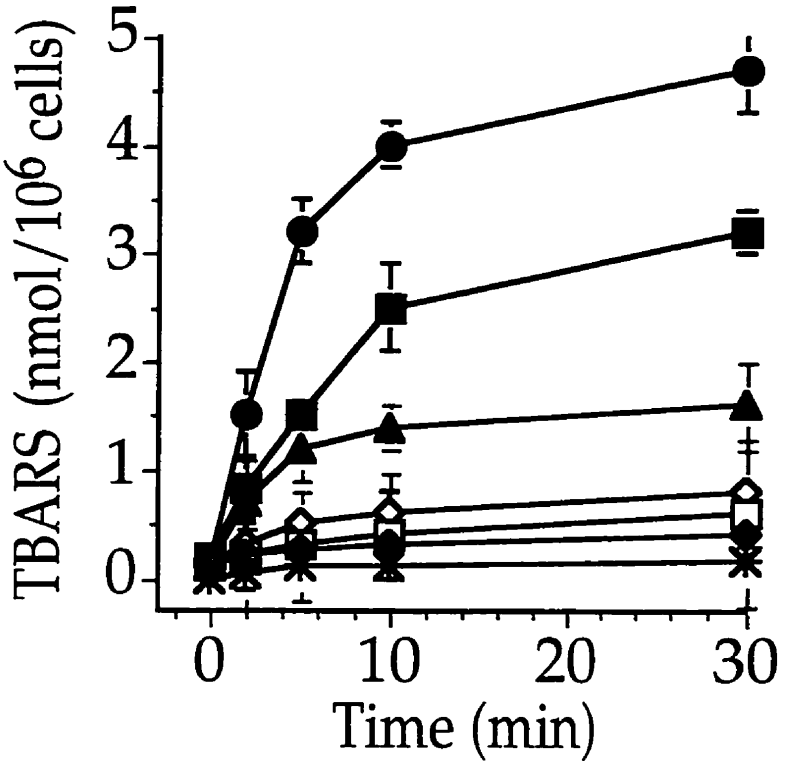

Figure 5-5: Effects of P450 Inhibitors/Substrates and Various Cytoprotective Agents on CumOOH-Induced Hepatocyte Lipid Peroxidation. Hepatocytes (10^6 cells/mL) from uninduced rats were incubated alone (✻) or with CumOOH (150 μM) (■), phenylimidazole (300 μM) + CumOOH (❑), chlorzoxazone (400 μM) + CumOOH (◇), deferoxamine (500 μM) + CumOOH (▲), TEMPO (100 μM) + CumOOH (◆), CumOOH in GSH-depleted hepatocytes (●) and the level of lipid hydroperoxide was measured as described in section 2.11. Values represent mean ± SE of three separate experiments.

prevented by various P450 substrates/inhibitors, e.g. chlorzoxazone and phenylimidazole (90-94% inhibition, $p<0.01$), or the radical trap TEMPO (92% inhibition, $p<0.01$). Deferoxamine, however did not prevent the initial rate of lipid peroxidation ($p>0.05$) measured during the first five min of incubation but inhibited subsequent lipid peroxidation (68% inhibition, $p<0.05$).

The effect of various P450 2E1 substrates/inhibitors on microsomal CumOOH-induced lipid peroxidation was investigated to determine if a correlation exists between the P450 2E1 activity and the metabolic activation of CumOOH. As shown in Table 5-3, hepatic microsomal P450 2E1 activity and CumOOH-induced lipid peroxidation were inhibited by various P450 2E1 substrates or anti-rat P450 2E1 serum. It should be noted that the concentrations of the P450 2E1 substrates required to inhibit hepatic microsomal P450 2E1 activity were the same as those required to inhibit CumOOH metabolism by hepatocytes. At the maximal tolerable concentrations used throughout the study, metyrapone inhibited partially the CumOOH-induced microsomal lipid peroxidation with no significant effect on microsomal P450 2E1 activity. SKF-525A neither inhibited markedly P450 2E1 activity nor prevented CumOOH-induced lipid peroxidation.

Table 5-3: Effect of Cytochrome P450 Inhibitors on the Microsomal CumOOH-Induced Lipid Peroxidation and NADPH-Supported *p*-Nitrophenol Hydroxylation[a] .

Treatment	% Inhibition of Microsomal TBARS[b]	%Inhibition of Microsomal PNPH[c]
Chlorzoxazone 400 μM	87	81
Phenylimidazole 300 μM	96	100
Isoniazid 1 mM	97	93
Metyrapone 0.7 mM	17	6
SKF-525A 100 μM	5	16
α- NF 10 μM	<1	4
Anti-rat P450 2E1 serum 50 μL	71	62

[a]Microsomes from control rat livers were either incubated with CumOOH in the presence of various P450 inhibitors for the analyses of microsomal TBARS or incubated with p-nitrophenol in the presence of various P450 inhibitors/substrates for analysis of PNPH as described in section 2.5. Inhibitors and anti-rat P450 2E1 serum (5.75 mg protein/mL incubation) were preincubated for 2 and 30 min respectively before the addition of CumOOH or p-nitrophenol.

[b]Level of lipid peroxidation in CumOOH-treated group was 17.5 nmol TBARS/mg microsomal protein/30 min incubation.

[c]NADPH-supported PNPH activity in control group was 105 nmol 4-nitrocatechol/mg microsomal protein/30 min incubation.

5.3.4. Effects of P450 Inhibitors and Other Cytoprotective Agents on CumOOH-Induced Hepatocyte Iron Release

Free intracellular iron was released within 5 min of CumOOH addition to hepatocytes and reached a maximum of 190 nmol free iron per 10^6 hepatocytes at 30 min (Figure 5-6). This hydroperoxide-enhanced iron release was also prevented (89-98% inhibition, $p<0.01$) in hepatocytes pretreated with P450 substrates/inhibitors e.g. phenylimidazole and chlorzoxazone. Deferoxamine and TEMPO also prevented the CumOOH-induced hepatocyte iron release.

5.3.5. Effects of P450 Inhibitors and Other Cytoprotective Agents on CumOOH-Induced Hepatocyte GSH and ATP Depletion

The oxidation of GSH to GSSG was determined so as to elucidate the involvement of P450s in initiation of extensive oxidative stress caused by a cytotoxic CumOOH concentration. As shown in Figure 5-7, addition of a cytotoxic CumOOH concentration rapidly oxidized hepatocyte GSH to GSSG (which effluxed the hepatocyte) so that hepatocyte GSH levels remained low until cell death. At lower nontoxic CumOOH concentrations, hepatocyte GSH was oxidized just as rapidly but was reduced back to GSH (without effluxing the cells) (Figures 5-7 and 5-8). A similar GSH recovery also occurred with a CumOOH concentration of 150 μM if hepatocyte cytochrome P450 was inhibited beforehand with phenylimidazole or chlorzoxazone. TEMPO or

deferoxamine had no effect on the rate of GSH oxidation though the GSH level was partially recovered by these cytoprotective agents.

The effects of cytochrome P450 inhibitors on the CumOOH-induced ATP depletion were investigated to determine whether the depletion of cellular ATP that has been attributed to hydroperoxide-mediated mitochondrial cytotoxicity (Saylor *et al.*, 1995) could result from hydroperoxide activation by P450s. As shown in Figure 5-9, hepatocyte ATP levels declined and reached 55% of its control levels following a 30 min incubation with 150 μM CumOOH. Hepatocyte ATP depletion was also prevented by chlorzoxazone, phenylimidazole (83-90% inhibition, $p<0.01$), the radical trap TEMPO (78% inhibition, $p<0.01$) or the ferric chelator deferoxamine (66% inhibition, $p<0.05$).

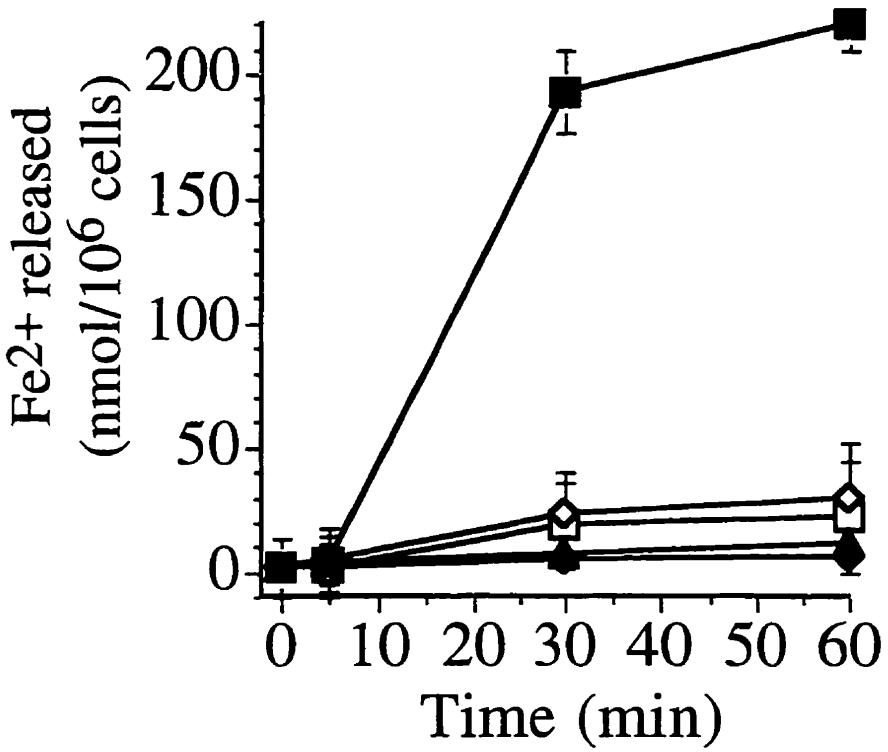

Figure 5-6: Effects of P450 Inhibitors/Substrates and Various Cytoprotective Agents on CumOOH-Induced Hepatocyte Iron Release. Hepatocytes (10^6 cells/mL) from untreated rats were incubated with CumOOH (150 μM) (■), phenylimidazole (300 μM) + CumOOH (❑), chlorzoxazone (400 μM) + CumOOH (◇), deferoxamine (500 μM) + CumOOH (▲), TEMPO (100 μM) + CumOOH (◆) and the level of hepatocyte free iron was measured as described in section 2.13. Values represent mean ± SE of three separate experiments.

