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Identification of catalase in human livers as a factor that enhances phenytoin dihydroxy metabolite formation by human liver microsomes

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Abstract

We have reported previously that the formation of a 3′,4′-dihydroxylated metabolite of phenytoin (3′,4′-diHPPH) by human liver microsomal cytochrome P450 (P450) is enhanced by the addition of human liver cytosol [Komatsu *et al.*, Drug Metab Dispos 2000;28:1361–8]. The enhancing factor was determined in this study. The addition of cytosolic proteins precipitated by 50% ammonium sulfate to incubation mixtures increased the rate of microsomal 3′,4′-diHPPH formation. This fraction was separated further by diethylaminoethyl-, carboxymethyl-, and hydroxyapatite-column chromatography. The amino acid sequence of the purified protein of ~55 kDa by electrophoresis revealed this protein to be a catalase. The addition of purified or authentic catalase to the incubation mixtures increased the rates of microsomal 3′,4′-diHPPH formation from 3′- and 4′-hydroxylated metabolites and from phenytoin in a concentration-dependent manner. In reconstituted systems containing CYP2C9, CYP2C19, and CYP3A4, the formation of 3′,4′-diHPPH was also enhanced by catalase to different extents. This is the first report that catalase in livers enhances drug oxidation activities catalyzed by P450 in human liver microsomes.

Keywords: Cytochrome P450; Human; Liver; Microsomes; Cytosol; Catalase; Hydrogen peroxide; Phenytoin; Enhancement

1. Introduction

Cytochrome P450 comprises a multigene family of hemoproteins responsible for the oxidation of numerous exogenous and endogenous compounds [1]. P450 enzymes that belong to the CYP1, CYP2, and CYP3 families play important roles in the metabolism of therapeutic drugs [2]. There are large interindividual variations in the contents and activities of several P450 forms, which affect the efficacy or toxicity of drugs [3].

Phenytoin (5,5-diphenylhydantoin) is widely used as an anticonvulsant agent. It has been reported that phenytoin shows non-linearity in its blood concentrations [4], teratogenicity [5], and hypersensitivity reactions including hepatitis [6]. Many drug interactions with phenytoin have also been reported [7–9]. The proposed metabolic pathways of phenytoin catalyzed by human liver microsomal P450 are shown in Fig. 1. It has been suggested that phenytoin is oxidized to 4′-HPPH, a main metabolite, mainly by CYP2C9 and to a minor extent by CYP2C19 [10,11]. The formation of 3′-HPPH is a minor pathway, and the P450s responsible for its formation are still unclear. Recently, we reported that CYP2C9, CYP2C19, and CYP3A4 had catalytic activities in 3′,4′-diHPPH formation from 4′-HPPH and that the contributions of these three enzymes differed in individual humans [11]. Although the human urinary concentration of 3′,4′-diHPPH is lower than that of 4′-HPPH [12], it has been demonstrated that 3′,4′-diHPPH could be oxidized to semiquinone and quinone derivatives, which may covalently bind to microsomal proteins and lead to P450 inactivation or produce autoantibodies [13]. Therefore, the liver microsomal formation of 3′,4′-diHPPH may be important in terms of the toxicity of phenytoin.

In our previous study, we reported that the rates of liver microsomal 3′,4′-diHPPH formation are enhanced by the addition of human liver cytosol [11]. There were large interindividual differences in the extent of the effects of cytosol. Serum albumin could not produce these effects

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Abbreviations: P450, general term for cytochrome P450; CYP, individual forms of P450; 4′-HPPH, 5-(4′-hydroxyphenyl)-5-phenylhydantoin; 3′-HPPH, 5-(3′-hydroxyphenyl)-5-phenylhydantoin; 3′,4′-diHPPH, 5-(3′,4′-dihydroxyphenyl)-5-phenylhydantoin; and HA, hydroxyapatite.

