Free Radical Research, 2002 Vol. 36 (8), pp. 893–903

Taylor & Francis
healthsciences

Differential Role of CYP2E1 Binders and Isoniazid on CYP2E1 Protein Modification in NADPH-dependent Microsomal Oxidative Reactions: Free Radical Scavenging Ability of Isoniazid

DAL WOONG CHOI[a,b], BRIGITTE LEININGER-MULLER[b], YOUNG CHUL KIM[c], PIERRE LEROY[a], GERARD SIEST[b] and MARIA WELLMAN[a,*]

[a]*Thiols et fonctions celluloizes;* [b]*INSERM U525, Equipe 4, Faculty of Pharmacy, Université Henri Poincaré Nancy 1, 30 rue Lionnois, 54000 Nancy, France;* [c]*College of Pharmacy, Seoul National University, Seoul, South Korea*

Accepted by Professor H. Sies

(Received 26 November 2001; In revised form 7 March 2002)

We evaluated the effect of "weak" CYP2E1 binders (ethanol, acetone and glycerol) "tight" CYP2E1 binders (4-methylpyrazole, imidazole, isoniazid and pyridine) and CCl_4 (suicide substrate of CYP2E1) on the NADPH-dependent production of microsomal reactive oxygen species (ROS), lipid peroxidation (LPO), and subsequent modification of microsomal and CYP2E1 proteins. The oxidation of 2′,7′-dichlorofluorescin diacetate (DCFHDA) was used as an index of formation of microsomal ROS and LPO-derived reactive species. Microsomal LPO was determined by malondialdehyde (MDA) HPLC measurement. Addition of NADPH to rat liver microsomes initiated DCFHDA oxidation and MDA formation, leading to further selective modification of microsomal proteins and proteases-independent degradation of CYP2E1 protein. Iron chelators prevented these processes whereas hydroxyl radical scavengers showed weak effects, suggesting an important role of LPO. Among the tested CYP2E1 binders, only isoniazid strongly inhibited NADPH-dependent DCFHDA oxidation, LPO and modification of microsomal proteins. Other CYP2E1 binders showed weak inhibitory effects of these processes. Concerning NADPH-dependent modification of CYP2E1 protein, all of the tested CYP2E1 binders, except glycerol, prevented this process with a different potency (isoniazid > 4-methylpyrazole = imidazole = pyridine ⩾ acetone > ethanol). "Tight" binders were more effective than "weak" binders. The CCl_4 stimulated the DCFHDA oxidation, LPO and CYP2E1 protein modification. Among the tested CYP2E1 binders, only isoniazid effectively scavenged 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals. In microsomes isolated from CYP2E1 transfected HepG2 cells, isoniazid inhibited the CYP2E1-dependent DCFHDA oxidation whereas other CYP2E1 binders did not inhibit this reaction although these compounds strongly inhibited CYP2E1 activity. The present study demonstrates that CYP2E1 binders and isoniazid differentially inhibit LPO-catalyzed oxidative modification of CYP2E1 protein in NADPH-dependent microsomal reactions. It seems that CYP2E1 binders protect CYP2E1 from the oxidative modification mainly by binding to the active site of the enzyme, rather than by blocking the reactive species production. The strong protective effect of isoniazid can be attributed to its ability to scavenge free radicals. These effects of CYP2E1 binders are considered to contribute to the regulation of hepatic CYP2E1 protein levels *via* stabilization of the protein.

Keywords: CYP2E1 protein modification; "Tight" binders; "Weak" binders; Isoniazid; LPO; ROS

Abbreviations: CYP2E1, cytochrome P-450 2E1; 4-MP, 4-methylpyrazole; P-450, cytochrome P-450; ER, endoplasmic reticulum; ROS, reactive oxygen species; LPO, lipid peroxidation; DCFHDA, 2′,7′-dichlorofluorescin diacetate; DCFH, 2′,7′-dichlorofluorescin; GSH, reduced glutathione; DPI, diphenyleneiodonium; EDTA, ethylenediamine tetraacetic acid; DCF, 2′,7′-dichlorofluorescein; MDA, malondialdehyde; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel-electrophoresis; DPPH, 2-2-diphenyl-1-picrylhydrazyl; DMSO, dimethylsulphoxide

*Corresponding author. Tel.: +33-3-83682152. Fax: +33-3-83682154. E-mail: wellman@pharma.uhp-nancy.fr

ISSN 1071-5762 print/ISSN 1029-2470 online © 2002 Taylor & Francis Ltd
DOI: 10.1080/1071576021000005339

INTRODUCTION

The regulation of cytochrome P-450 2E1 (CYP2E1) protein level is of considerable interest because of its role in the metabolic activation of a variety of toxic chemicals[1] and its high NADPH oxidase activity.[2] CYP2E1 protein level is elevated several folds in rat hepatic tissue in response to treatment with xenobiotics such as ethanol, acetone, 4-methylpyrazole (4-MP), isoniazid, and pyridine, with the notable exception of imidazole among the nitrogen heterocyclic compounds.[3–6] Similar increase in CYP2E1 protein levels in response to xenobiotics have been reported in primary cultured rat hepatocytes[7,8] and CYP2E1 transfected HepG2 cells.[9]

In contrast to most other cytochrome P-450 (P-450) isoforms, the xenobiotic induction of CYP2E1 does not generally involve transcriptional activation. Many studies have provided evidence that protein stabilization represents a mechanism for controlling CYP2E1 protein level in response to xenobiotics. *In vivo* labeling of protein and subsequent immunopurification demonstrated that native CYP2E1 protein turnover followed a biphasic pattern, and acetone or ethanol treatment prolonged the half-life of CYP2E1 in rat liver by eliminating the fast-phase component.[10,11] In primary cultures of rat hepatocytes, CYP2E1 protein was also maintained by ethanol or imidazole even when mRNA rapidly disappeared.[7]

CYP2E1 has a short life (4–7 h).[9–12] Several mechanisms for this rapid degradation of CYP2E1 have been suggested. One pathway may involve a cAMP-dependent phosphorylation of CYP2E1, followed by heme loss and subsequent degradation by serine proteases present in the endoplasmic reticulum (ER).[13] Other studies have shown that this rapid CYP2E1 degradation process involves the ubiquitin-dependent or -independent proteasome complex system.[10,12,14,15]

The P-450 enzymes, which utilize oxygen to oxidize their substrates, are important sources of reactive oxygen species (ROS) formation and lipid peroxidation (LPO). Superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) are formed by P-450s even in the absence of exogenous uncoupling substrate.[2,16,17] In the presence of microsomal non-heme iron, $O_2^{\bullet -}$ and H_2O_2 can be converted to reactive hydroxyl radical ($OH^{\bullet}$).[17,18] CYP2E1 is more active in producing ROS and catalyzing LPO than other P-450 isoforms in a substrate-free form.[2] Recently, several studies have suggested the involvement of oxidative reaction in the CYP2E1 turn-over process[10,15,19,20]. Roberts reported a 20 S proteasome-mediated degradation of CYP2E1, in a process that requires prior labilization of the enzyme.[15] The 20S proteasome has been showed to be involved in selective degradation of oxidatively modified proteins[21]. Goasduff and Cederbaum also suggested that NADPH-dependent production of microsomal ROS may result in oxidative modification of CYP2E1, followed by a rapid degradation of the labilized CYP2E1 by cytosolic proteasome complex.[20] However, it is not well known whether the increased level of CYP2E1 protein in response to xenobiotics is mediated by inhibition of oxidative modification of CYP2E1 or by increased resistance to proteolytic degradation of oxidatively modified CYP2E1.