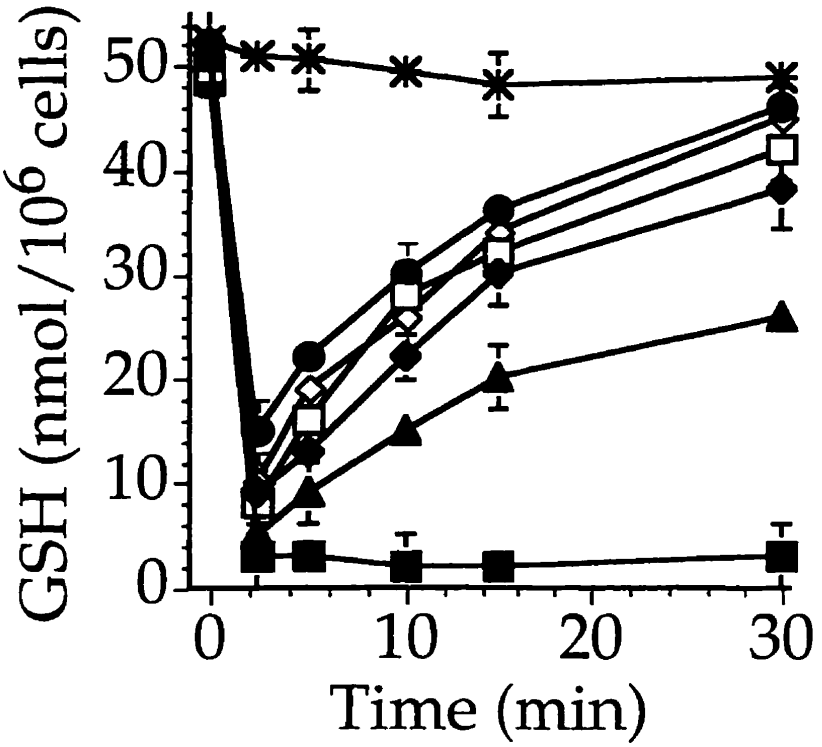

Figure 5-7: Effects of P450 Inhibitors/Substrates and Various Cytoprotective Agents on the Level of Hepatocyte GSH. Hepatocytes (10^6 cells/mL) from untreated rats were incubated alone (✻) or with CumOOH (80 μM) (●), CumOOH (150 μM) (■), phenylimidazole (300 μM) + CumOOH (150 μM) (❑), chlorzoxazone (400 μM) + CumOOH (150 μM) (◊), deferoxamine (500 μM) + CumOOH (150 μM) (▲), TEMPO (100 μM) + CumOOH (150 μM) (◆) and the level of glutathione and glutathione disulfide were measured as described in section 2.14. Values represent mean ± SE of three separate experiments.

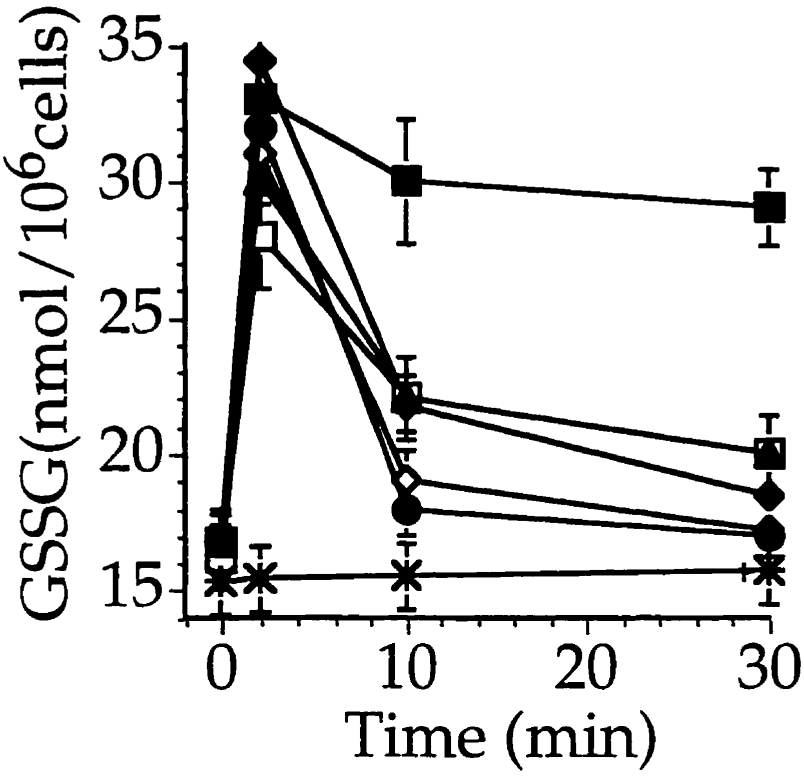

Figure 5-8: Effects of P450 Inhibitors/Substrates and Various Cytoprotective Agents on the Level of Hepatocyte GSSG. Hepatocytes (10^6 cells/mL) from untreated rats were incubated alone (✱) or with CumOOH (80 μM) (●), CumOOH (150 μM) (■), phenylimidazole (300 μM) + CumOOH (150 μM) (❑), chlorzoxazone (400 μM) + CumOOH (150 μM) (◊), deferoxamine (500 μM) + CumOOH (150 μM) (▲), TEMPO (100 μM) + CumOOH (150 μM) (◆) and the level of glutathione and glutathione disulfide were measured as described in section 2.14. Values represent mean ± SE of three separate experiments.

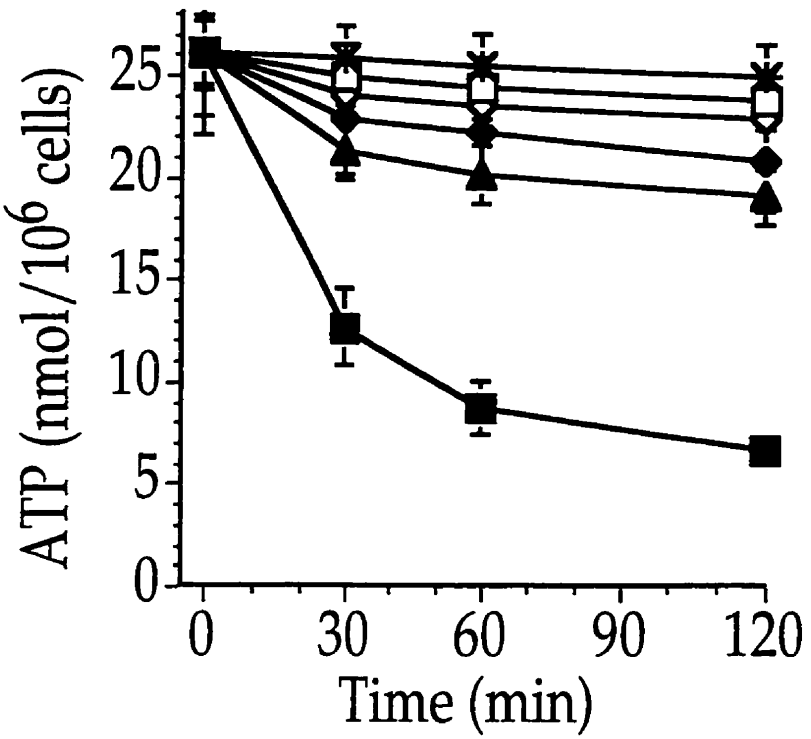

Figure 5-9: Inhibition of CumOOH-Induced ATP Depletion by P450 Inhibitors/Substrates and Antioxidants. Hepatocytes (10^6 cells/mL) from untreated rats were incubated alone (✱) or with CumOOH (150 μM) (■), phenylimidazole (300 μM) + CumOOH (❑), chlorzoxazone (400 μM) + CumOOH (◊), deferoxamine (500 μM) + CumOOH (▲), TEMPO (100 μM) + CumOOH (◆) and the level of ATP was measured as described in section 2.15. Values represent mean ± SE of three separate experiments.

5.4. DISCUSSION

Since hydroperoxide-induced cytotoxicity was widely used as a model to study the mechanisms of oxidative stress cell injury, previous investigators have addressed the cellular cytotoxic targets rather than the metabolic pathways involved in hydroperoxide bioactivation (Bellomo *et al.*, 1982; Bhatnagar, 1994; Caprari *et al.*, 1995; Glascott *et al.*, 1992; Imberti *et al.*, 1993; Jones *et al.*, 1981; Masaki *et al.*, 1989; Richter and Frei, 1988; Richter and Kass, 1991; Rush *et al.*, 1986; Saylor *et al.*, 1995). The cytotoxic mechanisms identified included lipid peroxidation-induced disruption of the cytoskeleton (Caprari *et al.*, 1995; Glascott *et al.*, 1992), mitochondrial membrane depolarization (Saylor *et al.*, 1995), ATP depletion (Imberti *et al.*, 1993) and the Ca^{2+}-dependent activation of proteases and/or phospholipases (Bellomo *et al.*, 1982; Richter and Frei, 1988; Rush *et al.*, 1986). The metabolic basis of the hydroperoxide bioactivation which triggered these cytotoxic cascades however is not clear in intact cells although it is widely believed that the metabolic activation and detoxification steps were mediated by non-heme iron and GSH peroxidase, respectively. In the present study, using freshly isolated rat hepatocytes as a cell model with high P450 content, we have demonstrated that CumOOH metabolism and cytotoxicity can be inhibited by non-toxic concentrations of various P450 substrates/inhibitors.

The inhibition of hepatocyte P450s, e.g. P450 2E1 in uninduced and P450 1A in 3MC-treated hepatocytes, resulted in a marked inhibition of CumOOH

metabolism and cytotoxicity. The extent of hepatocyte CumOOH metabolism by P450s was nearly equal to that of GSH peroxidase, and accounted for up to 50% of the total hepatocyte CumOOH decomposition. We have previously shown that liver microsomal fractions catalyzed a P450-mediated metabolism of cumene hydroperoxide (Hrycay and O'Brien, 1971a; Hrycay and O'Brien, 1973; Hrycay and O'Brien, 1974; O'Brien, 1978; O'Brien and Rahimtula, 1975). Furthermore reduced P450 2E1 was previously shown to be the most efficient P450 isoform in catalyzing the homolytic cleavage of fatty acid hydroperoxides (Vaz *et al.*, 1990). This is consistent with our findings that P450 2E1 inhibitors/substrates prevent CumOOH metabolism and cytotoxicity in untreated hepatocytes. Furthermore, the high correlation found between the inhibition of P450 2E1 activity in uninduced hepatic microsomes and the prevention of microsomal CumOOH-induced lipid peroxidation suggests that P450 2E1 metabolically bioactivates CumOOH in untreated rat hepatocytes. The P450 1A inhibitor, α–NF did not affect CumOOH metabolism or cytotoxicity in untreated hepatocytes which could be attributed to the negligible levels of P450 1A in untreated rat hepatocytes (Guengerich *et al.*, 1982). The inhibition of CumOOH metabolism or cytotoxicity by the non-selective P450 inhibitors metyrapone, phenylimidazole, and SKF-525A in untreated rat hepatocytes may well indicate the involvement of other P450 enzymes in CumOOH metabolism. The CumOOH-supported P450 3A-catalyzed peroxygenation reaction has been recently reported (Brian *et al.*,

1990). SKF-525A, a broad P450 enzyme inhibitor (particularly P450 2C and 2B) inhibited CumOOH metabolism but enhanced CumOOH cytotoxicity in the present study. In untreated rat hepatocytes, P450 2B levels are negligible while the expression of P450 2C11, 2C6, and 2C7 accounts for over 50% of the total P450 content (Waxman *et al.*, 1985). This suggests that P450 2C enzymes are involved in metabolic detoxification of CumOOH in untreated rat hepatocytes. It is likely that P450 enzymes can either bioactivate or detoxify hydroperoxides depending on the hydroperoxide substrate/P450 isoform involved as metyrapone was recently found to markedly enhance butylated hydroxytoluene hydroperoxide cytotoxicity towards isolated hepatocytes even though it delayed CumOOH cytotoxicity (Thompson *et al.*, 1995), in agreement with the results of the present study.