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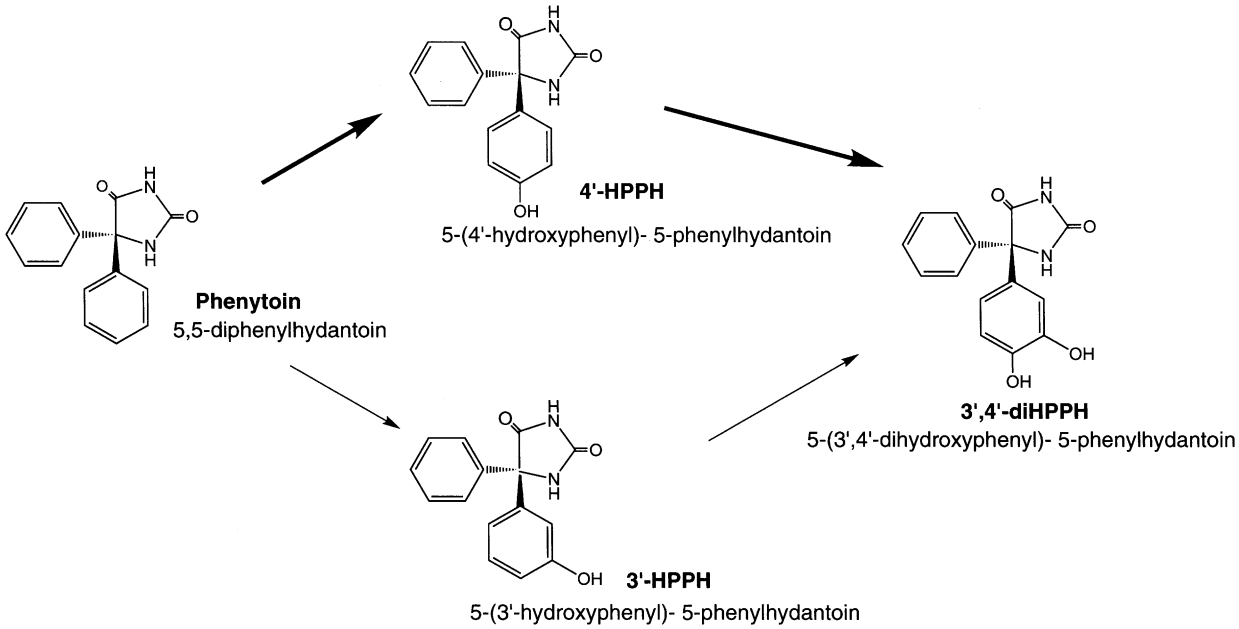

Fig. 1. Proposed pathways of phenytoin metabolism in humans.

[11]. The enhancing effects of cytosolic protein on microsomal P450 systems have been reported previously with regard to the *N*-demethylation reactions of drugs and the mutagenic activation of polycyclic aromatic hydrocarbons, aminoazo dyes, or *N*-nitrosamines [14–19]. However, the cytosolic factor(s) responsible for activation has not been identified.

In this study, we demonstrated that catalase is one factor in human liver cytosol that enhances the liver microsomal formation of 3′,4′-diHPPH from phenytoin.

2. Materials and methods

2.1. Chemicals

Phenytoin, 3-amino-1,2,4-triazole, and phenylhydrazine were obtained from Wako Pure Chemicals. 4′-HPPH and 3′-HPPH were purchased from the Aldrich Chemical Co. Ion exchange chromatographic supports were obtained from Pharmacia Biotech. Catalase from human erythrocytes (50,000 units/mg protein, Lot 120K1402) was purchased from the Sigma Chemical Co. and was used without further treatment. Anti-human catalase immunoglobulin G fraction was obtained from Oxis International. Other reagents used in this study were obtained from sources described previously [11] or were of the highest quality commercially available.

2.2. Enzyme preparations

Human liver microsomes and cytosol were prepared as described previously [20]. Liver samples designated HL-1, -3, -4, -5, -6, -9, and -10 corresponded to those reported previously [11]. A mixture of equal volumes of these seven samples was used as pooled human liver microsomes or cytosol. Recombinant human CYP2C9, CYP2C19, and CYP3A4 were purified from *Escherichia coli* membranes as described previously [21], and NADPH-cytochrome P450 reductase and cytochrome b_5 were from rabbit liver microsomes [22,23]. Catalytic activities of CYP2C9, CYP2C19, and CYP3A4 in the reconstituted systems were validated by *S*-warfarin 7-hydroxylation, *S*-mephenytoin 4′-hydroxylation, and nifedipine oxidation activities, respectively [24].

The concentrations of P450 [25] and protein [26] were estimated as described previously.

2.3. Enzyme assays

The hydroxylation of phenytoin, 4′-HPPH, and 3′-HPPH was determined as described previously [11]. Substrates (100 μM) were incubated at 37° for 30 min with human liver microsomes (1.0 mg protein/mL) and liver cytosol (5.0 mg protein/mL) in 50 mM potassium phosphate buffer (pH 7.4) containing an NADPH-generating system. In reconstituted systems, recombinant human P450 (0.20 μM), NADPH-cytochrome P450 reductase (0.80 μM), cytochrome b_5 (0.20 μM), cholate (0.25 mM), and a phospholipid mixture (20 μg/mL) were used as described previously [11]. After extraction with methyl-*tert*-butyl ether, the product formation was determined by a high performance liquid chromatography system [11]. Assignment of the 3′,4′-diHPPH peak was made by liquid chromatography/mass spectrometry analysis as in our previous study [27]. We present the activities for the formation of 3′,4′-diHPPH on the basis of the chromatographic peak height, using 4′-HPPH as a standard. The results presented in this study are the means of

duplicate determinations, and the ranges in these values were less than 10% of the means.