To evaluate the role of xenobiotics in the modification of CYP2E1 protein in oxidative reactions, we examined whether xenobiotics can protect CYP2E1 protein from oxidative modification in NADPH-dependent microsomal reactions, and whether the observed effect of xenobiotics is mediated by inhibition of NADPH-dependent production of microsomal ROS and LPO. We also compared the effect of xenobiotics with that of CCl_4, which is known to accelerate CYP2E1 proteolysis *in vivo*.[14] In these experiments, we divided the xenobiotics into two groups by their properties, which are "tight" binders to CYP2E1 (4-MP, imidazole, isoniazid and pyridine) and "weak" binders to CYP2E1 (ethanol, acetone and glycerol).[9,22–25]

MATERIALS AND METHODS

Materials

Polyclonal rat anti-CYP2E1 antibody was obtained from Calbiochem (La Jolla, CA, USA). This antibody has no cross-reactivity with other P-450 enzymes. All other chemicals were Sigma Chemical (St. Louis, MO, USA). Solutions were prepared using Milli-Q water (18.2 MΩ cm).

Animals and Microsome Preparation

Male Sprague–Dawley rats (150–180 g, Iffa-Credo, France) were housed in a controlled environment room with a 12 h light/dark photoperiod. The rats were fasted overnight prior to sacrifice. Liver microsomes were prepared by differential centrifugation as previously described.[2] Protein concentrations were determined[26] with bovine serum albumin as a standard.

Cell Lines and Cell Culture

A HepG2 subclone overexpressing CYP2E1, HepG2-CYP2E1-E47 (E47 cells), was generously supplied by Dr Cederbaum (Mount Sinai School of Medicine, NY, USA).[27] E47 cells contain the human CYP2E1 cDNA inserted into the EcoRI restriction site of the pCI-neo expression vector in the sense orientation. C34 cells

(control HepG2 cells) contain the pCI-neo vector alone. The HepG2 transduced clones C34 and E47 were cultured in minimal essential medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine in a humidified atmosphere in 5% CO_2 at 37°C. Cells were maintained in the presence of 0.4 mg/ml of geneticin. Microsomes were prepared by differential centrifugation of the sonicated cell extracts and resuspended in phosphate buffer.

Measurement of Generation of Microsomal ROS and LPO-derived Reactive Species

The rate of generation of ROS and LPO-derived species was quantified with the fluorescent probe precursor, 2′,7′-dichlorofluorescin diacetate (DCFHDA), a probe that has been utilized extensively in microsomal system.[16,17] This assay has been shown to detect the fluorescence associated with the oxidation of 2′,7′-dichlorofluorescin (DCFH) by several reactive intermediates, including $O_2^{\bullet -}$, H_2O_2, $OH^{\bullet}$ and LPO-derived reactive species in microsomes.[17] Microsomes (rat, 0.5 mg/ml; E47 cells, 1.0 mg/ml) were incubated for 15 min with 4 μM DCFHDA for de-esterification by endogenous esterase in a reaction system containing 50 mM phosphate buffer, pH 7.4 at 37°C and then for an additional 30 min in the absence or presence of 0.5 mM NADPH. The microsomes were preincubated for 5 min in the presence of "tight" binders to CYP2E1 (2 mM), "weak" binders (200 mM), reduced glutathione (GSH) (2 mM), ascorbic acid (2 mM), diphenyleneiodonium (DPI) (25 μM), mannitol (0.1 M), dimethylsulphoxide (DMSO) (50 mM), ethylenediamine tetraacetic acid (EDTA) (0.2 mM), desferrioxamine (0.2 mM) or CCl_4 (2 mM) prior to the addition of NADPH. At the end of incubation fluorescent 2′,7′-dichlorofluorescin (DCF) formation was monitored using a microplate fluorimeter (Cytofluor 2350; Millipore; Bedford, MA, USA) at an excitation and emission wavelength of 485 and 530 nm, respectively. The rate of DCFHDA oxidation was linear over the incubation period. A standard curve using 0.01–0.10 μM DCF was prepared and the results were expressed as picomoles of DCF formed per minute per milligram of protein.

Measurement of Modification of Microsomal and CYP2E1 Proteins

The modification of microsomal and CYP2E1 proteins was followed in a reaction system containing 50 mM phosphate buffer, pH 7.4 and rat liver microsomes (0.5 mg/ml). The microsomes were preincubated for 5 min in the presence of "tight" binders to CYP2E1 (2 mM), "weak" binders (200 mM), GSH (2 mM), ascorbic acid (2 mM), DPI (25 μM), mannitol (0.1 mM), DMSO (50 mM), EDTA (0.2 mM), desferrioxamine (0.2 mM) CCl_4 (2 mM) or phenylmethylsulfonyl fluoride (PMSF) (0.6 mM) prior to NADPH (0.5 mM) addition. DMSO (10 or 50 mM) was used to dissolve PMSF. The sample incubation was carried out at 37°C for 4 h in the absence or presence of NADPH and the reaction was stopped by adding one volume of the sodium dodecyl sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE) running buffer followed by heating at 100°C. The modification of microsomal proteins and CYP2E1 protein was monitored by 12% SDS-PAGE under reducing conditions followed by Coomassie Brilliant Blue staining or Western blot analysis. Western blot analysis was carried out as described previously[28] using polyclonal rat anti-CYP2E1 antibody. The blots were quantified by densitometry using Biomax 1D software (Kodak).

Measurement of Microsomal LPO

Microsomal LPO was monitored using a reverse-phase HPLC method devoted to the measurement of malondialdehyde (MDA) after precolumn derivatization with thiobarbituric acid (TBA).[29] After 30 min microsomal incubation (rat liver microsomes, 0.5 mg/ml), with tested compounds, a 20 μl aliquot of microsomal incubation mixture diluted 15-fold was added to 0.25 ml of 1.22 mM H_3PO_4 and 0.25 ml of 0.67% TBA. The mixture was heated for 60 min at 95°C in vials closed under nitrogen. To 200 μl of the resulting mixture, 340 μl of methanol and 60 μl of 1 mM NaOH were added and samples were centrifuged at 9500*g* for 5 min at 4°C. HPLC chromatography was performed with a Nucleosil 100A C18 (5 μM) 125 mm × 4 mm ID (Macherey-Nagel; Düren, Germany) column equipped with a LiChrospher RP-18 end-capped (5 μm) 4 mm × 4 mm ID (Merck) precolumn. The mobile phase was 25 mM phosphate buffer pH 6.5–methanol (60:40, v/v) at a flow rate of 0.8 ml/min and at 35°C. Spectrofluorimetric detection was operated at an excitation and emission wavelength at 532 and 553 nm, respectively. For quantification, the calibration curve was made with MDA prepared from 1,1,3,3-tetraethoxypropane hydrolyzed in acidic medium.

Measurement of Free Radical Scavenging Capacity

The free radical scavenging capacity of CYP2E1 binders was determined with widely used stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH).[30,31] The reaction involves a color change from violet to yellow that can easily be monitored by measuring the decrease in absorbance at 515 nm. A 0.02 ml aliquot of the tested compound at the indicated final concentration was mixed with 0.18 ml of 0.1 mM DPPH solution (in 50% ethanol) in a 96 well

microplate and the absorbance change was determined at 515 nm on a microplate spectrophotometer reader (Power Wave X, Bio-Tek).

Measurement of Microsomal CYP2E1 Activity

CYP2E1 activity was determined on microsomes from E47 cells using the hydroxylation of chlorzoxazone measured by HPLC.[32]

Statistical Analysis

Each experiment was performed at least three times. The results were compared using one-way ANOVA followed by Newman–Keuls multiple range test, $p < 0.05$.