The P450-dependent metabolism of hydroperoxides by liver microsomes was previously shown to markedly induce microsomal oxygen uptake and lipid peroxidation which could be prevented by antioxidant radical traps or P450 inhibitors (O'Brien and Rahimtula, 1975; Weiss and Estabrook, 1986). In hepatocytes it was found that the radical trap TEMPO or P450 inhibitors prevented CumOOH-induced antimycin A-resistant respiration and lipid peroxidation, whereas TEMPO had no effect on CumOOH metabolism. The alkoxyl radicals generated through the P450-dependent homolytic cleavage of CumOOH (Barr *et al.*, 1996) in the absence of xenobiotics could be responsible for initiation of lipid peroxidation. Most of

the CumOOH metabolism by P450 is heterolytic and results in the formation of cumyl alcohol and its oxygenated metabolite 2-phenyl-1,2-propanediol (Wand and Thompson, 1986; Weiss and Estabrook, 1986). Lipid peroxidation could also be initiated by the intermediate ferryl cytochrome P450 oxidation states formed in the absence of xenobiotics as has been shown with ferrylmyoglobin (Maiorino *et al.*, 1994).

Non-heme iron appears to play a minor role in the metabolism of CumOOH by freshly isolated hepatocytes as the ferric chelator deferoxamine had no effect on CumOOH metabolism and was still cytoprotective when added after CumOOH had been metabolized. Deferoxamine also did not affect CumOOH-induced antimycin A-resistant respiration or the initial rate of lipid peroxidation which further suggests that free iron is not involved in CumOOH metabolic activation. In fact hepatocyte free iron release occurred when at least 80% of the CumOOH had been metabolized. The CumOOH-mediated iron release was also prevented by the P450 substrates phenylimidazole and chlorzoxazone. This suggests that iron was mobilized following hydroperoxide metabolic activation by cytochrome P450 and that iron contributes to CumOOH cytotoxicity. It is possible that the iron is released as a result of P450 heme oxidative destruction (O'Brien, 1978). The critical role of iron in the production of reactive oxygen species and induction of oxidative cell injuries by various toxins has been well documented (Minotti and Aust, 1989). Normally the levels of free intracellular iron,

especially ferric ion (10^{-17} M), are extremely low since it is immobilized in the ferritin core (Williams, 1990). Recent reports also suggest the existence of redox active iron binding sites in hepatocytes that participate in hydroperoxide-mediated cytotoxicity (Shertzer *et al.*, 1992).

The rapid oxidation of GSH catalyzed by GSH peroxidase and the subsequent reduction of GSSG catalyzed by NADPH-dependent GSSG-reductase provides an efficient enzymatic system for hydroperoxide metabolic detoxification (Eklow *et al.*, 1981; Jones *et al.*, 1981; Sies, 1985). The analysis of GSH/GSSG levels following addition of a nontoxic concentration of CumOOH also demonstrated a complete recovery of GSH in isolated hepatocytes. At cytotoxic CumOOH concentrations, most of the GSSG formed by the rapid oxidation of GSH effluxes the hepatocytes but some of the GSSG reacts with protein thiols to form mixed protein disulfides (Brigelius *et al.*, 1983). By using a toxic concentration of CumOOH, the ratio of GSH/GSSG rapidly declined following hydroperoxide addition to hepatocytes and remained low until cell death. However the GSSG was reduced back to GSH with little GSSG efflux if hepatocytes were pretreated with P450 inhibitors or radical traps. This suggests that GSSG reductase is probably inactivated by the radicals or oxidizing species formed following cytochrome P450 catalyzed CumOOH metabolism.

Hepatocyte ATP levels also declined following CumOOH metabolism, in confirmation of previously published reports on hydroperoxide-mediated

mitochondrial cytotoxicity (Saylor *et al.*, 1995). This was also prevented by cytochrome P450 substrates/inhibitors. Furthermore TEMPO or deferoxamine prevented this loss of cellular ATP induced by CumOOH.

Freshly isolated hepatocytes seem to be nearly an order of magnitude more susceptible to organic hydroperoxides than primary cultured hepatocytes (Glascott *et al.*, 1992; Masaki *et al.*, 1989; Yamamoto *et al.*, 1993). One explanation could be the higher level of P450s in freshly isolated hepatocytes (Moldeus *et al.*, 1978) as P450s including P450 2E1 are rapidly down-regulated following hepatocyte cultivation (Wu *et al.*, 1990).

5.5. SIGNIFICANCE

In conclusion, the present study suggests that CumOOH is metabolically bioactivated or inactivated in hepatocytes by certain P450 enzymes. Bioactivation appears to involve the formation of oxidizing radical species which initiate lipid peroxidation and membrane disruption. The effectiveness of various P450 inhibitors in prevention of hydroperoxide-induced oxidative stress *in vivo* requires further investigation. This could have significant biological implications as P450 inhibitors may be used therapeutically to prevent the initiation and propagation of hydroperoxide-induced injuries during the course of oxidative stress in tissues with substantial levels of P450 enzymes.

Chapter 6

SUMMARY, CONCLUSION, FUTURE PROSPECTIVE AND IMPLICATION

6.1. SUMMARY

P450 enzymes can catalyze the oxidation of various substrates independent of molecular oxygen and reducing enzymes if alkyl or lipid hydroperoxides are used as the oxidant source (Hrycay *et al.*, 1976; Hrycay and O'Brien, 1971a; Hrycay and O'Brien, 1971b; O'Brien, 1978; Rahimtula and O'Brien, 1974; Rahimtula and O'Brien, 1975; Rahimtula and O'Brien, 1977a). Analysis of the microsomal hydroperoxide-supported oxidation reaction has provided valuable insights into the mechanism of oxygen activation by P450 (Guengerich and MacDonald, 1990; O'Brien, 1978; Ortiz de Montellano, 1995b), but the significance of this pathway in intact cells remained unresolved. Characterization of the functional role of the P450 peroxygenase pathway at a cellular level in catalyzing xenobiotic oxidations was therefore the primary objective of this. This topic was addressed in Chapters 3 and 4.

In **Chapter 3**, the cytochrome P450-catalyzed xenobiotic metabolism and cytotoxicity towards isolated rat hepatocytes was found to be markedly enhanced by the addition of nontoxic concentrations of an alkyl hydroperoxide, *tert*-butyl hydroperoxide (Anari *et al.*, 1995b). The molecular mechanism of hydroperoxide-enhanced xenobiotic cytotoxicity was shown to

be due to an increased hepatocyte metabolism of xenobiotics to cytotoxic metabolites. The reactive metabolites formed were identified by the LC/MS analysis of the metabolite-GSH conjugates. The hydroperoxide-supported cellular bioactivation of 4-HA, a hepatotoxic antimelanoma agent, revealed that the 4-HA reactive intermediates formed were *p*-benzoquinone and *o*-quinone.

In **Chapter 4**, the toxicological significance of the P450 peroxygenase pathway has been assessed by using hydroperoxides known to be formed in vivo with human P450 enzyme systems and dietary xenobiotics. The bioactivation of the typical procarcinogen/promutagen arylamine P450 1A2 substrate, IQ, was shown to be efficiently supported by low concentrations of hydrogen peroxide through the P450 peroxygenase pathway by both human and rat P450 1A2 (Anari *et al.*, 1996a). The enhanced-genotoxicity of IQ in the intact cells, expressing human NAT enzymatic activity, was shown to occur parallel to the formation of DNA-reactive metabolites.

Cytochromes P450 are also known to metabolize hydroperoxides through a homolytic cleavage of the hydroperoxide O-O bound. This P450 "peroxidase" type of reaction can catalyze a variety of novel transformations of fatty acid hydroperoxides in plants, platelets, and endothelial cells via radical intermediates to form important biological mediators, e.g., prostacyclin and thromboxane (Song *et al.*, 1993; Ullrich and Graf, 1984). The toxicological significance of this intracellular radical formation by P450

"peroxidase" activity has not been investigated, even though peroxides and hydroperoxides have been widely used as model oxidants to study the mechanisms of oxidative stress cell injury. Until now, it has been widely believed that hydroperoxides were detoxified by GSH peroxidase and activated by non-heme iron. **Chapter 5** described how the metabolism and cytotoxicity of organic hydroperoxides in intact cells is highly dependent on the activity of P450 enzymes, particularly P450 2E1 for CumOOH in untreated rat hepatocytes and P450 1A for CumOOH in 3-MC-treated rat hepatocytes (Anari *et al.*, 1996b). Hydroperoxide-induced oxidative stress (analyzed by GSH/GSSG ratio, antimycin A-resistance respiration, lipid peroxidation, ATP depletion, and cell membrane damage) was prevented by various P450 substrates/inhibitors, radical trap, and iron chelator treatment. This indicates for the first time that cytochrome P450 modulates hydroperoxide-induced oxidative stress in intact cells.

6.2. CONCLUSION

We have shown that both the acute cytotoxicity and genotoxicity of P450 substrates can be markedly enhanced by hydroperoxides through the cytochrome P450 peroxygenase pathway. The former can occur at non-cytotoxic hydroperoxide concentrations that do not overwhelm the cellular antioxidant defense system and directly cause cell death. It remains to be determined whether *in vivo* "oxidative stress", which leads to the formation

of cellular hydroperoxides (Sies, 1991), increases xenobiotic metabolic detoxification or bioactivation *in vivo*. A significantly enhanced xenobiotic biotransformation could, in turn, affect the disposition of endobiotics or xenobiotics as well as their pharmacodynamic/toxicodynamic potential *in vivo*.

The enhanced genotoxicity of P450 substrates that could occur at low physiological concentrations of hydroperoxides is also of toxicological significance, particularly in the bioactivation of chemical carcinogens. The xenobiotic reactive metabolites formed through the P450 peroxygenase pathway could potentially contribute to the induction of organ selective toxicity and carcinogenesis.

The role of cytochrome P450 enzymes in the metabolic activation of xenobiotics as a partial explanation for their organ selective cytotoxicity or carcinogenesis is well documented (Guengerich, 1988). However a careful examination of the process usually reveals the underlying existence of extensive cellular oxidative stress in the target tissue. For example, ethanol, estrogens, polycyclic aromatic hydrocarbons and many xenobiotics that are known P450 substrates, induce cytochrome P450-dependent metabolism of xenobiotics and organ selective toxicity parallel to the induction of lipid peroxidation and oxidative stress. As yet the possibility has not been considered that the endogenous hydroperoxides formed could serve as

cofactors for P450 peroxygenase/peroxidase xenobiotic metabolic activation *in vivo*.

6.3. FUTURE PROSPECTIVE AND IMPLICATION

The use of heterologous expression systems to study structural and functional aspects of various forms of cytochrome P450 has found wide acceptance in recent years (Gonzalez *et al.*, 1991; Langenbach *et al.*, 1992). A panel of human cell lines each expressing a single human P450 isoform (Crespi *et al.*, 1991) could be used to characterize the role of various individual human P450 isoforms in catalyzing xenobiotic metabolism. Hydroperoxides may then be useful for following xenobiotic metabolism in cells which do not have active P450 reductase and NADPH generating systems.