2.4. Ammonium sulfate fractionation

Pooled human liver cytosol (~10 mg/mL, 100 mL) was subjected to 1–25, 25–50, and 50–75% ammonium sulfate fractionation. After the addition of ammonium sulfate and standing at 4° for 30 min, the liver cytosolic fraction was centrifuged at 9000 *g* for 30 min. Each pellet was suspended in 10 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA. These fractions were dialyzed using cellulose tubing (Spectrum Laboratories) for about 20 hr against 100 vol. of the suspended buffer.

2.5. Ion exchange chromatography

Part of the 50% ammonium sulfate fraction (~40 mg/mL, ~9 mL) was loaded onto a 2.5 × 8 cm DEAE-Sephacel column equilibrated with 10 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA. The column was washed successively with the equilibration buffer and buffer containing 50, 100, 150, 200, 250, 300, 400, and 500 mM KCl. Each eluate fraction (4 mL) was collected, and protein elution was monitored by measuring A_{280}. A portion of each protein peak fraction (50, 100, and 150 μL) was added to an incubation mixture (250 μL) as described in Section 2.3 and was examined for enhancing 3′,4′-diHPPH formation catalyzed by human liver microsomes. Fractions 66–76 eluted from DEAE-Sephacel (~0.5 mg/mL, ~44 mL) were combined and further separated by cation exchange chromatography on a 1.5 × 4 cm CM-Sepharose column equilibrated with 10 mM potassium phosphate buffer (pH 6.5) containing 1 mM EDTA. Proteins were eluted with the equilibration buffer and buffer containing 50, 100, 300, and 500 mM KCl. Each eluate fraction (4 mL) was collected, and the enhancing effects of each A_{280} peak fraction on microsomal 3′,4′-diHPPH formation were examined. Fractions 5–15 eluted from CM-Sepharose were combined (~44 mL) and concentrated to 5 mL (~3.5 mg/mL) by ultra-filtration (Amicon YM-10, Millipore). The concentrated sample was loaded onto a 1.5 × 1.5 cm HA column equilibrated with 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA. The column was washed with equilibration buffer and with buffer containing increasing concentrations of potassium phosphate (50, 100, 300, and 500 mM). Each eluate fraction (2 mL) was collected, and the enhancing effects of the three A_{280} peak fractions on 3′,4′-diHPPH formation were examined. Fractions 27–33 eluted from HA were combined (~14 mL) and concentrated to 1.5 mL (~0.8 mg/mL) by ultra-filtration as described above.

2.6. SDS–PAGE

The proteins in individual A_{280} peak fractions were separated by SDS–PAGE according to the methods described previously [28] with a 3% stacking gel and a 7.5% separating gel. After electrophoresis, proteins were visualized by Coomassie Brilliant Blue staining [29].

2.7. Analysis of amino acid sequence

The concentrated HA fraction (~4 μg) was separated by SDS–PAGE as described above, and the Coomassie-stained major protein band (~55 kDa) was excised. After incubation with lysyl endopeptidase at 35° for about 20 hr, digested peptide mixtures were separated by reverse-phase high performance liquid chromatography with a TSKgel ODS-80Ts QA column (2.0 × 250 mm, TOSOH). Buffer A consisted of 0.1% trifluoroacetic acid, and buffer B was 90% CH_3CN and 0.09% trifluoroacetic acid. The condition of the gradient was as follows: 0–2 min, 0%; 2–7 min, 10%; 7–82 min, 50%; 82–87 min, 100%; 87–92 min, 100%; 92–97 min, 0% of buffer B. The eluate was monitored at 210 and 280 nm at a flow rate of 0.2 mL/min. As a control, protein-free gel pieces of approximately the same size were processed identically. Fractions of 0.2 mL were collected, and the 10 amino acid sequences of fraction 64 were analyzed with an HP G1005A Protein Sequencing System.