RESULTS

NADPH-dependent Microsomal Oxidation of DCFHDA

The generation of microsomal ROS and LPO-derived reactive species was measured by oxidation of DCFHDA.[16,17] Addition of NADPH to the rat liver microsomes initiated DCFHDA oxidation which was elevated more than ten times in the presence of 0.5 mM NADPH (570 ± 51 pmol/min/mg/protein) (Fig. 1). The addition of GSH (2 mM) or ascorbic acid (2 mM), which are known to inhibit microsomal LPO and scavenge ROS, decrease the DCFHDA oxidation by 63 or 73%, respectively (Fig. 1). When we added 25 μM DPI (an inhibitor of flavin oxidoreductase such as NADPH-cytochrome P-450 reductase),[33] which suppresses NADPH-dependent electron transfer, the oxidation of DCFHDA was inhibited by 85%. These results demonstrate that inhibitors of LPO, ROS scavengers, and inhibitors of microsomal enzyme can prevent the NADPH-dependent oxidation of DCFHDA. We used iron chelators in order to evaluate the involvement of metal ion in this reaction. This NADPH-dependent oxidation of DCFHDA was strongly inhibited by EDTA or desferrioxamine treatment (Fig. 1). However, mannitol (100 mM) or DMSO (50 mM), $OH^{\bullet}$ scavengers, exhibited weak inhibitory effects, although the effect of DMSO was significant (Fig. 1).

The microsomal NADPH-dependent oxidation of DCFHDA was also measured in the presence of "tight" binders (4-methylpyrazole, imidazole, isoniazid, or pyridine) or "weak" binders to CYP2E1 (ethanol, acetone or glycerol). The process was inhibited in the presence of "tight" binders (Fig. 1). The inhibition was about 40% and isoniazid appeared to have stronger inhibitory effect (60%) than other "tight" binders. The "weak" binders exhibited little inhibitory effect on DCFHDA oxidation. Both acetone and glycerol had no significant effect, while ethanol showed a weak, but significant inhibitory effect. When CCl_4, a suicide substrate of CYP2E1, was present in the

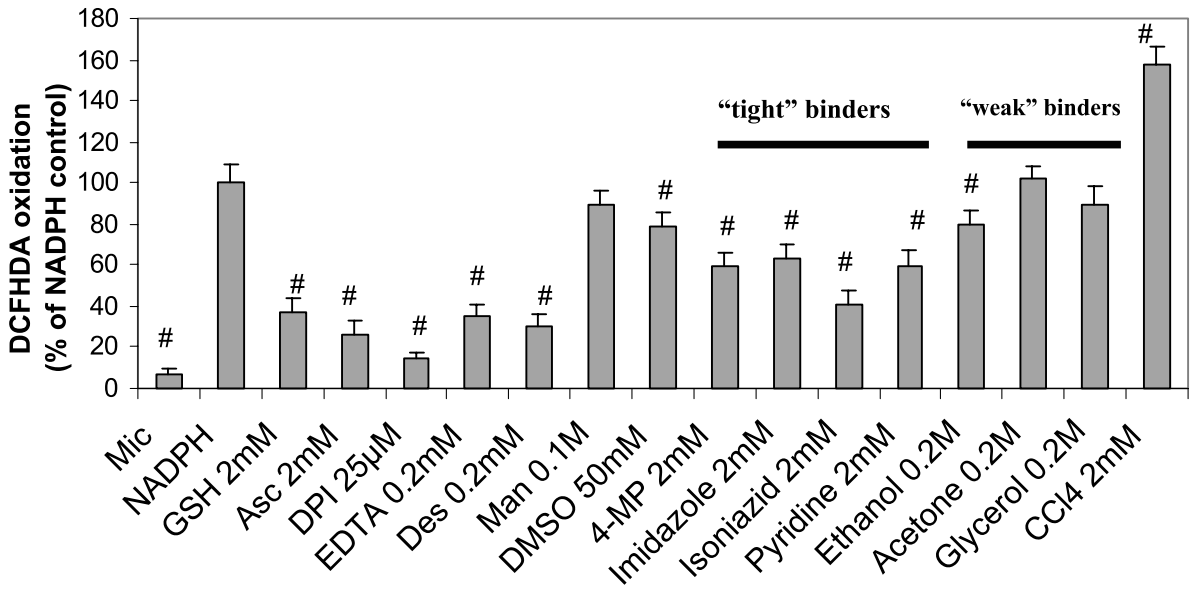

FIGURE 1 NADPH-initiated microsomal oxidation of DCFHDA and its modification in the presence of GSH, ascorbic acid, DPI, chelating agents, hydroxyl radical scavenger, "tight" binders to CYP2E1, "weak" binders to CYP2E1 and CCl_4. Microsomal oxidation of DCFHDA was measured as described under "Materials and Methods" section with rat liver microsomes in the presence of indicated additions. Values are the mean ± SD of three individual determinations. Number signs indicate a significant difference from the NADPH control (One-way ANOVA followed by Newman–Keuls multiple range test, $p < 0.05$) [(Mic: microsomes (0.5 mg/ml) alone), (NADPH: microsomes + NADPH (0.5 mM)), other samples contains microsomes, NADPH and indicated additions. (Asc: ascorbic acid), (Des: desferrioxamine), (Man: Mannitol)].