Hydroperoxides could also have implications in the detoxification and clean up of contaminated soil using P450 enriched fungi, yeast, and bacteria (Shah *et al.*, 1992).

Chapter 7

REFERENCES

Adamson, R. H., Thorgeirsson, U. P., Snyderwine, E. G., Thorgeirsson, S. S., Reeves, J., Dalgard, D. W., Takayama, S., and Sugimura, T. (1990) Carcinogenicity of 2-amino-3-methylimidazo[4,5-f]quinoline in nonhuman primates: induction of tumors in three macaques. *Jpn. J. Cancer Res.* **81,** 10-4.

Anari, M. R., Cribb, A. E., and Renton, K. W. (1995a) The duration of induction and species influences the downregulation of cytochrome P450 by the interferon inducer polyinosinic acid-polycytidylic acid. *Drug. Metab. Dispos.* **23,** 536-41.

Anari, M. R., Josephy, P. D., Henry, T., and O'Brien, P. J. (1996a) Hydrogen peroxide supports human and rat cytochrome P450 1A2-catalyzed 2-amino-3-methylimidazo[4,5f]quinoline bioactivation to mutagenic metabolites: significance of cytochrome P450 peroxygenase. *Chem. Res. Toxicol.*, submitted.

Anari, M. R., Khan, S., Liu, Z. C., and O'Brien, P. J. (1995b) Cytochrome P450 peroxidase/peroxygenase mediated xenobiotic metabolic activation and cytotoxicity in isolated hepatocytes. *Chem. Res. Toxicol.* **8,** 997-1004.

Anari, M. R., Khan, S., and O'Brien, P. J. (1996b) The involvement of cytochrome P450 peroxidase in the metabolic bioactivation of cumene hydroperoxide by isolated rat hepatocytes. *Chem. Res. Toxicol.* **9,** 924-931.

Baillie, T. A., and Davis, M. R. (1993) Mass spectrometry in the analysis of glutathione conjugates. *Biol. Mass Spectrom.* **22,** 319-325.

Barr, D. P., Martin, M. V., Guengerich, F. P., and Mason, R. P. (1996) Reaction of cytochrome P450 with cumene hydroperoxide: ESR spin-trapping evidence

for the homolytic scission of the peroxide O-O bond by ferric cytochrome P450 1A2. *Chem. Res. Toxicol.* **9,** 318-325.

Beaune, P. H., Kremers, P. G., Kaminsky, L. S., De Graeve, J., Albert, A., and Guengerich, F. P. (1986) Comparison of monooxygenase activities and cytochrome P-450 isozyme concentrations in human liver microsomes. *Drug Metab. Dispos.* **14,** 437-42.

Belcher, H. J. C. R., Nizam, N., and O'Neill, T. J. (1992) Intra-arterial 4-hydroxyanisole chemotherapy for locally recurrent malignant melanoma: a re-appraisal. *Br. J. Plast. Surg.* **45,** 208-210.

Bellomo, G., Jewell, S. A., Thor, H., and Orrenius, S. (1982) Regulation of intracellular calcium compartmentation: studies with isolated hepatocytes and t-butyl hydroperoxide. *Proc. Natl. Acad. Sci. U.S.A.* **79,** 6842-6846.

Bhatnagar, A. (1994) Biochemical mechanism of irreversible cell injury caused by free radical-initiated reactions. *Mol. Cell. Biochem.* **137,** 9-11.

Biasi, F., Chiarpotto, E., Lanfranco, G., Capra, A. Z., U., Chiappino, I., Scavazza, A., Albano, E., and Poli, G. (1994) Oxidative stress in the development of human ischemic hepatitis during circulatory shock. *Free Rad. Biol. Med.* **17,** 225-233.

Black, S. D., and Coon, M. J. (1985) Studies on the identity of the heme-binding cysteinyl residue in rabbit liver microsomal cytochrome P-450 isozyme 2. *Biochem. Biophys. Res. Commun.* **128,** 82-89.

Black, S. D., and Coon, M. J. (1987) P-450 cytochromes: structure and function. *Adv. Enzymol. Rel. Areas Mol. Biol.* **60,** 35-87.

Blake, R. C., and Coon, M. J. (1980) On the mechanism of action of cytochrome P-450. Spectral intermediates in the reaction of $P450_{LM2}$ with peroxy compounds. *J. Biol. Chem.* **255,** 4100-4111.

Blake, R. C., and Coon, M. J. (1981a) On the mechanism of action of cytochrome P-450. Cytochrome P-450. Evaluation of homolytic and heterolytic mechanisms of oxygen-oxygen bond cleavage during substrate hydroxylation by peroxides. *J. Biol. Chem.* **256,** 12127-12133.

Blake, R. C., and Coon, M. J. (1981b) On the mechanism of action of cytochrome P-450. Role of peroxy spectral intermediates in substrate hydroxylation. *J. Biol. Chem.* **256,** 5755-5763.

Bocker, P. R. 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.

Boobis, A. R., and Davies, D. S. (1984) Human cytochromes P-450. *Xenobiotica* **14,** 151-185.

Boyd, J. A. (1990) Prostaglandin H synthase. *Methods Enzymol.* **186,** 283-287.

Bray, H. G., Craddock, V. M., and Thrope, W. V. (1955) Metabolism of ethers in the rabbit. *Biochem. J.* **60,** 225-232.

Brian, W. R., Sari, M. A., Iwasaki, M., Shimada, T., Kaminsky, L. S., and Guengerich, F. P. (1990) Catalytic activities of human liver cytochrome P-450 IIIA4 expressed in Saccharomyces cerevisiae. *Biochemistry* **29,** 11280-92.

Brigelius, R., Mucked, C., Akerboom, T. P. M., and Sies, H. (1983) Identification and quantification of glutathione in hepatic protein mixed disulfides and its relationship to glutathione disulfide. *Biochem. Pharmacol.* **32,** 2529-2539.

Brown, R. C., and Bidlack, W. R. (1991) Dietary magnesium depletion: *p*-Nitroanisole metabolism and glucuronidation in rat hepatocytes and hepatic microsomal membranes. *Proc. Soc. Exp. Biol. Med.* **197,** 85-91.

Bruice, T. C., Balasubramanian, P. N., Lee, R. W., and Lindsay Smith, J. R. (1988) The mechanism of hydroperoxide O-O bond scission on reaction of hydroperoxides with iron (III) porphyrins. *J. Amer. Chem. Soc.* **110,** 7890-7892.

Brumby, P. E., and Massey, V. (1967) Determination of nonheme iron, total iron and copper. *Methods Enzymol.* **10,** 463-474.

Bui, Q., and Weisz, J. (1988) Identification of microsomal, organic hydroperoxide-dependent catechol estrogen formation: comparison with NADPH-dependent mechanism. *Pharmacology* **36,** 356-64.

Burke, M. D., and Mayer, R. T. (1975) Inherent specificities of purified cytochrome P450 and P448 toward biphenyl hydroxylation and ethoxyresorufin deethylation. *Drug Metab. Dispos.* **3,** 245-253.

Burke, M. D., and Orrenius, S. (1978) The effect of albumin on the metabolism of ethoxyresorufin through O-demethylation and sulphate-conjugation using isolated rat hepatocytes. *Biochem. Pharmacol.* **27,** 1533-1538.

Butler, M. A., Guengerich, F. P., and Kadlubar, F. F. (1989) Metabolic oxidation of the carcinogens 4-aminobiphenyl and 4,4'-methylene-bis(2-chloroaniline) by human hepatic microsomes and by purified rat hepatic cytochrome P-450 monooxygenases. *Cancer Res.* **49,** 25-31.

Capdevila, J., Estabrook, R. W., and Prough, R. A. (1980) Differences in the mechanism of NADPH- and cumene hydroperoxide supported reactions of cytochrome P450. *Arch. Biochem. Biophys.* **200,** 186-195.

Caprari, P., Bozzi, A., Malorni, W., Bottini, A., Iosi, F., Santini, M. T., and Salvati, A. M. (1995) Junctional sites of erythrocyte skeletal proteins are specific targets of tert-butylhydroperoxide oxidative damage. *Chem,-Biol. Interact.* **94,** 245-258.

Caughey, W. S., and Ibers, J. A. (1977) Crystal and molecular structure of the free base porphyrin, protoporphyrin IX dimethyl ester. *J. Amer. Chem. Soc.* **99,** 6639-6645.

Cavalieri, E. L., Rogan, E. G., Cremonesi, P., and Devanesan, P. D. (1988) Radical cations as precursors in the metabolic formation of quinones from benzo[a]pyrene and 6-fluorobenzo[a]pyrene. *Biochem. Pharmacol.* **37,** 2173-2182.

Chaudhary, A. K., Nokubo, M., Reddy, G. R., Yeola, S. N., Morrow, J. D., Blair, I. A., and Marnett, L. J. (1994) Detection of endogenous malondialdehyde-deoxyguanosine adducts in human liver. *Science* **265,** 1580-1582.

Cholerton, S., Daly, A. K., and Idle, J. R. (1992) The role of individual human cytochrome P450 in drug metabolism and clinical response. *Trends Pharmacol. Sci.* **13,** 434-439.

Coon, M. J. (1978) Reconstitution of the cytochrome P450-containing mixed-function oxidase system of liver microsomes. *Methods Enzymol.* **52,** 200-206.

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

Coon, M. J., and Porter, T. D. (1988) The cytochrome P-450 gene superfamily. In *Toxicological and Immunological Aspects of Drug Metabolism and Environmental chemicals* (Estabrook, R. W., Lindenlaub, E., Oesch, F., and de Weck, A. L., ed.) pp. 11-25, Schattauer, Stuttgart.

Cornish-Bowden, A. (1979) *Fundamentals of enzyme kinetics.* Butterworth & Co Ltd, Toronto.

Crespi, C. L., Penman, B., Steimel, D. T., Gelboin, H. V., and Gonzalez, F. J. (1991) The development of a human cell line stably expressing human

CYP3A4: role in the metabolic activation of aflatoxin B1 and comparison to CYP1A2 and CYP2A3. *Carcinogenesis* **12,** 355-359.

Cstro, C. E. (1980) Mechanisms of reaction of hemoproteins with oxygen and hydrogen peroxide in the oxidation of organic substrates. *Pharmacol. Ther.* **10,** 171-189.

Dallner, G. (1978) Isolation of microsomal subfractions by use of density gradients. *Methods Enzymol.* **52,** 71-83.

Dargel, R. (1992) Lipid peroxidation--a common pathogenic mechanism? *Exp. Toxicol. Pathol.* **44,** 169-181.

Dawson, J. H., Andersson, L. A., and Sono, M. (1982) Spectroscopic investigations of ferric cytochrome P-450cam ligand complex. *J. Biol. Chem.* **257,** 3606-3617.

Eckert, K.-G., Eyer, P., Sonnenbichler, J., and Zetl, I. (1990) Activation and detoxification of aminophenols, III. Synthesis and structural elucidation of various glutathione addition products to 1,4-benzoquinone. *Xenobiotica* **20,** 351-361.