2.8. Catalase activity

The catalase activities of human liver cytosol or each separated fraction were determined by the catalytic reduction of hydrogen peroxide at 240 nm [30]. The final reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0) and 10 mM H_2O_2. The units of catalase activity were calculated from the extinction coefficient of H_2O_2 at 240 nm ($\epsilon = 39.4\ M^{-1}\ cm^{-1}$). One unit of activity was defined as the amount of liver cytosolic or fractionated protein, in mg, required to decompose 1.0 μmol of H_2O_2 per min at pH 7.0 at 25°.

3. Results

3.1. Effects of human liver cytosol on phenytoin, 4′-HPPH, and 3′-HPPH hydroxylation activities catalyzed by human liver microsomes

Phenytoin was incubated with human liver microsomes alone or with liver cytosol in the presence of an NADPH-generating system. Based on the ratio in the liver 9000 *g* supernatant fractions, a 5-fold concentration of cytosolic protein was added to that of microsomal protein in this study. The microsomal formation of 4′-HPPH was not affected by the addition of the liver cytosol (Table 1). On the other hand, the formation of 3′-HPPH was enhanced sufficiently to become detectable, and the 3′,4′-diHPPH formation was increased. The rate of 3′,4′-diHPPH formation from 4′-HPPH or 3′-HPPH was increased ~1.3-fold by

Table 1
Enhancing effects of human liver cytosol of 3′,4′-diHPPH formation catalyzed by human liver microsomes

Pooled human liver microsomes (mg/mL)	Pooled human liver cytosol (mg/mL)	Substrate (100 μM)	Metabolite (pmol/min/mg microsomal protein)		
			4′-HPPH	3′-HPPH	3′,4′-diHPPH
1	–	Phenytoin	6.4 (100)	<0.1	0.2 (100)
	5		6.7 (105)	0.3	0.7 (350)
1	–	4′-HPPH			11 (100)
	5				14 (128)
1	–	3′-HPPH			48 (100)
	5				64 (133)
0.25	–	3′-HPPH			81 (100)
	1.25				108 (133)

Results are the means of duplicate determinations. Values in parentheses indicate percent of control activities (in the absence of cytosol).

the addition of cytosol. When 0.25 mg microsomal protein/mL was used, the rate of 3′,4′-diHPPH formation from 3′-HPPH was higher than that with 1 mg protein/mL, and the enhancement of 3′,4′-diHPPH formation by cytosol was observed to the same extent of ∼1.3-fold. From these results, we determined that the rate of 3′,4′-diHPPH formation from 100 μM 3′-HPPH could be used as a marker reaction to screen an enhancing factor in human liver cytosol.

3.2. Fractionation of human liver cytosol

Pooled human liver cytosol (∼1000 mg protein) was fractionated by the ammonium sulfate precipitation method. The protein contents of each fraction were 84, 366, and 277 mg in 25, 50, and 75% ammonium sulfate pellets, respectively. When a portion of the 50% ammonium sulfate fraction was added to the incubation mixtures at 1.25, 2.5, and 5 mg/mL, the rates of 3′,4′-diHPPH formation from 3′-HPPH catalyzed by human liver microsomes were increased to 128, 133, and 140%, respectively. Other fractions or the 50% ammonium sulfate supernatant did not enhance the rate of microsomal 3′,4′-diHPPH formation (data not shown).

The 50% ammonium sulfate fraction was separated by DEAE-Sephacel anion exchange chromatography using 10 mM Tris–HCl buffer (pH 7.4) followed by a discontinuous KCl gradient for elution (Fig. 2A). By monitoring A_{280}, five major protein peaks were observed. A portion of each A_{280}peak fraction (50, 100, and 150 μL) was added to an incubation mixture (250 μL) and examined for enhancing effects on the rate of liver microsomal 3′,4′-diHPPH formation from 3′-HPPH. The effect of 150 μL of each fraction examined on 3′,4′-diHPPH formation is shown in Fig. 2. Although the rate of 3′,4′-diHPPH formation was increased by the addition of some fractions, fraction 72 from the DEAE-Sephacel column most strongly enhanced the microsomal 3′,4′-diHPPH formation to ∼140%. No enhancing effect was observed with the KCl solution (up to 500 mM). Fraction 72 contained three major bands and three minor bands visualized by Coomassie Brilliant Blue staining (Fig. 3, lane 4).

Fractions 66–76 eluted from DEAE-Sephacel (∼44 mL) were combined and separated further by CM-Sepharose cation exchange chromatography using 10 mM potassium phosphate buffer (pH 6.5) followed by elution with a discontinuous KCl gradient (Fig. 2B). Four protein peaks were detected, and liver microsomal 3′,4′-diHPPH formation was increased in a concentration-dependent manner to ∼130% by the addition of the CM-Sepharose unabsorbed fraction (CM 11). There were some Coomassie Brilliant Blue-staining bands in this fraction (Fig. 3, lane 5).