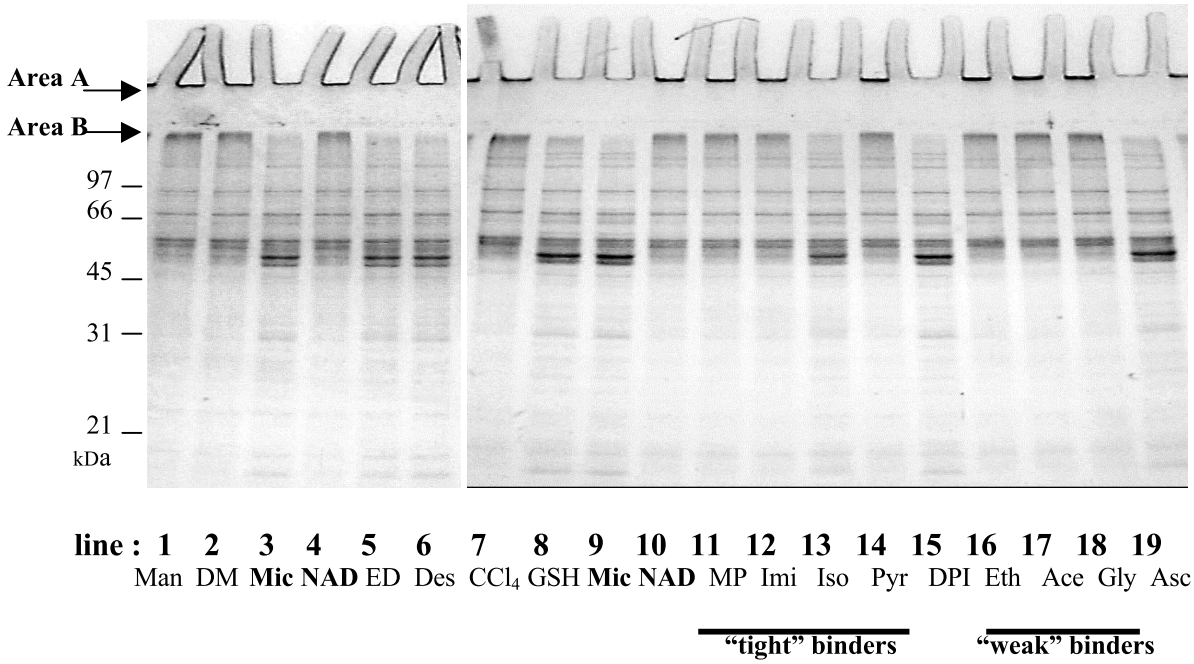

FIGURE 2 Effect of hydroxyl radical scavenger, chelating agents, CCl_4, GSH, "tight" binders to CYP2E1, DPI, "weak" binders to CYP2E1, and ascorbic acid on NADPH-dependent modifications of microsomal proteins. The rat liver microsomes (0.5 mg/ml) were incubated with NADPH (0.5 mM) at 37°C for 4 h in the presence of indicated additions, followed by SDS-PAGE and Coomassie Brilliant Blue staining [(Mic: microsomes (5 μg) alone), (NAD: microsomes + NADPH), other samples contain microsomes, NADPH and indicated additions: (Man: Mannitol, 100 mM), (DM: DMSO, 50 mM), (ED: EDTA, 0.2 mM), (Des: desferrioxamine, 0.2 mM), (CCl_4: CCl_4, 2 mM), (GSH: GSH, 2 mM), (MP: 4-MP, 2 mM), (Imi: imidazole, 2 mM), (Iso: Isoniazid, 2 mM), (Pyr: Pyridine, 2 mM), (DPI: DPI, 25 μM), (Eth: Ethanol, 200 mM), (Ace: Acetone, 200 mM), (Gly: Glycerol, 200 mM), (Asc: Ascorbic acid, 2 mM)].

incubation mixture, the DCFHDA oxidation was stimulated by about 50% (Fig. 1).

NADPH-dependent Modification of Microsomal Proteins

To demonstrate the biological significance of this NADPH-dependent microsomal LPO-derived reactive species and ROS production measured by DCFHDA oxidation, we examined the effect of NADPH on the modification of microsomal proteins. The Coomassie Brilliant Blue SDS-PAGE profile of the microsomal proteins modification is shown in Fig. 2. The electrophoretical profiles of microsomal proteins incubated with NADPH for 4 h at 37°C (Fig. 2, lines 4 and 10) were selectively modified at various degrees, when the protein profiles were compared to NADPH non-treated microsomes (Fig. 2, lines 3 and 9). The 50–55 kDa microsomal proteins markedly disappeared and high molecular weight aggregates appeared on the surface of stacking gel (Fig. 2, Area A) or on the junction of stacking and separating gel (Fig. 2, Area B) without penetrating into the 12% separating gel layer. These protein aggregates may result from the polymerization of low molecular weight proteins (under 55 kDa), which markedly disappeared. Those in the 60–100 kDa molecular weight zone remained unchanged after incubation with NADPH.

In the presence of GSH, ascorbic acid or DPI, we did not observe the strong modification of 50–55 kDa microsomal proteins and the high molecular weight aggregates, thus suggesting a protection of microsomal proteins from the LPO-derived reactive species and ROS (Fig. 2, lines 8, 15 and 19). Addition of 0.2 mM EDTA or desferrioxamine in the incubation mixture completely prevented the NADPH-dependent modification of microsomal proteins (Fig. 2, lines 5 and 6). These results suggests a requirement for iron and therefore the $OH^{\bullet}$ or LPO-derived reactive products are responsible. Mannitol (100 mM) and DMSO (50 mM) failed to prevent this modification (Fig. 2, lines 1 and 2), suggesting a LPO-dependent modification, rather than the effects of $OH^{\bullet}$.

We evaluated whether CYP2E1 binders influence the degree of the observed modification. Treatment with CYP2E1 binders did not protect the microsomal proteins from NADPH-dependent modification, with the exception of isoniazid (Fig. 2, lines 11–14 and 16–18). Incubation in the presence of isoniazid at a concentration of 2 mM prevented the modification of the low molecular weight bands (in the 50–55 kDa zone), which is approximately 80% of their initial total optical band intensity as quantified by densitometry (Fig. 2, line 13). There was also a decrease in the polymers at the surface of the gel (Fig. 2, Area A, line 13) and at the junction of the both gels (Area B).

TABLE I Effects of GSH, ascorbic acid, DPI, chelating agents, "tight" binders to CYP2E1, "weak" binders to CYP2E1 and CCl_4 on NADPH-dependent lipid peroxidation in rat liver microsomes. Incubations (30 min) were performed with rat liver microsomes (0.5 mg/ml) in 50 mM phosphate buffer, pH 7.4 as outlined under "Materials and Methods" section

	MDA (pmol/min/mg)	Percentage of NADPH control
Mic	23.8 ± 10.2#	9
Mic + NADPH (0.5 mM)	256.0 ± 40.3	100
Mic + NADPH + GSH (2 mM)	133.0 ± 41.2#	52
Mic + NADPH + Ascorbic acid (2 mM)	117.8 ± 35.4#	46
Mic + NADPH + DPI (25 μM)	107.5 ± 13.6#	42
Mic + NADPH + EDTA (0.2 mM)	84.5 ± 16.1#	33
Mic + NADPH + Desferrioxamine (0.2 mM)	110.1 ± 17.4#	43
Mic + NADPH + 4-MP (2 mM)	221.4 ± 39.3	87
Mic + NADPH + Imidazole (2 mM)	245.0 ± 45.1	96
Mic + NADPH + Isoniazid (2 mM)	133.1 ± 39.2#	52
Mic + NADPH + Pyridine (2 mM)	225.0 ± 40.2	88
Mic + NADPH + Ethanol (0.2 mM)	291.8 ± 51.6	114
Mic + NADPH + Acetone (0.2 mM)	307.2 ± 39.8	120
Mic + NADPH + Glycerol (0.2 mM)	350.7 ± 29.2#	137
Mic + NADPH + CCl_4 (2 mM)	527.4 ± 30.7#	206

Values are the mean ± SD of three individual determinations. #Sign indicates a significant difference from microsomes + NADPH. (One-way ANOVA followed by Newman–Keuls multiple range test, $p < 0.05$) (Mic: microsomes).

Ethanol, glycerol and 4-MP, which are also known as $OH^{\bullet}$ scavengers,[34] did not show any protective effect. Treatment with CCl_4 did not modify the degree of the NADPH-dependent modification of microsomal proteins (Fig. 2, line 7).

NADPH-dependent Microsomal LPO

Since LPO-derived reactive species are considered to play an important role in these NADPH-dependent oxidations, microsomal LPO was evaluated using the HPLC method for the measurement of MDA. Table I demonstrates the effect of several different compounds on NADPH-dependent microsomal MDA formation. Addition of NADPH initiated microsomal LPO. In the presence of GSH, ascorbic acid, DPI, EDTA and desferrioxamine, the NADPH-dependent LPO was strongly inhibited by about 50–70%. Among the tested CYP2E1 binders, only isoniazid showed a significant inhibition (50%) whereas other CYP2E1 binders did not significantly inhibit this reaction (Table I). When CCl_4 was present in the incubation mixture, the LPO was stimulated by about 100%.

NADPH-dependent Modification of CYP2E1 Protein

Since the modification of CYP2E1 protein cannot be evaluated with Coomassie Brilliant Blue staining, we performed Western blot analysis to study the modification of CYP2E1 protein. The specific anti-CYP2E1 antibody recognized in microsomes only one major protein band with a molecular weight of 52 kDa corresponding to that of CYP2E1 (Fig. 3A, lines 1 and 7). After 4 h incubation with NADPH, the intensity of the CYP2E1 protein band was decreased by 94% as quantified by densitometry (Fig. 3A, lines 2 and 8; Fig. 3B). In the presence of EDTA, desferrioxamine, ascorbic acid, GSH and DPI, the diminution of the CYP2E1 band intensity was strongly counteracted, suggesting that these compounds protected the CYP2E1 proteins from modifications (Fig. 3A, lines 3–6 and 13; Fig. 3B).