Eklow, L., Thor, H., and Orrenius, S. (1981) Formation and efflux of glutathione disulfide studied in isolated hepatocytes. *FEBS Lett.* **127,** 125-128.

Estabrook, R. W., Chacos, N., Martin-Wixtrom, C., and Capdevilla, J. (1982) Cytochrome P-450: a versatile vehicle of variable veracity. In *Oxygenases and Oxygen Metabolism* (Nozaki, M., Yamamoto, S., Ishimura, Y., Coon, M. J., Ernster, L., and Estabrook, R. W., ed.) pp. 371-381, Academic Press, New York.

Estabrook, R. W., Martin-Wixtrom, C., Saeki, Y., Renneberg, R., Hildebrandt, A., and Werringloer, J. (1984) The peroxidatic function of liver microsomal cytochrome P-450: comparison of hydrogen peroxide and NADPH-catalysed N-demethylation reactions. *Xenobiotica* **14,** 87-104.

Frandsen, H., Grivas, S., Turesky, R. J., Andersson, R., Dragsted, L. O., and Larsen, J. C. (1994) Formation of DNA adducts by the food mutagen 2-amino-3,4,8-trimethyl-3H-imidazo[4,5-f]quinoxaline (4,8-DiMeIQx) in vitro and in vivo. Identification of a N2-(2'-deoxyguanosin-8-yl)-4,8-DiMeIQx adduct. *Carcinogenesis* **15,** 2553-8.

Gander, J. E., and Mannering, G. J. (1980) Kinetics of hepatic cytochrome P-450 dependent monooxygenase streams. *Pharmacol. Ther.* **10,** 191-221.

Glascott, P. A., Gilfor, E., and Farber, J. L. (1992) Effects of vitamin E on the killing of cultured hepatocytes by tert-butyl hydroperoxide. *Mol. Pharmacol.* **4,** 1155-1162.

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

Gonzalez, F. J. (1990) Molecular genetics of the P-450 superfamily. *Pharmacol. Ther.* **45,** 1-38.

Gonzalez, F. J., Crespi, C. L., and Gelboin, H. V. (1991) DNA-expressed human cytochrome P450s: a new age of molecular toxicology and human risk assessment. *Mutat. Res.* **247,** 113-127.

Gonzalez, F. J., and Gelboin, H. V. (1994) Role of human cytochrome P450 in the metabolic activation of chemical carcinogens and toxins. *Drug Metab. Rev.* **26,** 165-183.

Gotz, M. E., Kunig, G., Riederer, P., and Youdim, M. B. (1994) Oxidative stress: free radical production in neural degeneration. *Pharmacol. Ther.* **63,** 37-122.

Groves, J. T., McClusky, G. A., White, R. E., and Coon, M. J. (1978) Aliphatic hydroxylation by highly purified liver microsomal cytochrome P-450.

Evidence for a carbon radical intermediate. *Biochem. Biophys. Res. Commun.* **81,** 154-160.

Guengerich, F. P. (1988) Roles of cytochrome P-450 enzymes in chemical carcinogenesis and cancer chemotherapy. *Cancer Res.* **48,** 2946-2954.

Guengerich, F. P. (1990) Characterization of roles of human cytochrome P-450 enzymes in carcinogen metabolism. *Asia Pacific J. Pharmacol.* **5,** 327-345.

Guengerich, F. P. (1991) Reactions and significance of cytochrome P-450 enzymes. *J. Biol. Chem.* **266,** 10019-22.

Guengerich, F. P. (1992a) Characterization of human cytochrome P450 enzymes. *FASEB J.* **6,** 745-748.

Guengerich, F. P. (1992b) Human cytochrome P-450 enzymes. *Life Sci.* **50,** 1471-1478.

Guengerich, F. P., Dannan, G. A., Wright, S. T., Martin, M. V., and Kaminsky, L. S. (1982) Purification and characterization of liver microsomal cytochromes P-450: electrophoretic, spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or beta-naphthoflavone. *Biochemistry* **21,** 6019-6030.

Guengerich, F. P., and MacDonald, T. L. (1990) Mechanisms of cytochrome P-450 catalysis. *FASEB J.* **4,** 2453-2459.

Guengerich, F. P., and Shimada, T. (1991) Oxidation of toxic and carcinogenic chemicals by human cytochrome P450 enzymes. *Chem. Res. Toxicol.* **4,** 391-401.

Gustafsson, J. A., Rondahl, L., and Bergman, J. (1979) Iodosylbenzene derivatives as oxygen donors in cytochrome P-450 catalyzed steroid hydroxylations. *Biochemistry* **18,** 865-870.

Hahn, J. E., Hodgson, K. O., Andersson, L. A., and Dawson, J. H. (1982) Endogenous cysteine ligation in ferric and ferrous cytochrome P-450. Direct evidence from X-ray absorption spectroscopy. *J. Biol. Sci.* **257,** 10934-10941.

Hamor, G. H. (1989) Nonsteroidal anti-inflammatory drugs. In *Principals of medicinal chemistry* (Foye, W. O., ed.) pp. 503-530, Lea & Febiger, Philadelphia.

Haroldsen, P. E., Reilly, M. H., Hughes, H., Gaskell, S. J., and Porter, C. J. (1988) Characterization of glutathione conjugates by fast atom bombardment /tandem mass spectrometry. *Biomed. Environ. Mass Spectrom.* **15,** 615-621.

Hasegwa, T. (1983) The recognition of a soluble cytochrome P450 in rat liver. *FEBS Lett.* **155,** 257-262.

Haurand, M., and Ullrich, V. (1985) Isolation and characterization of thromboxane synthase from human platelets as a cytochrome P450 enzyme. *J. Biol. Chem.* **260,** 15059-15067.

Hlavica, P., Golly, I., and Mietaschk, J. (1983) Comparative studies on the cumene hydroperoxide and NADPH-supported N-oxidation of 4-chloroaniline by cytochrome P-450. *Biochem. J.* **212,** 539-547.

Holton, T. A., Brugliera, F., Lester, D. R., Tanaka, Y., Hyland, C. D., Menting, J. G. T., Lu, C. Y., Fracy, E., Stevenson, T. W., and Cornish, E. C. (1993) Cloning and expression of cytochrome P450 genes controlling flower colour. *Nature* **366,** 276-279.

Hrycay, E. G., Gustafsson, J-A., and Ingelman-Sundberg, M (1976) The involvement of cytochrome P450 in hepatic microsomal steroid hydroxylation reactions supported by sodium periodate, sodium chlorite, and organic hydroperoxide. *Eur. J. Biochem.* **61,** 43-52.

Hrycay, E. G., and O'Brien, P. J. (1971a) Cytochrome P450 as a microsomal peroxidase utilizing a lipid peroxide substrate. *Arch. Biochem. Biophys.* **147,** 14-27.

Hrycay, E. G., and O'Brien, P. J. (1971b) Peroxidase nature of cytochrome P450 utilizing a lipid peroxide substrate. *Arch. Biochem. Biophys.* **147,** 28-33.

Hrycay, E. G., and O'Brien, P. J. (1972) Cytochrome P450 as a microsomal peroxidase in steroid hydroperoxide reduction. *Arch. Biochem. Biophys.* **153,** 480-494.

Hrycay, E. G., and O'Brien, P. J. (1973) Microsomal electron transport, I. Reduced nicotinamide adenine phosphate-cytochrome c reductase and cytochrome P450 as electron carriers in microsomal NADPH-peroxidase activity. *Arch. Biochem. Biophys.* **157,** 7-22.

Hrycay, E. G., and O'Brien, P. J. (1974) Microsomal electron transport II. Reduced nicotinamide adenine dinucleotide-cytochrome b5 reductase and cytochrome P450 as electron carriers in microsomal NADH-peroxidase activity. *Arch. Biochem. Biophys.* **160,** 230-245.

Ikeya, k., Jaiswal, A. K., Owens, R. A., Jones, J. E., Nebert, D. W., and Kimura, S. (1989) Human CYP1A2: sequence, gene structure, comparison with the mouse and rat orthologous gene, and differences in liver 1A2 mRNA expression. *Mol. Endocrinol.* **3,** 1399-1408.

Imberti, R., Nieminen, A. L., Herman, B., and Lemasters, J. J. (1993) Mitochondrial and glycolytic dysfunction in lethal injury to hepatocytes by t-butylhydroperoxide: protection by fructose, cyclosporin A and trifluoperazine. *J. Pharmacol. Exp. Ther.* **265,** 392-400.

Janig, G. R., Makower, A., Rabe, H., Bernhardt, R., and Ruckpaul, K. (1984) Chemical modification of cytochrome P450 LM2. Characterization of tyrosine as axial heme iron ligand *trans* to thiolate. *Biochem. Biophys. Acta* **787,** 8-18.

Jones, D. P., Eklow, L., Thor, H., and Orrenius, S. (1981) Metabolism of hydrogen peroxide in isolated hepatocytes: relative contributions of catalase and glutathione peroxidase in decomposition of endogenously generated H_2O_2. *Arch. Biochem. Biophys.* **210,** 505-516.

Jonsson, K. H., and Lindeke, B. (1990) Cytochrome P-455 nm complex formation in the metabolism of phenylalkylamines. XI. Peroxygenase vs monooxygenase function of cytochrome P-450 in rat liver microsomes. *Chem.-Biol. Interact.* **75,** 276-279.

Josephy, P. D., Chiu, A. L., and Eling, T. E. (1989) Prostaglandin H synthase-dependent mutagenic activation of benzidine in a Salmonella typhimurium Ames tester strain possessing elevated N-acetyltransferase levels. *Cancer Res.* **49,** 853-6.

Kadlubar, F. F., Morton, K. C., and Ziegler, D. M. (1973) Microsomal-catalyzed hydroperoxide-dependent C-oxidation of amines. *Biochem. Biophys. Res. Commun.* **54,** 1255-1261.

Kapke, G. F., and Baron, J. (1980) Hepatic mitochondrial cholesterol hydroxylase activity-a cytochrome P-450-catalyzed monooxygenation refractory to cobaltous chloride. *Biochem. Pharmacol.* **29,** 845-847.

Kedderis, G. L., Dwyer, L. A., Rickert, D. E., and Hollenberg, P. F. (1983) Source of the oxygen atom in the product of cytochrome P450-catalyzed N-demethylation reactions. *Mol. Pharmacol.* **23,** 758-760.

Khan, S., and O'Brien, P. J. (1991) 1-Bromoalkanes as new potent nontoxic glutathione depletors in isolated rat hepatocytes. *Biochem. Biophys. Res. Comm.* **179,** 436-441.

Knickle, L. C., and Bend, J. R. (1994) Bioactivation of arachidonic acid by the cytochrome P450 monooxygenases of guinea pig lung: the orthologue of

cytochrome P450 2B4 is solely responsible for formation of epoxyeicosatrienoic acids. *Mol. Pharmacol.* **45,** 1273-1280.

Koop, D. R. (1986) Hydroxylation of p-nitrophenol by ethanol-inducible cytochrome P-450 isozyme 3a. *Mol. Pharmacol.* **29,** 399-404.