Fractions 5–15 eluted from CM-Sepharose (∼44 mL) were combined, concentrated by ultra-filtration, and separated further by HA column chromatography with potassium phosphate buffer (pH 7.4) gradient elution (Fig. 2C). Three major protein peaks were eluted, and the addition of HA fraction 29 (eluted by 50 mM potassium phosphate buffer) enhanced microsomal 3′,4′-diHPPH formation to ∼150%. In this fraction, one major band was detected by Coomassie Brilliant Blue staining (Fig. 3, lane 6). Thus, this ∼55 kDa protein band was identified as a factor enhancing 3′,4′-diHPPH formation activity. The yield of protein and the specific enhancing ratio of 3′,4′-diHPPH formation in each step of the fractionation procedure described above are shown in Table 2.

3.3. Identification of the enhancing factor on 3′,4′-diHPPH formation

Fractions 27–33 eluted from the HA column were combined (∼14 mL) and concentrated. A portion of the concentrated HA fraction (∼4 μg) was separated by SDS–PAGE and transferred to a polyvinylidene difluoride membrane. The Coomassie-stained protein band (∼55 kDa) was excised from the membrane and subjected to N-terminal Edman sequencing. This protein was found to have a blocked N-terminus. Therefore, after incubation with lysyl endopeptidase, digested peptide mixtures were separated by reverse-phase high performance liquid chromatography (data not shown), and the 10 amino acid sequences of the fraction were analyzed. The sequence obtained corresponded to the amino acid sequence of catalase (78–87) using the SWISS-PROT database (Table 3). As shown in Table 2, the catalase activity of the purified HA fraction was determined to be 12,000 units/mg protein. The molecular