We evaluated whether "tight" and "weak" binders to CYP2E1 could protect CYP2E1 protein in the NADPH-dependent microsomal oxidative reaction. All of tested compounds added to the incubation medium prevented the modification of CYP2E1 protein with the exception of glycerol (Fig. 3A, lines 9–12 and 15–17). Each compound showed a different potency in protection (Fig. 3B), isoniazid being the most effective whereas ethanol the least effective (isoniazid $>$ 4-MP = imidazole = pyridine $\geqslant$ acetone $>$ ethanol). "Tight" binders were more effective than "weak" binders. 4-MP, imidazole and pyridine exhibited similar protective potency. The treatment with CCl_4 accelerated the CYP2E1 modification (Fig. 3A, line 14 and B).

Effect of Protease Inhibitor on NADPH-dependent Modification of CYP2E1 Protein

To study if proteases are involved in the observed CYP2E1 modifications, we employed PMSF, an inhibitor of serine proteases.[35] The NADPH-dependent modification of CYP2E1 protein was not inhibited in the presence of 0.6 mM PMSF (Fig. 4), suggesting a direct oxidative degradation of CYP2E1 rather than protease-mediated degradation.

Free Radical Scavenging Capacity

Since only isoniazid prevented the NADPH-dependent microsomal proteins modification and LPO among the tested CYP2E1 binders, we explored the

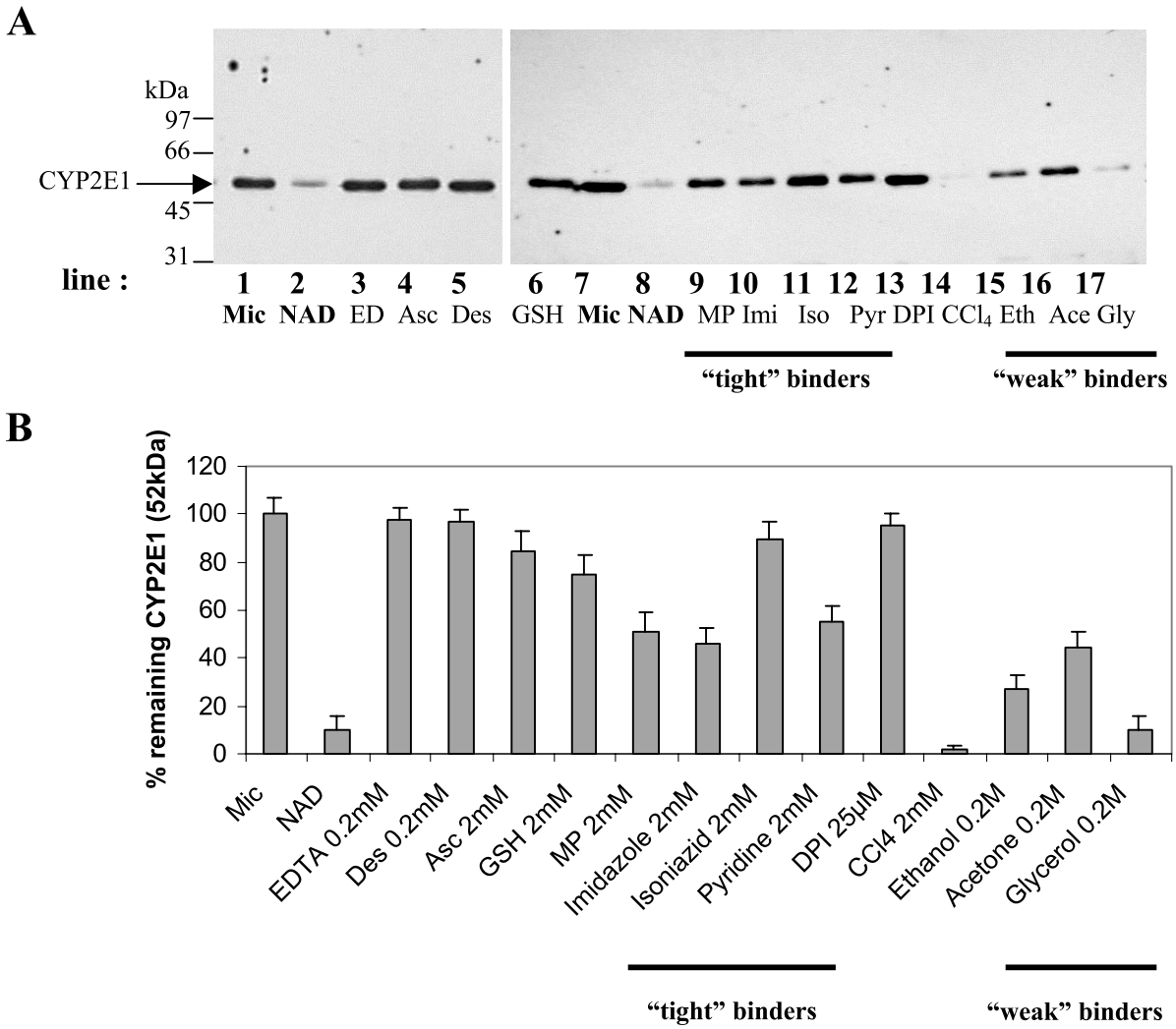

FIGURE 3 Influence of chelating agents, ascorbic acid, GSH, "tight" binders to CYP2E1 DPI, "weak" binders to CYP2E1, and CCl_4 on NADPH-dependent modifications of CYP2E1 protein. The rat liver microsomes (0.5 mg/ml) were incubated with NADPH (0.5 mM) at 37°C for 4 h in the presence of indicated additions, followed by SDS-PAGE and Western blot analysis using anti-P450 antibodies (A) as described in "Materials and Methods" section and quantified by densitometry analysis of remaining CYP2E1 protein (B). Values are the mean ± SD of three individual determinations [(Mic: microsomes (5 μg) alone), (NAD: microsomes + NADPH), other samples contain microsomes, NADPH and indicated additions (ED: EDTA, 0.2 mM), (Asc: Ascorbic acid, 2 mM), (Des: desferrioxamine, 0.2 mM), (MP: 4-MP, 2 mM), (Imi: imidazole, 2 mM), (Iso: Isoniazid, 2 mM), (Pyr: Pyridine, 2 mM), (DPI: DPI, 25 μM), (CCl_4: CCl_4, 2 mM), (Eth: Ethanol, 200 mM), (Ace: Acetone, 200 mM), (Gly: Glycerol, 200 mM)].

possibility that this compound might have an antioxidant property. We thus compared its free radical scavenging ability with that of GSH and ascorbic acid by using the DPPH assay. Isoniazid, GSH and ascorbic acid were powerful scavengers of DPPH free radicals whereas other CYP2E1 binders, DPI and EDTA did not show any effect (Fig. 5A and B). The free radical scavenging ability of isoniazid was as effective as that of GSH.

Experiments with HepG2 Cells Transfected by CYP2E1

We also evaluated whether the CYP2E1 binders can inhibit CYP2E1-dependent production of ROS and LPO-derived reactive species. Since it is not clear how much of these reactive species production is due to CYP2E1 in rat liver microsomes, we used microsomal fractions prepared by CYP2E1 transfected HepG2 (E47) cells and control HepG2 (C34) cells. We compared DCFHDA oxidation in microsomal fractions from E47 and C43 cells. This result showed a 6.1-fold increase in DCFHDA oxidation in microsomes from E47 cells as compared to C34 cells in the presence of NADPH (E47 cells, 48.1 ± 7.5 pmol/min/mg; C34 cells, 7.9 ± 2.3 pmol/min/mg), supporting strong participation of the transfected CYP2E1. Among the tested CYP2E1 binders, only isoniazid significantly (60%) inhibited DCFHDA oxidation in E47 cells microsomes (Fig. 6). This result correlates with the antioxidant property of isoniazid observed in experiments with rat liver microsomes. Other binders did not significantly inhibit the reaction.