Koop, D. R., and Hollenberg, P. F. (1980) Kinetics of the hydroperoxide-dependent dealkylation reactions catalyzed by rabbit liver microsomal cytochrome P-450. *J. Biol. Chem.* **255,** 9685-9692.

Koymans, L., Donne-Op den Kelder, G. M., te Koppele, J. M., and Vermeulen, N. P. E. (1993) Cytochromes P450: Their active-site structure and mechanism of oxidation. *Drug Metab. Rev.* **25,** 325-387.

Kreamer, B. L., Staecker, J. L., Sawada, N., Sattler, G. L., Hsia, M. T. S., and Pitot, H. C. (1986) Use of low speed, iso-density percoll centrifugation method to increase the viability of isolated rat hepatocyte preparations. *In Vitro Cell Dev. Biol.* **22,** 201-211.

Kumaki, K., and Nebert, D. W. (1978) Spectral evidence for weak ligand in sixth position of hepatic microsomal cytochrome P-450 low spin ferric iron *in vivo*. *Pharmacol.* **17,** 262-279.

LaBella, F. S. (1991) Cytochrome P450 enzymes: ubiquitous "receptors" for drugs. *Can. J. Physiol. Pharmacol.* **69,** 1129-1132.

Langenbach, R., Smith, P. B., and Crespi, C. (1992) Experimental carcinogenesis for the development of metabolic systems used in in vitro toxicology. *Mutat. Res.* **277,** 251-275.

Lau, S. S., Hill, B. A., Highet, R. J., and Monks, T. J. (1989) Sequential oxidation and glutathione addition to 1,4-benzoquinone: correlation of toxicity with increased glutathione substitution. *Mol. Pharmacol.* **34,** 829-836.

Layton, D. W., Bogen, K. T., Knize, M. G., Hatch, F. T., Johnson, V. M., and Felton, J. S. (1995) Cancer risk of heterocyclic amines in cooked foods: an analysis and implications for research. *Carcinogenesis* **16,** 39-52.

Lichtenberger, F., Nastainczyk, W., and Ullrich, V. (1976) Cytochrome P-450 as an oxene transferase. *Biochem. Biophys. Res. Commun.* **70,** 939-946.

Loughrey, C. M., Young, I. S., Lightbody, J. H., McMaster, D., McNamee, P. T., and Trimble, E. R. (1994) Oxidative stress in haemodialysis. *Q. J. Med.* **87,** 679-683.

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

Maiorino, M., Ursini, F., and Cadenas, E. (1994) Reactivity of metmyoglobin towards phospholipid hydroperoxides. *Free Rad. Biol. Med.* **16,** 661-667.

Marker, E. K., and Kulkarni, A. P. (1986) Cumene hydroperoxide-supported denitrification of 2-nitropropane in uninduced mouse liver microsomes. *Int. J. Biochem.* **18,** 595-601.

Maron, D. M., and Ames, B. N. (1983) Revised methods for the *Salmonella* mutagenicity test. *Mutat. Res.* **113,** 173-215.

Masaki, N., Kyle, M. E., and Farber, J. L. (1989) tert-Butyl hydroperoxide kills cultured hepatocytes by peroxidizing membrane lipids. *Arch. Biochem. Biophys.* **269,** 390-399.

McCarthy, M. B., and White, R. E. (1983a) Competing modes of peroxyacid flux through cytochrome P-450. *J. Biol. Chem.* **258,** 11610-11616.

McCarthy, M. B., and White, R. E. (1983b) Functional differences between peroxidase compound I and the cytochrome P-450 reactive oxygen intermediates. *J. Biol. Chem.* **258,** 9153-9158.

Mesnil, M., and Testa, B. (1984) Xenobiotic metabolism by brain monooxygenases and other enzymes. In *Advances in Drug Research* (Testa, B., ed.), Academic Press, London.

Miller, E., and Miller, J. A. (1981) Mechanisms of chemical carcinogenesis. *Cancer* **47,** 1055-1064.

Miners, J., Birkett, D. J., Drew, R., and McManus, M. (1988) *Microsomes and Drug Oxidations*. Taylor & Francis, London.

Minotti, G., and Aust, S. D. (1989) The role of iron in oxygen radical mediated lipid peroxidation. *Chem.-Biol. Interact.* **71,** 1-19.

Minotti, G., and Aust, S. D. (1992) Redox cycling of iron and lipid peroxidation. *Lipids* **27,** 219-226.

Moldeus, P., Hogberg, J., and Orrenius, S. (1978) Isolation and use of liver cells. *Methods Enzymol.* **52,** 60-70.

Morrison, L. D., Eling, T. E., and Josephy, P. D. (1993) Prostaglandin H synthase-dependent formation of the direct-acting mutagen 2-nitro-3-methylimidazo[4,5-f]quinoline (nitro-IQ) from IQ. *Mutat. Res.* **302,** 45-52.

Muindi, J. F., and Young, C. W. (1993) Lipid hydroperoxides greatly increase the rate of oxidative catabolism of all-trans-retinoic acid by human cell culture microsomes genetically enriched in specified cytochrome P450 isoforms. *Cancer Res.* **53,** 1226-1229.

Nebert, D. W., Adesnik, M., Coon, M. J., Estabrook, R. W., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Kemper, B., Levin, W., Phillips, I. R., Sato, R., and Waterman, M. R. (1987) The P450 gene superfamily: recommended nomenclature. *DNA* **6,** 1-11.

Nebert, D. W., and Gonzalez, F. J. (1985) Cytochrome P450 gene expression and regulation. *Trends Pharmacol. Sci.* **6,** 160-164.

Nebert, D. W., and Gonzalez, F. J. (1987) P450 genes: Structure, evolution, and regulation. *Ann. Rev. Biochem.* **56,** 949993.

Nebert, D. W., and Negishi, M. (1982) Multiple forms of cytochrome P-450 and the importance of molecular biology and evolution. *Biochem. Pharmacol.* **31,** 2311-2317.

Nebert, D. W., Nelson, D. R., Adesnik, M., Coon, M. J., Estabrook, R. W., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Kemper, B., Levin, W., Phillips, I. R., Sato, R., and Waterman, M. R. (1989a) The P450 superfamily: updated listing of all genes and recommended nomenclature for the chromosomal loci. *DNA* **8,** 1-13.

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 Cell Biol.* **10,** 1-14.

Nebert, D. W., Nelson, D. R., and Feyereisen, R. (1989b) Evolution of cytochrome P450 genes. *Xenobiotica* **19,** 1149-1160.

Nelson, D. R., Kamataki, T., Waxman, D. J., Guengerich, F. P., Estabrook, R. W., Feyereisen, R., Gonzalez, F. J., Coon, M. J., Gunsalus, I. C., Gotoh, O., and Nebert, D. W. (1993) The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA & Cell Biology* **12,** 1-51.

Nelson, D. R., Koymans, L., Kamataki, T., Stegeman, J. J., Feyereisen, R., Waxman, D. J., Waterman, M. R., Gotoh, O., Coon, M. J., Estabrook, R. W., Gunsalus, I. C., and Nebert, D. W. (1996) P450 superfamily : update on new

sequences, gene-mapping, accession numbers and nomenclature. *Pharmacogenetics* **6,** 1-42.

Nordblom, G. D., White, R. E., and Coon, M. J. (1976) Studies of hydroperoxide-dependent substrate hydroxylation by purified liver microsomal cytochrome P450. *Arch. Biochem. Biophys.* **175,** 524-533.

Nordmann, R. (1994) Alcohol and antioxidant system. *Alcohol Alcohol.* **29,** 513-522.

O'Brien, P. J. (1978) Hydroperoxides and superoxides in microsomal oxidations. *J. Pharmacol. Ther.* **A2,** 517-536.

O'Brien, P. J. (1991) Molecular mechanism of quinone cytotoxicity. *Chem,-Biol. Interact.* **80,** 1-14.

O'Brien, P. J., and Rahimtula, A. (1975) Involvement of cytochrome P-450 in the intracellular formation of lipid peroxides. *Agricul. Food Chem.* **23,** 154-158.

Omura, T., Sadano, H., Hasegawa, T., Yoshida, Y., and Komonami, S. (1984) Hemoprotein H-450 having an endogenous ligand at the 6th coordination position in the heme. *J. Biochem.* **96,** 1491-1500.

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

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

Ortiz de Montellano, P. R. (1995a) *Cytochrome P450. Structure, Mechanism, and Biochemistry.* 2nd. edition, Plenum Press, New York.

Ortiz de Montellano, P. R. (1995b) Oxygen activation and reactivity. In *Cytochrome P450. Structure, Mechanism, and Biochemistry.* (Ortiz de Montellano, P. R., ed.) pp. 245-303, Plenum Press, New York.

Penneberg, R., Scheller, F., Ruckpaul, K., Pirrwitz, J., and Mohr, P. (1978) NADPH and H_2O_2-dependent reactions of cytochrome $P450_{LM}$ compared with peroxidase catalysis. *FEBS Lett.* **96,** 349-353.

Pereira, B., Wu, K. K., and Wang, L. H. (1993) Bovine prostacyclin synthase: purification and isolation of partial cDNA. *Biochem. Biophys. Res. Commun.* **197,** 1041-1048.

Petry, T. W., Eling, T. E., Chiu, A. L., and Josephy, P. D. (1988) Ram seminal vesicle microsome-catalyzed activation of benzidine and related compounds: dissociation of mutagenesis from peroxidase-catalyzed formation of DNA-reactive material. *Carcinogenesis* **9,** 51-7.

Petry, T. W., Josephy, P. D., Pagano, D. A., Zeiger, E., Knecht, K. T., and Eling, T. E. (1989) Prostaglandin hydroperoxidase-dependent activation of heterocyclic aromatic amines. *Carcinogenesis* **10,** 2201-7.

Potter, D. W., and Hinson, J. A. (1987) Mechanisms of acetaminophen oxidation to N-acetyl-p-benzoquinone imine by horseradish peroxidase and cytochrome P-450. *J. Biol. Chem.* **262,** 966-973.

Poulos, T. A., and Raag, R. (1992) Cytochrome P-450cam: crystallography, oxygen activation, and electron transfer. *FASEB J.* **6,** 674-679.

Precious, W. Y., and Barrett, J. (1986) Xenobiotic metabolism in helminths. *Parasitol. Today* **5,** 156-160.

Puga, A., and Nebert, D. W. (1990) Evolution of the P450 gene superfamily and regulation of the murine *Cyp1a1* gene. *Biochem. Soc. Trans.* **18,** 7-10.

Rahimtula, A. D., and O'Brien, P. J. (1974) Hydroperoxide catalyzed liver microsomal aromatic hydroxylation reactions involving cytochrome P450. *Biochem. Biophys. Res. Commun.* **60,** 440-447.

Rahimtula, A. D., and O'Brien, P. J. (1975) Hydroperoxide-dependent O-dealkylation reactions catalyzed by liver microsomal cytochrome P450. *Biochem. Biophys. Res. Commun.* **62,** 268-275.

Rahimtula, A. D., and O'Brien, P. J. (1977a) The hydroperoxide dependent microsomal oxidation of alcohols. *Eur. J. Biochem.* **77,** 201-211.