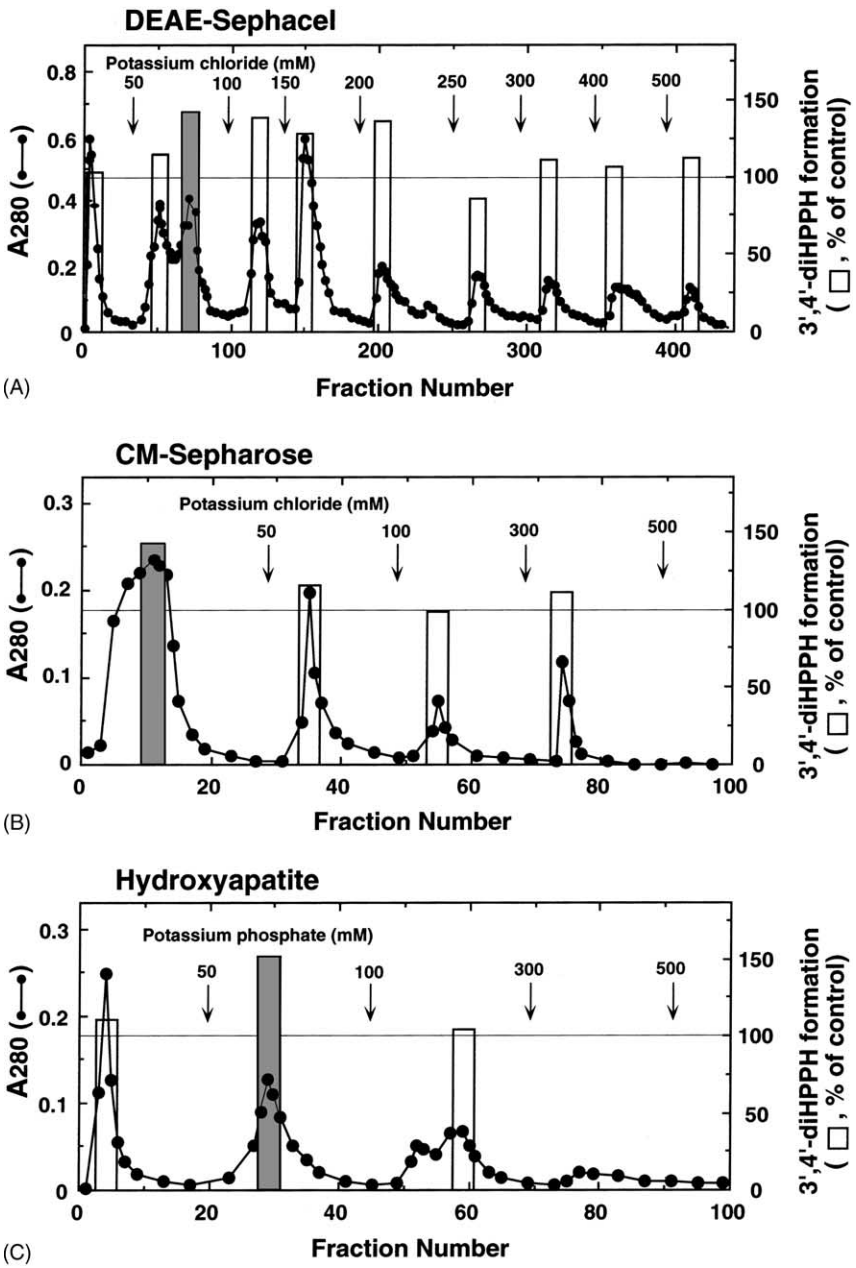

Fig. 2. Separation of human liver cytosol: protein (A_{280}) elution profiles and enhancement of microsomal 3′,4′-diHPPH formation activity. The 50% ammonium sulfate fraction of human liver cytosol was further separated by DEAE-Sephacel (A), CM-Sepharose (B), and HA (C) column chromatography as described under Section 2. A portion of each protein peak fraction (150 μL) was added to an incubation mixture (250 μL), and the 3′,4′-diHPPH formation activities by human liver microsomes are shown as a percent of control. Control experiments were performed, and they confirmed that there were no effects from the addition of the corresponding elution buffer alone. Results are the means of duplicate determinations. The control activity of 3′,4′-diHPPH formation from 3′-HPPH catalyzed by pooled human liver microsomes was 81 pmol/min/mg protein. Some fractions near the shaded column were combined and subjected to the next step. The combined fraction containing the shaded column eluted from HA was defined as the final purified material.

mass of this protein in the HA fraction determined by SDS–PAGE (∼55 kDa, Fig. 3, lane 6) was consistent with that of commercially available catalase (data not shown).

3.4. Enhancement of 3′,4′-diHPPH formation by catalase in human liver microsomes and in reconstituted systems containing purified recombinant P450

The enhancing effects of the purified HA fraction and catalase were examined on human liver microsomal 3′,4′-diHPPH formation from 3′-HPPH (Fig. 4). Addition of the HA fraction (corresponding to 250–500 units of catalase activity) increased the rate of 3′,4′-diHPPH formation 1.3-fold. The addition of catalase (250–1000 units) increased the rate of 3′,4′-diHPPH formation to the same extent as the HA fraction. However, inactivated catalase (pretreated at 100° for 2 min) did not enhance the microsomal activity of 3′,4′-diHPPH formation (Fig. 4B). In separate experiments, the effects of catalase inhibitors, 3-amino-1,2,4-triazole [31], and phenylhydrazine [32], on the enhancing

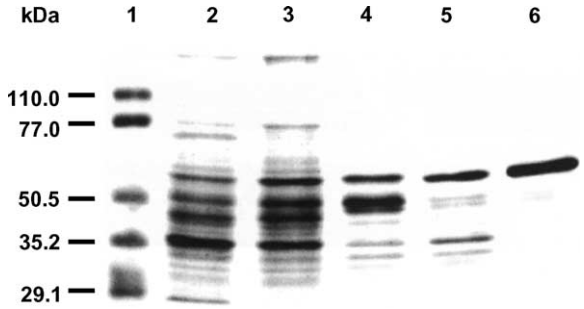

Fig. 3. Protein profiles of human liver cytosol fractionated by ammonium sulfate and column chromatography. The proteins were separated by SDS–PAGE with a 3% stacking gel and a 7.5% separating gel. After electrophoresis, proteins were visualized by Coomassie Brilliant Blue staining: lane 1, molecular mass standard; lane 2, pooled human liver cytosol (10 μg protein); lane 3, ammonium sulfate (50%) precipitated fraction (10 μg); lane 4, DEAE-Sephacel fraction 72 (5 μg); lane 5, CM-Sepharose fraction 11 (5 μg); and lane 6, HA fraction 29 (5 μg).

effects of human liver cytosol on microsomal 3′,4′-diHPPH formation from 3′-HPPH were examined. The enhanced activity of microsomal 3′,4′-diHPPH formation by human liver cytosol (127% of control) was inhibited to 113 and 108% by 1 and 2 mM 3-amino-1,2,4-triazole, respectively. Similarly, phenylhydrazine inhibited the enhanced activity to 114 and 103% at 1 and 5 μM, respectively. These concentrations of 3-amino-1,2,4-triazole and phenylhydrazine did not affect the microsomal activity of 3′,4′-diHPPH formation. The commercially available anti-catalase antibody for immunoblotting did not inhibit the cytosolic enhancing effects (data not shown).