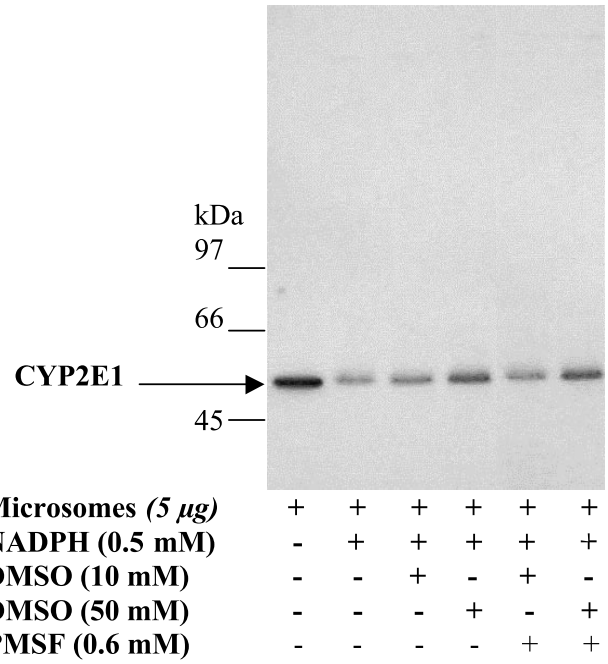

FIGURE 4 Influence of PMSF on NADPH-dependent modifications of microsomal CYP2E1 protein. The rat liver microsomes (0.5 mg/ml) were incubated with NADPH (0.5 mM) at 37°C for 4 h in the presence of indicated additions, followed by SDS-PAGE and Western blot analysis using anti-P450 2E1 antibodies. DMSO (10 or 50 mM) was used to dissolve PMSF.

For the following experiments, the CYP2E1 activity was measured in the presence of CYP2E1 binders using chlorzoxazone as a specific CYP2E1 substrate in E47 microsomes. In the presence of ethanol (20 mM), acetone (20 mM), 4-MP (0.2 mM), or isoniazid (0.2 mM), the CYP2E1 activity was inhibited by 65, 55, 85 or 84%, respectively. "Tight" CYP2E1 binders were more effective in inhibition of CYP2E1 activity that "weak" binders.

DISCUSSION

The results of the present investigation demonstrate that LPO plays a major role in the NADPH-dependent modification of microsomal proteins. The prevention of microsomal protein modification by EDTA and desferrioxamine suggests a requirement for iron and therefore the hydroxyl radical or LPO is responsible for this modification, rather than $O_2^{\bullet -}$ or H_2O_2. The weak effects of $OH^{\bullet}$ scavengers (DMSO, mannitol, ethanol, glycerol, and 4-MP)[34] suggest LPO.

Therefore, it is believed that LPO-derived reactive species cause the observed NADPH-dependent modification of CYP2E1 protein. It is known that LPO induces destruction of heme enzymes.[36] A direct relationship between LPO and breakdown of P-450 was demonstrated in previous studies.[36] Isoform-specific degradation of P-450 by LPO in rat liver microsomes was also reported.[37] The results from the present experiment with PMSF also support, proteases-independent, direct oxidative modification of CYP2E1 protein.

The present study demonstrates that CYP2E1 binders and isoniazid differentially inhibit LPO-catalyzed oxidative modification of CYP2E1 protein in NADPH-dependent microsomal reactions. It seems that CYP2E1 binders protect CYP2E1 protein from the oxidative modification mainly by binding to its active site, which provides a steric protection of some sensitive structural elements, rather than by blocking the reactive species production, because these compounds, except isoniazid, did not inhibit NADPH-dependent LPO. In the case of isoniazid, this compound exerted its strong protective effect on CYP2E1 protein modification by its ability to scavenge free radicals, besides its binding affinity to CYP2E1.

Another experimental support for the role of binding in CYP2E1 protein protection is based on previous study monitoring the effects of ethanol, 2-propanol, and DMSO on the loss of CYP2E1 in cultured hepatocytes.[7] Their ability to maintain CYP2E1 levels was correlated with the spectral binding constant (Kd) for the purified enzyme. In these studies, "tight" binders showed stronger protective effects on CYP2E1 modification than "weak" binders. The strong binding affinity of *N*-based ligands to CYP2E1 has been shown in the previous studies[22–24] and it is known that the nitrogen lone pair of electrons has a strong affinity for heme of CYP2E1.[22]

The observed inability of glycerol to prevent CYP2E1 modification can be explained by absence of direct CYP2E1 binding ability of this compound and its stimulation of the microsomal LPO. Glycerol is not a direct substrate for oxidation to formaldehyde by CYP2E1, but it is a substrate for an oxidant derived from interaction of iron with H_2O_2 generated by P-450.[25] No distinct substrate binding spectrum was observed when 2 M glycerol was added to microsomal systems in the study of Voznesensky and Schenkman.[38]

The CCl_4 is known to be metabolized by CYP2E1 to trichloromethyl radical ($^{\bullet}CCl_3$), a carbon-centered radical, which will increase microsomal LPO.[39] Tierney *et al.*[14] using an *in vivo* mouse model, showed that CYP2E1 inactivated by CCl_4 was rapidly removed from the ER. Our results from the experiment with CCl_4 demonstrate that its LPO stimulating metabolism accelerates the modification of CYP2E1 protein.

The observed inhibition of DCFHDA oxidation by "tight" binders in rat liver microsomes seems to have a limited biological significance under these experimental conditions because "tight" binders were not able to prevent NADPH-dependent modification of microsomal proteins. Although CYP2E1 binders