Rahimtula, A. D., and O'Brien, P. J. (1977b) The peroxidase nature of cytochrome P450. In *Microsomes and Drug Oxidations* (Ullrich, V., Roots, I., Hildebrandt, A., Estabrook, R. W., and Conney, A. H., ed.) pp. 210-217, Pergamon Press, Oxford.

Rahimtula, A. D., and O'Brien, P. J. (1977c) The role of cytochrome P-450 in the hydroperoxide-catalyzed oxidation of alcohols by rat liver microsomes. *Eur. J. Biochem.* **77,** 201-208.

Rahimtula, A. D., O'Brien, P. J., Seifried, H. E., and Jerina, D. M. (1978) The mechanism of action of cytochrome P450. Occurrence of the "NIH" shift during hydroperoxide dependent aromatic hydroxylation. *Eur. J. Biochem.* **89,** 133-141.

Reed, D. J., Babson, J. R., Beaty, B. W., Brodie, A. E., Ellis, W. W., and Potter, D. W. (1980) High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. *Anal. Biochem.* **106,** 55-62.

Richter, C., and Frei, B. (1988) Calcium release from mitochondria induced by prooxidants. *Free Rad. Biol. Med.* **4,** 365-375.

Richter, G., and Kass, G. E. N. (1991) Oxidative stress in mitochondria: its relationship to cellular Ca^{2+} homeostasis, cell death, proliferation, and differentiation. *Chem.-Biol. Interact.* **77,** 1-23.

Rodrigues, A. D., and Prough, R. A. (1991) Induction of cytochrome P450IA1 and P450IA2 and measurement of catalytic activities. *Methods Enzymol.* **206,** 423-431.

Roy, D., Bernhardt, A., Strobel, H. W., and Liehr, J. G. (1992) Catalysis of the oxidation of steroid and stilbene estrogens to estrogen quinone metabolites by the beta-naphthoflavone-inducible cytochrome P450 IA family. *Arch. Biochem. Biophys.* **296,** 450-456.

Roy, D., and Liehr, J. G. (1992) Target organ-specific inactivation of drug metabolizing enzymes in kidney of hamsters treated with estradiol. *Mol. Cell. Biochem.* **110,** 31-39.

Rush, G. F., Yodis, L. A., and Alberts, D. (1986) Protection of rat hepatocytes from tert-butyl hydroperoxide induced injury by catechol. *Toxicol. Appl. Pharmacol.* **68,** 607-616.

Savard, S., and Josephy, P. D. (1986) Synthesis and mutagenicity of 3,3'-dihalogenated benzidines. *Carcinogenesis* **7,** 1239-41.

Saylor, A. K., Tesfal, S. A., Herman, B., and Lemasters, J. J. (1995) Contribution of the mitochondrial permeability transition to lethal injury after exposure of hepatocytes to t-butylhydroperoxide. *Biochem. J.* **307,** 99-106.

Schraufstatter, I. U., Hinshaw, D. B., Hyslop, P. A., Spragg, R. G., and Cochrane, C. G. (1988) Oxidant injury of cells. DNA strand-breaks activate polyadenosine diphosphate-ribose polymerase and lead to depletion of nicotinamide adenine dinucleotide. *J. Clin. Invest.* **77,** 1312-1320.

Shah, M. M., Barr, D. P., Chung, N., and Aust, S. D. (1992) Use of white rot fungi in the degradation of environmental chemicals. *Toxicol. Lett.* **64,** 493-501.

Shertzer, H. G., Bannenberg, G. L., and Moldeus, P. (1992) Evaluation of iron binding and peroxide-mediated cytotoxicity in rat hepatocytes. *Biochem. Pharmacol.* **44,** 1367-1373.

Shimada, T., Iwasaki, M., Martin, M. V., and Guengerich, F. P. (1989) Human liver microsomal cytochrome P-450 enzymes involved in the bioactivation of procarcinogens detected by umu gene response in Salmonella typhimurium TA 1535/pSK1002. *Cancer Res.* **49,** 3218-28.

Shimizu, T., Murakami, Y., and Hatano, M. (1994) Glu318 and Thr319 mutations of cytochrome P450 1A2 remarkably enhance homolytic O-O cleavage of alkyl hydroperoxides. An optical absorption spectral study. *J. Biol. Chem.* **269,** 13296-304.

Sies, H. (1985) *Oxidative stress*. Academic Press, London.

Sies, H. (1991) *Oxidative stress. Oxidants and antioxidants*. Academic Press, London.

Sinal, C. J., Zhu, L. F., Zhong, R., Cherian, M. G., and Bend, J. R. (1995) Liver transplantation induces cytochrome P450 1A1 dependent monooxygenase activity in rat lung and kidney. *Can. J. Physiol. Pharmacol.* **73,** 146-52.

Sipe, H. J. J., Jordan, S. J., Hanna, P. M., and Mason, R. P. (1994) The metabolism of 17 beta-estradiol by lactoperoxidase: a possible source of oxidative stress in breast cancer. *Carcinogenesis* **15,** 2637-2643.

Smith, B. J., DeBruin, L., Josephy, P. D., and Eling, T. E. (1992) Mutagenic activation of benzidine requires prior bacterial acetylation and subsequent

conversion by prostaglandin H synthase to 4-nitro-4'-(acetylamino)biphenyl. *Chem. Res. Toxicol.* **5,** 431-9.

Smith, M. T., Evans, C. G., Thor, H., and Orrenius, S. (1985) Quinone-induced oxidative injury to cells and tissues. In *Oxidative Stress* (Sies, H., ed.) pp. 91-113, Academic press, London.

Smith, M. T., Thor, H., Hartzell, P., and Orrenius, S. (1982) The measurement of lipid peroxidation in isolated hepatocytes. *Biochem. Pharmacol.* **31,** 19-26.

Snyder, R., Chepiga, T., Yang, C. S., Platt, K., and Oesch, F. (1993) Benzene metabolism by reconstituted cytochromes P450 2B1 and 2E1 and its modulation by cytochrome b5, microsomal epoxide hydrolase, and glutathione transferases: evidence for an important role of microsomal epoxide hydrolase in the formation of hydroquinone. *Toxicol. Appl. Pharmacol.* **122,** 172-181.

Snyderwine, E. G., Roller, P. P., Adamson, R. H., Sato, S., and Thorgeirsson, S. S. (1988a) Reaction of N-hydroxylamine and N-acetoxy derivatives of 2-amino-3-methylimidazolo[4,5-f]quinoline with DNA. Synthesis and identification of N-(deoxyguanosin-8-yl)-IQ. *Carcinogenesis* **9,** 1061-5.

Snyderwine, E. G., Yamashita, K., Adamson, R. H., Sato, S., Nagao, M., Sugimura, T., and Thorgeirsson, S. S. (1988b) Use of the 32P-postlabeling method to detect DNA adducts of 2-amino-3-methylimidazolo[4,5-f]quinoline (IQ) in monkeys fed IQ: identification of the N-(deoxyguanosin-8-yl)-IQ adduct. *Carcinogenesis* **9,** 1739-43.

Song, W. C., and Brash, A. R. (1991) Purification of an allene oxide synthase and identification of the enzyme as a cytochrome P-450. *Science* **253,** 781-784.

Song, W. C., Funk, C. D., and Brash, A. R. (1993) Molecular cloning of an allene oxide synthase: A cytochrome P450 specialized for the metabolism of fatty acid hydroperoxides. *Proc. Natl. Acad. Sci.* **90,** 8519-8523.

Soucek, P., and Gut, I. (1992) Cytochromes P-450 in rats: structures, functions, properties and relevant human forms. *Xenobiotica* **22,** 83-103.

Stocchi, V., Cucchiarini, L., Magnani, M., Chiarantini, P. P., and Crescentini, G. (1984) Simultaneous extraction and reverse-phase high-performance liquid chromatographic determination of adenine and pyridine nucleotides in human red blood cells. *Anal. Biochem.* **146,** 118-124.

Sugimura, T. (1986) Studies on environmental chemical carcinogenesis in Japan. *Science* **233,** 312-318.

Sugimura, T. (1988) Successful use of short-term tests for academic purposes: their use in identification of new environmental carcinogens with potential risk for humans. *Mutat. Res.* **205,** 33-39.

Teng, J. I., and Smith, L. L. (1985) High-performance liquid chromatography of linoleic acid hydroperoxides and their corresponding alcohol derivatives. *J. Chromatogr.* **350,** 445-451.

Testa, B., and Jenner, P. (1981) Inhibitors of cytochrome P450s and their mechanism of action. *Drug Metabol. Rev.* **12,** 1-117.

Thies, R. L., and Autor, A. P. (1991) Reactive oxygen injury to cultured pulmonary artery endothelial cells: Mediation by poly(ADP-ribose) polymerase activation causing NAD depletion and altered energy balance. *Arch. Biochem. Biophys.* **286,** 353-363.

Thompson, J. A., Schullek, K. M., and Turnipseed, S. B. (1995) Role of cytochrome P450 in the metabolism and toxicity of hydroperoxides in isolated rat hepatocytes. *Arch. Biochem. Biophys.* **323,** 463-470.

Thompson, J. A., and Yumibe, N. P. (1989) Mechanistic aspects of cytochrome P-450-hydroperoxide interactions: substitute effects on degradative pathways. *Drug Metab. Rev.* **20,** 365-378.

Thurman, R. G., Ley, H. G., and Scholz, R. (1972) Hepatic microsomal ethanol oxidation. Hydrogen peroxide formation and the role of catalase. *Eur. J. Biochem.* **25,** 420-430.

Toyokuni, S., Okamoto, K., Yodoi, J., and Hiai, H. (1995) Persistent oxidative stress in cancer. *FEBS Letters* **358,** 1-3.

Traylor, T., Fann, W., and Bandyopadhtah, D. (1989) A common heterolytic mechanism for reactions of iodosobenzenes, peracids, hydroperoxides and hydrogen peroxide with iron (III) porphyrins. *J. Am. Chem. Soc.* **111,** 8009-8010.

Traylor, T. G., and Xu, F. (1990) Mechanisms of reactions of iron (III) porphyrins with hydrogen peroxide and hydroperoxides: solvent and solvent isotope effects. *J. Am. Chem. Soc.* **112,** 178-186.

Turesky, R. J., Gremaud, E., Markovic, J., and Snyderwine, E. G. (1996a) DNA adduct formation of the food-derived mutagen 2-amino-3-methylimidazo[4,5-f]quinoline in nohuman primates undergoing carcinogen bioassay. *Chem. Res. Toxicol.* **9,** 403-408.

Turesky, R. J., and Markovic, J. (1995) DNA adduct formation of the food carcinogen 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) in liver, kidney and colo-rectum of rats. *Carcinogenesis* **16,** 2275-9.

Turesky, R. J., Markovic, J., and Aeschlimann, J.-R. (1996b) Formation and differential removal of C-8 and N2-guanine adducts of the food carcinogen 2-amino-3-methylimidazo[4,5-f]quinoline in the liver, kidney, and colorectum of the rat. *Chem. Res. Toxicol.* **9,** 397-402.