Table 3
Amino acid sequence of human liver cytosolic protein that enhances liver microsomal 3′,4′-diHPPH formation activity

Sample			Catalase	
Cycle no.	Found	Recovered (pmol)	Position	Expected
1	G	13.9	78	G
2	A	14.3	79	A
3	G	10.8	80	G
4	A	14.5	81	A
5	F	12.4	82	F
6	G	10.2	83	G
7	Y	13.0	84	Y
8	F	12.4	85	F
9	E	9.6	86	E
10	V	9.7	87	V

The lysyl endopeptidase-digested peptide mixture was separated by reverse-phase high performance liquid chromatography, and the 10 amino acid sequences of the fraction were analyzed with the HP G1005A Protein Sequencing System. The amino acid position number of catalase indicates that from the translation initiation site.

Table 2
Purification of a cytosolic factor that enhances 3′,4′-diHPPH formation from 3′-HPPH catalyzed by human liver microsomes

	Total protein (mg)	Enhancement of 3′,4′-diHPPH formation			Catalase activity (units/mg protein)	Yield of protein (%)
		Added protein to the reaction mixture (mg)	Relative activity (% of control)	Specific enhancing ratio (%/mg protein)		
Human liver cytosol	1,000	0.312	133	110	160	100
50% Ammonium sulfate	366	0.312	128	90	220	36.6
DEAE fraction	22.1	0.064	142	660	1,300	2.21
CM fraction	8.9	0.025	142	1,680	5,600	0.89
HA fraction	1.2	0.019	151	2,680	12,000	0.12

The specific enhancing ratio was calculated by the increased ratio of activity per cytosolic protein added to the reaction mixture.

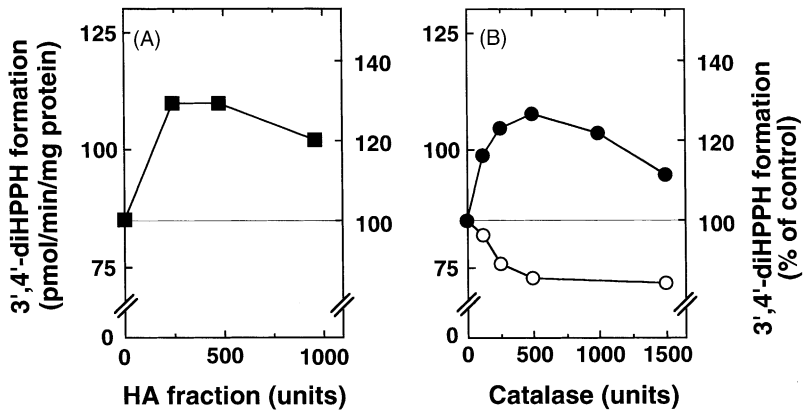

Fig. 4. Enhancement of 3′,4′-diHPPH formation activities from 3′-HPPH catalyzed in human liver microsomes by HA fraction (■), catalase (●), and heat-inactivated catalase (○). 3′-HPPH (100 μM) was incubated with pooled human liver microsomes (0.25 mg protein/mL) in the absence or presence of HA fraction, catalase, and heat-inactivated catalase. The contents of the HA fraction and catalase that were added to the incubation mixtures are indicated as catalase activity. One unit of activity was defined as the amount of the HA fraction or catalase protein, in mg, required to decompose 1.0 μmol of H_2O_2 per min at pH 7.0 at 25°. Inactivated catalase was prepared by pretreatment at 100° for 2 min. Results are the means of duplicate determinations.