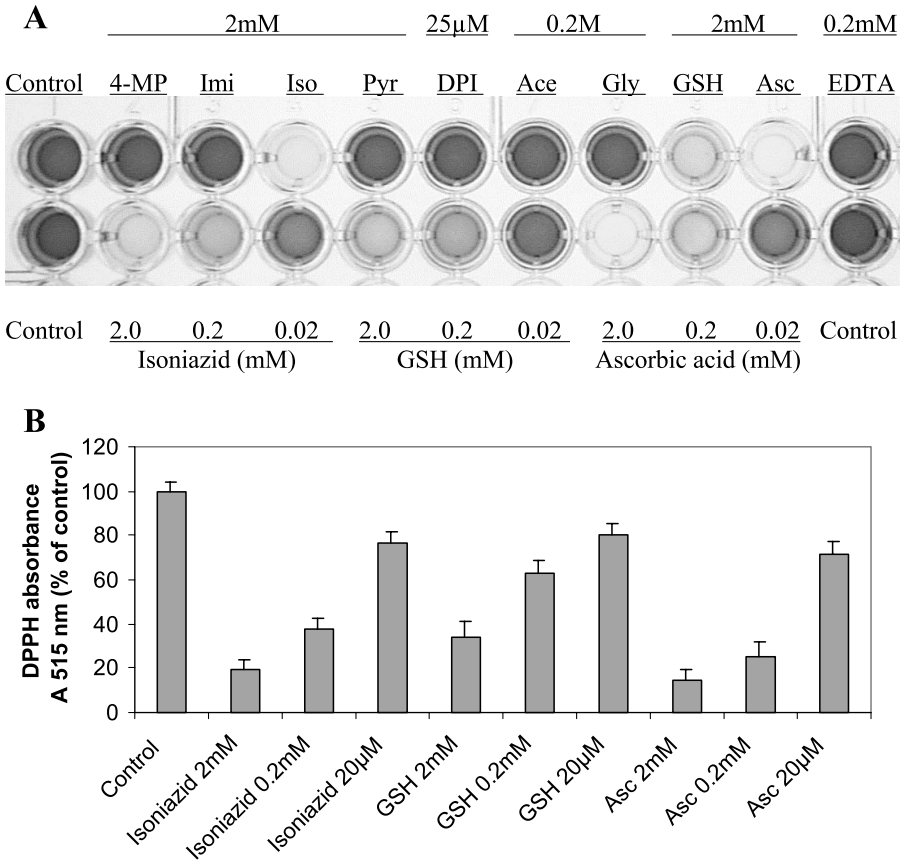

FIGURE 5 Free radical scavenging capacity of isoniazid, GSH and ascorbic acid. The free radical scavenging capacity of CYP2E1 binders, DPI, GSH, ascorbic acid, and EDTA was measured using DPPH (0.1 mM) as described under "Materials and Methods" section (A): photograph of 96 wells micro plate and (B): quantification of DPPH absorbance at 515 nm [(Control: DPPH alone), other samples contain DPPH and indicated additions: (Imi: imidazole), (Iso: Isoniazid), (Pyr: Pyridine), (Ace: Acetone), (Gly: Glycerol), (Asc: Ascorbic acid)].

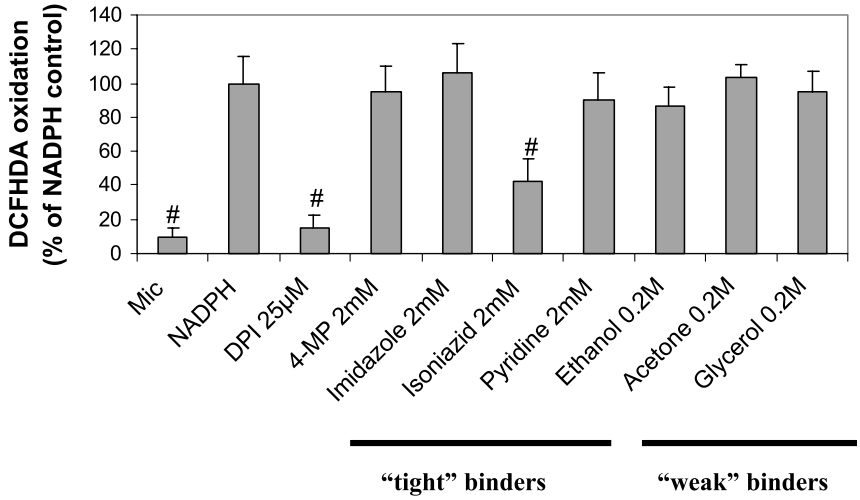

FIGURE 6 Effect of CYP2E1 binders, DPI on NADPH-dependent oxidation of DCFHDA in microsomal fractions from CYP2E1 transfected HepG2 cells. Microsomal oxidation of DCFHDA was measured as described under "Materials and Methods" section with E47 cells microsomes in the presence of indicated additions. Values are the mean ± SD of three individual determinations. Number signs indicate a significant difference from the NADPH control. (One-way ANOVA followed by Newman–Keuls multiple range test, $p < 0.05$) [(Mic: microsomes (1.0 mg/ml) alone), (NADPH: microsomes + NADPH (0.5 mM)], other samples contain microsomes, NADPH and indicated additions.

strongly inhibited CYP2E1 activity in E47 cells microsomes, these compounds were not able to inhibit CYP2E1-mediated DCFHDA oxidation, reflecting the loose coupling associated with this enzyme.[40,41] Therefore, the observed inhibition of DCFHDA oxidation by "tight" binders in rat liver microsomes can be considered to be a consequence of non-specific inhibition of other P-450 isoforms.

It has been postulated in many studies that CYP2E1 binders stabilize CYP2E1 protein because these compounds inhibit proteolytic degradation of the enzyme.[7–12] Recently, several studies suggested that P-450-dependent oxidative reaction plays an important role in the rapid CYP2E1 turn-over process.[10,15,19,20] However, it is not well known whether the stabilization effect of CYP2E1 binders is mediated either by inhibition of the oxidative modification of CYP2E1 or by prevention of the proteolytic degradation of xenobiotic-bound CYP2E1. The present study showed that CYP2E1 binders inhibit oxidative modification of CYP2E1 protein in protease-independent oxidative reactions. Therefore, these effects of CYP2E1 binders are considered to contribute to the regulation of hepatic CYP2E1 protein level in response to these compounds via stabilization of the protein.

Acknowledgements

The authors would like to thank Dr A.I. Cederbaum for providing with HepG2 C34 and E47 cell subclones. We thank Professor N.E. Huseby for manuscript reading. This work was partially supported by Association Régionale pour 1'Enseignement et la Recherche Scientifique (ARERS) and Biologie Prospective.