Turesky, R. J., Rossi, S. C., Welti, D. H., Lay, J. O., Jr., and Kadlubar, F. F. (1992) Characterization of DNA adducts formed in vitro by reaction of N-hydroxy-2-amino-3-methylimidazo[4,5-f]quinoline and N-hydroxy-2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline at the C-8 and N2 atoms of guanine. *Chem. Res. Toxicol.* **5,** 479-90.

Ullrich, V., and Graf, H. (1984) Prostacyclin and thromboxane synthase as P-450 enzymes. *Trends Pharmacol. Sci.* **5,** 352-355.

Vaz, A. D., and Coon, M. J. (1987) Hydrocarbon formation in the reductive cleavage of hydroperoxides by cytochrome P-450. *Proc. Natl. Acad. Sci. USA* **84,** 1172-1176.

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

Veldink, G. A., and Vliegenthart, J. F. (1984) Lipoxygenases, nonheme iron-containing enzymes. *Adv. Inorg. Biochem.* **6,** 139-161.

Voest, E. E., van Faassen, E., and Marx, J. J. (1993) An electron paramagnetic resonance study of the antioxidant properties of the nitroxide free radical TEMPO. *Free Rad. Biol. Med.* **15,** 589-595.

Vuillaume, M. (1987) Reduced oxygen species, mutations, induction and cancer initiation. *Mutat. Res.* **186,** 43-72.

Wand, M. D., and Thompson, J. A. (1986) Cytochrome P-450-catalyzed rearrangement of a peroxyquinol derived from butylated hydroxytoluene. *J. Biol. Chem.* **261,** 14049-14056.

Wang, M. Y., and Liehr, J. G. (1995) Lipid hydroperoxide-induced endogenous DNA adducts in hamsters: possible mechanism of lipid hydroperoxide-mediated carcinogenesis. *Arch. Biochem. Biophys.* **316,** 38-46.

Watanabe, M., Ishidate, M. J., and Nohmi, T. (1990) Sensitive method for the detection of mutagenic nitroarenes and aromatic amines: new derivatives of Salmonella typhimurium tester strain possessing elevated O-acetyltransferase levels. *Mutat. Res.* **234,** 337-348.

Waterman, M. R., and Johnson, E. F. (1991) *Cytochrome P450.* In *Methods in Enzymology* (Packer, L., ed.), Academic Press, San Diego.

Waxman, D. J., Dannan, G. A., and Guengerich, F. P. (1985) Regulation of rat hepatic cytochrome P-450: age-dependent expression, hormonal imprinting, and xenobiotic inducibility of sex-specific isoenzymes. *Biochemistry* **24,** 4409-17.

Weinbach, E. C., and Garbus, J. (1965) The interaction of uncoupling phenols with mitochondria and with mitochondrial protein. *J. Biol. Chem.* **240,** 1811-1819.

Weiss, R. H., and Estabrook, R. W. (1986) The mechanism of cumene hydroperoxide-dependent lipid peroxidation: the function of cytochrome P-450. *Arch. Biochem. Biophys.* **251,** 348-360.

Weisz, J., Bui, Q. D., Roy, D., and Liehr, J. G. (1992) Elevated 4-hydroxylation of estradiol by hamster kidney microsomes: a potential pathway of metabolic activation of estrogens. *Endocrinology* **131,** 655-661.

Weli, A. M., and Lindeke, B. (1986) Peroxidative N-oxidation and N-dealkylation reactions of pargyline. *Xenobiotica* **16,** 281-288.

Wheeler, E. L. (1983) Cytochrome P-450 mediated interaction between hydroperoxide and molecular oxygen. *Biochem. Biophys. Res. Commun.* **110,** 646-653.

White, R. E., and Coon, M. J. (1980) Oxygen activation by cytochrome P-450. *Ann. Rev. Biochem.* **49,** 315-356.

White, R. E., and Coon, M. J. (1982) Heme ligand replacement reactions of cytochrome P-450. Characterization of the bonding atom of the axial ligand trans to thiolate as oxygen. *J. Biol. Chem.* **257,** 3073-3083.

Wickramasinghe, R. H., and Ville, C. A. (1975) Early role during chemical evolution for cytochrome P-450 in oxygen detoxification. *Nature* **256,** 509-511.

Wilkinson, C. F., Hetnarski, k., Denison, M. S., and Guengerich, F. P. (1983) Selectivity of 1-phenylimidazole as a ligand for cytochrome P-450 and as an inhibitor for microsomal oxidation. *Biochem. Pharmacol.* **32,** 997-1003.

Williams, R. J. P. (1990) An introduction to the nature of iron transport and storage. In *Iron transport and storage* (Ponca, P., Schulman, H. M., and Woodworth, R. C., ed.) pp. 1-15, CRC Press, Boca Raton, FL.

Wringhton, S. A., and Stevens, J. C. (1992) The human hepatic cytochrome P450 involved in drug metabolism. *Crit. Rev. Toxicol.* **22,** 1-21.

Wu, D. F., Clejan, L., Potter, B., and Cederbaum, A. I. (1990) Rapid decrease of cytochrome P-4502E1 in primary hepatocyte culture and its maintenance by added 4-methylpyrazole. *Hepatology* **12,** 1379-1389.

Yamamoto, K., Tsukidate, K., and Farber, J. L. (1993) Differing effects of the inhibition of poly(ADP-ribose) polymerase on the course of oxidative cell injury in hepatocytes and fibroblasts. *Biochem. Pharmacol.* **46,** 483-491.

Yamamoto, Y., Frei, B., and Ames, B. N. (1990) Assay of lipid hydroperoxides using high-performance liquid chromatography with isoluminal chemiluminescence detection. *Methods Enzymol.* **186,** 371-380.

Yamazoe, Y., Shimada, M., Kamataki, T., and Kato, R. (1983) Microsomal activation of 2-amino-3-methylimidazo[4,5-f]quinoline, a pyrolysate of sardine and beef extracts, to a mutagenic intermediate. *Cancer Res.* **43,** 5768-74.

Yang, H. Y., Namkung, M. J., and Juchau, M. R. (1988) Cytochrome P-450-dependent biotransformation of a series of phenoxazone ethers in the rat conceptus during early organogenesis: evidence for multiple P-450 isoenzymes. *Mol Pharmacol.* **34,** 67-73.

Yumibe, N. P., and Thompson, J. A. (1988) Fate of free radicals generated during one-electron reductions of 4-alkyl-1,4-peroxyquinols by cytochrome P-450. *Chem. Res. Toxicol.* **1,** 385-390.

Zand, R., Nelson, S. D., Slattery, J. T., Thummel, K. E. K., T.F., Adams, S. P., and Wright, J. M. (1993) Inhibition and induction of cytochrome P4502E1-catalyzed oxidation by isoniazid in humans. *Clin. Pharmacol. Ther.* **54,** 142-149.

Zimmerman, B. J., and Granger, D. N. (1994) Mechanisms of reperfusion injury. *Am. J. Med. Sci.* **307,** 284-292.

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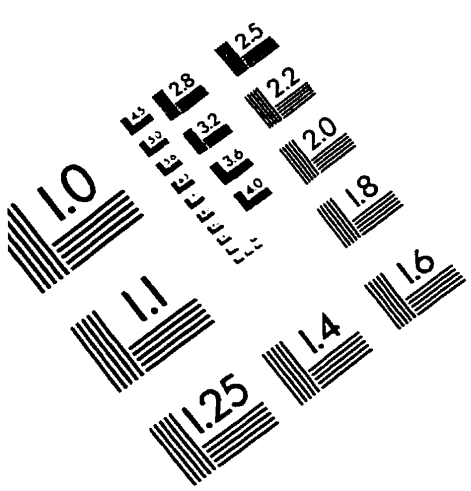

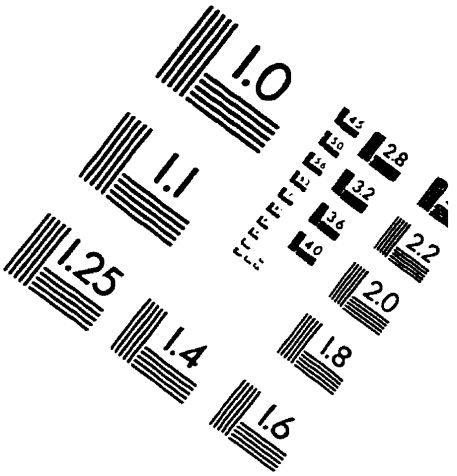

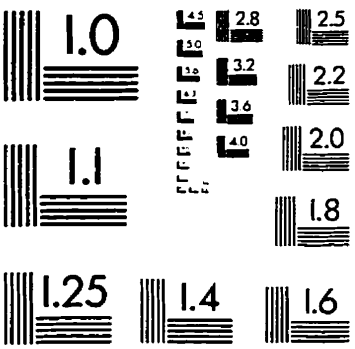

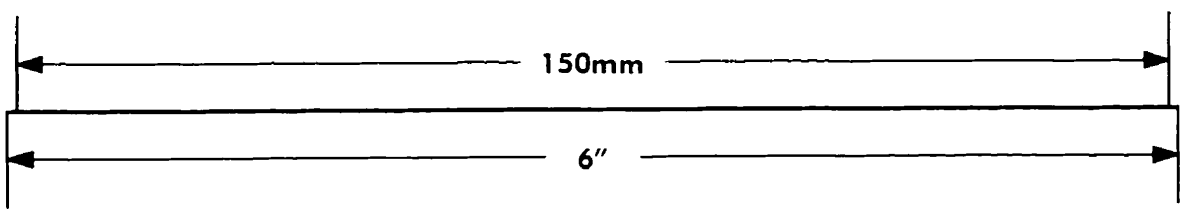

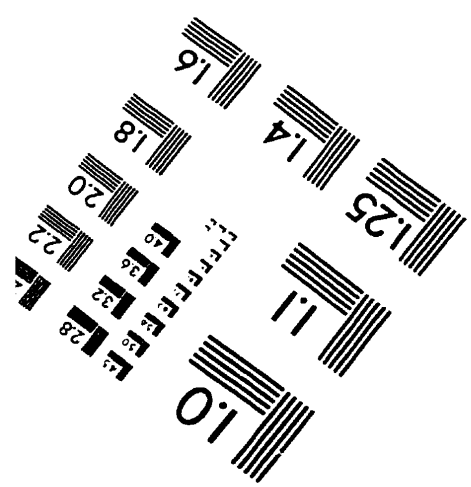

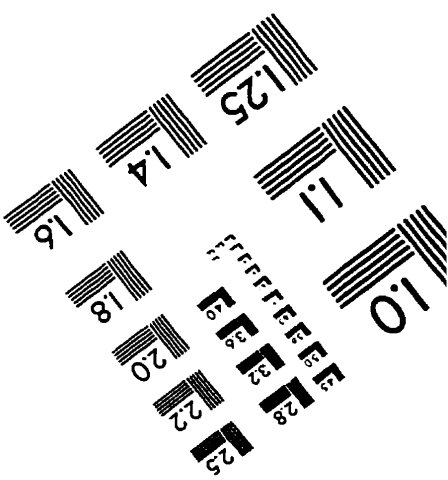

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