References

[1] Lieber, C.S. (1997) "Cytochrome P-4502E1: its physiological and pathological role", *Physiol. Rev.* **77**, 17–44.
[2] Ekstrom, G. and Ingelman-Sundberg, M. (1989) "Rat liver microsomal NADPH-supported oxidase activity and lipid peroxidation dependent on ethanol-inducible cytochrome P-450 (P-450IIE1)", *Biochem. Pharmacol.* **38**, 1313–1319.
[3] Song, B.J., Gelboin, H.V., Park, S.S., Yang, C.S. and Gonzalez, F.J. (1986) "Complementary DNA and protein sequences of ethanol-inducible rat and human cytochrome P-450s. Transcriptional and post-transcriptional regulation of the rat enzyme", *J. Biol. Chem.* **261**, 16689–16697.
[4] Ryan, D.E., Koop, D.R., Thomas, P.E., Coon, M.J. and Levin, W. (1986) "Evidence that isoniazid and ethanol induce the same microsomal cytochrome P-450 in rat liver, an isozyme homologous to rabbit liver cytochrome P-450 isozyme 3a", *Arch. Biochem. Biophys.* **246**, 633–644.
[5] Kim, S.G., Williams, D.E., Schuetz, E.G., Guzelian, P.S. and Novak, R.F. (1988) "Pyridine induction of cytochrome P-450 in the rat: role of P-450j (alcohol-inducible form) in pyridine *N*-oxidation", *J. Pharmacol. Exp. Ther.* **246**, 1175–1182.
[6] Reinke, L.A., Sexter, S.H. and Rikans, L.E. (1985) "Comparison of ethanol and imidazole pretreatments on hepatic monooxygenase activities in the rat", *Res. Commun. Chem. Pathol. Pharmacol.* **47**, 97–106.
[7] Eliasson, E., Johansson, I. and Ingelman-Sundberg, M. (1988) "Ligand-dependent maintenance of ethanol-inducible cytochrome P-450 in primary rat hepatocyte cell cultures", *Biochem. Biophys. Res. Commun.* **150**, 436–443.
[8] Wu, D.F., Clejan, L., Potter, B. and Cederbaum, A.I. (1990) "Rapid decrease of cytochrome P-450IIE1 in primary hepatocyte culture and its maintenance by added 4-methylpyrazole", *Hepatology* **12**, 1379–1389.
[9] Yang, M.X. and Cederbaum, A.I. (1997) "Glycerol increases content and activity of human cytochrome P-4502E1 in a transduced HepG2 cell line by protein stabilization", *Alcohol: Clin. Exp. Res.* **21**, 340–347.
[10] Roberts, B.J., Song, B.J., Soh, Y., Park, S.S. and Shoaf, S.E. (1995) "Ethanol induces CYP2E1 by protein stabilization. Role of ubiquitin conjugation in the rapid degradation of CYP2E1", *J. Biol. Chem.* **270**, 29632–29635.
[11] Song, B.J., Veech, R.L., Park, S.S., Gelboin, H.V. and Gonzalez, F.J. (1989) "Induction of rat hepatic *N*-nitrosodimethylamine demethylase by acetone is due to protein stabilization", *J. Biol. Chem.* **264**, 3568–3572.
[12] Yang, M.X. and Cederbaum, A.I. (1997) "Characterization of cytochrome P-4502E1 turnover in transfected HepG2 cells expressing human CYP2E1", *Arch. Biochem. Biophys.* **341**, 25–33.
[13] Eliasson, E., Mkrtchian, S. and Ingelman-Sundberg, M. (1992) "Hormone- and substrate-regulated intracellular degradation of cytochrome P-450 (2E1) involving MgATP-activated rapid proteolysis in the endoplasmic reticulum membranes", *J. Biol. Chem.* **267**, 15765–15769.
[14] Tierney, D.J., Haas, A.L. and Koop, D.R. (1992) "Degradation of cytochrome P-450 2E1: selective loss after labilization of the enzyme", *Arch. Biochem. Biophys.* **293**, 9–16.
[15] Roberts, B.J. (1997) "Evidence of proteasome-mediated cytochrome P-450 degradation", *J. Biol. Chem.* **272**, 9771–9778.
[16] Serron, S.C., Dwivedi, N. and Backes, W.L. (2000) "Ethylbenzene induces microsomal oxygen free radical generation: antibody-directed characterization of the responsible cytochrome P-450 enzymes", *Toxicol. Appl. Pharm.* **164**, 305–311.
[17] Puntarulo, S. and Cederbaum, A.I. (1998) "Production of reactive oxygen species by microsomes enriched in specific human cytochrome P-450 enzymes", *Free Radic. Biol. Med.* **24**, 1324–1330.
[18] McCay, P.B., Reinke, L.A. and Rau, J.M. (1992) "Hydroxyl radicals are generated by hepatic microsomes during NADPH oxidation: relationship to ethanol metabolism", *Free Radic. Res. Commun.* **15**, 335–346.
[19] Zhukov, A. and Ingelman-Sundberg, M. (1999) "Relationship between cytochrome P-450 catalytic cycling and stability: fast degradation of ethanol-inducible cytochrome P-450 2E1 (CYP2E1) in hepatoma cells is abolished by inactivation of its electron donor NADPH-cytochrome P-450 reductase", *Biochem. J.* **340**, 453–458.
[20] Goasduff, T. and Cederbaum, A.I. (1999) "NADPH-dependent microsomal electron transfer increases degradation of CYP2E1 by the proteasome complex: role of reactive oxygen species", *Arch. Biochem. Biophys.* **370**, 258–270.
[21] Grune, T., Reinheckel, T. and Davies, K.J. (1997) "Degradation of oxidized proteins in mammalian cells", *FASEB J.* **11**, 526–534.
[22] Hargreaves, M.B., Jones, B.J., Smith, D.A. and Gescher, A. (1994) "Inhibition of *p*-nitrophenol hydroxylase in rat liver microsomes by small aromatic and heterocyclic molecules", *Drug Metab. Dispos.* **22**, 806–810.
[23] Kim, S.G. and Novak, R.F. (1993) "The induction of cytochrome P-4502E1 by nitrogen- and sulfur-containing heterocycles: expression and molecular regulation", *Toxicol. Appl. Pharmacol.* **120**, 257–265.
[24] Lewis, D.F. (2000) "Structural characteristics of human P-450s involved in drug metabolism: QSARs and lipophilicity profiles", *Toxicology* **144**, 197–203.
[25] Clejan, L.A. and Cederbaum, A.I. (1992) "Role of cytochrome P-450 in the oxidation of glycerol by reconstituted systems and microsomes", *FASEB J.* **6**, 765–770.
[26] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) "Protein measurement with folin phenol reagent", *J. Biol. Chem.* **193**, 265–275.

[27] Chen, Q. and Cederbaum, A.I. (1998) "Cytotoxicity and apoptosis produced by cytochrome P-450 2E1 in Hep G2 cells", *Mol. Pharmacol.* **53**, 638–648.
[28] Jolivalt, C., Leininger-Muller, B., Betrand, P., Herber, R., Christen, Y. and Siest, G. (2000) "Differential oxidation of apolipoprotein E isoforms and interaction with phospholipids", *Free Radic. Biol. Med.* **28**, 129–140.
[29] Brown, R.K. and Kelly, F.J. (1996) "Peroxides and other products: detection of malondialdehyde by HPLC", In: Punchard, N.A. and Kelly, F.J., eds, Free Radicals: A Practical Approach (Oxford University Press, Oxford, UK), pp 128–130.
[30] Blois, M.S. (1958) "Antioxidant determination by the use of a stable free radical", *Nature* **181**, 1199–1200.
[31] Belinky, P.A., Aviram, M., Mahmood, S. and Vaya, J. (1998) "Structural aspects of the inhibitory effect of glabridin on LDL oxidation", *Free Radic. Biol. Med.* **24**, 1419–1429.
[32] Peter, R., Bocker, R., Beaune, P.H., Iwasaki, M., Guengerich, F.P. and Yang, C.S. (1990) "Hydroxylation of chlorzoxazone as a specific probe for human liver cytochrome P-450IIE1", *Chem. Res. Toxicol.* **3**, 566–573.
[33] McGuire, J.J., Anderson, D.J., McDonald, B.J., Narayanasami, R. and Bennett, B.M. (1998) "Inhibition of NADPH-cytochrome P-450 reductase and glyceryl trinitrate biotransformation by diphenyleneiodonium sulfate", *Biochem. Pharmacol.* **56**, 881–893.
[34] Cederbaum, A.I. and Berl, L. (1982) "Pyrazole and 4-methylpyrazole inhibit oxidation of ethanol and dimethyl sulfoxide by hydroxyl radicals generated from ascorbate, xanthine oxidase, and liver microsomes", *Arch. Biochem. Biophys.* **216**, 530–543.
[35] Turini, P., Kurooka, S., Steer, M., Corbascio, A.N. and Singer, T.P. (1969) "The action of phenylmethylsulfonyl fluoride on human acetylcholinesterase, chymotrypsin and trypsin", *J. Pharmacol. Exp. Ther.* **167**, 98–104.
[36] Levin, W., Lu, A.Y., Jacobson, M., Kuntzman, R., Poyer, J.L. and McCay, P.B. (1973) "Lipid peroxidation and the degradation of cytochrome P-450 heme", *Arch. Biochem. Biophys.* **158**, 842–852.
[37] Kitada, M., Komori, M., Ohi, H., Imaoka, S., Funae, Y. and Kamataki, T. (1989) "Form-specific degradation of cytochrome P-450 by lipid peroxidation in rat liver microsomes", *Res. Commun. Chem. Pathol. Pharmacol.* **63**, 175–188.
[38] Voznesensky, A.I. and Schenkman, J.B. (1992) "Inhibition of cytochrome-P-450 reductase by polyols has an electrostatic nature", *Eur. J. Biochem.* **210**, 741–746.
[39] McGregor, D. and Lang, G. (1996) "Carbon tetrachloride: genetic effects and other modes of action", *Mutat. Res.* **366**, 181–195.
[40] Gorsky, L.D., Koop, D.R. and Coon, M.J. (1984) "On the stiochiometry of the oxidase and monooxygenase reactions catalyzed by liver microsomal cytochrome P-450. Products of oxygen reduction", *J. Biol. Chem.* **259**, 6812–6817.
[41] Dai, Y., Rashba-Step, J. and Cederbaum, A.I. (1993) "Stable expression of human cytochrome P4502E1 in HepG2 cells: characterization of catalytic activities and production of reactive oxygen intermediates", *Biochemistry* **32**, 6928–6937.