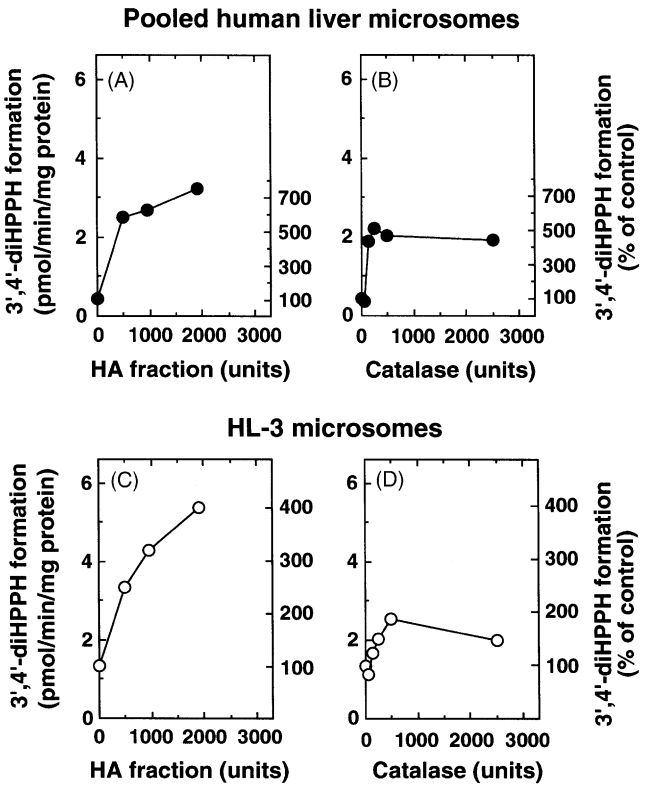

Fig. 5. Enhancement by HA fraction or catalase of 3′,4′-diHPPH formation from phenytoin catalyzed by human liver microsomes. Phenytoin (100 μM) was incubated with pooled human liver microsomes (1 mg protein/mL) (A, B) and HL-3 microsomes (C, D) in the absence or presence of HA fraction or catalase. The contents of the HA fraction and catalase added to the incubation mixtures are indicated as catalase activity. One unit of activity was defined as the amount of the HA fraction or catalase protein, in mg, needed to decompose 1.0 μmol of H_2O_2 per min at pH 7.0 at 25°. Results are the means of duplicate determinations.

The enhancement by catalase of 3′,4′-diHPPH formation from phenytoin is shown in Fig. 5. Addition of the HA fraction increased 3′,4′-diHPPH formation activity by pooled liver microsomes in a concentration-dependent manner (Fig. 5A). On the other hand, although the rates of microsomal 3′,4′-diHPPH formation were enhanced about 5-fold by the addition of 250 units of catalase, there was no additional increase of 3′,4′-diHPPH formation by further additions of catalase (Fig. 5B). In the case of an individual human liver microsomal preparation (HL-3), the addition of the HA fraction greatly increased the rate of 3′,4′-diHPPH formation (Fig. 5C) compared with pooled liver microsomes (Fig. 5A). The enhancing effects of catalase on HL-3 microsomal 3′,4′-diHPPH formation (Fig. 5D) were found to be similar to those in pooled liver microsomes (Fig. 5B). The rates of 4′-HPPH and 3′-HPPH formation from phenytoin catalyzed by human liver microsomes were not affected by the addition of the HA fraction or catalase (data not shown).

The enhancing effects of catalase were also determined in reconstituted systems containing purified recombinant CYP2C9, CYP2C19, and CYP3A4. These P450 forms were demonstrated to play important roles in 3′,4′-diHPPH formation [11]. When 3′-HPPH was used as a substrate, CYP2C9-mediated 3′,4′-diHPPH formation was increased by catalase to ∼250%, followed by CYP2C19 and CYP3A4 (Table 4). Although the enhancement of 3′,4′-diHPPH formation from 4′-HPPH was smaller than that from 3′-HPPH, catalase increased the 3′,4′-diHPPH formation from 4′-HPPH catalyzed by CYP2C9 and CYP2C19. In the presence of catalase, the rates of 3′,4′-diHPPH formation from phenytoin catalyzed by CYP2C9 and CYP2C19 clearly were increased.

4. Discussion

It has been reported that cytosolic proteins enhance several microsomal P450-mediated mutagenic activations of polyaromatic hydrocarbons, aminoazo dyes, or *N*-nitrosamines [16–19]. Some of the enhancing effects of the cytosol in mutagenicity can be explained by the conversion of a microsome-generated metabolite to a more mutagenic species. For example, oxidative metabolites

Table 4
Enhancing effects of catalase on 3′,4′-diHPPH formation in a reconstituted system containing purified P450 enzymes

P450	Catalase (units/pmol P450)	3′-HPPH (pmol/min/nmol P450)	4′-HPPH (pmol/min/nmol P450)	Phenytoin (pmol/min/nmol P450)		
		3′,4′-diHPPH	3′,4′-diHPPH	4′-HPPH	3′-HPPH	3′,4′-diHPPH
CYP2C9	–	50 (100)	8.6 (100)	178 (100)	<0.1	<0.1
	35	74 (148)	8.5 (99)	160 (90)	<0.1	5.4
	70	124 (247)	11.2 (133)	162 (91)	<0.1	8.4
CYP2C19	–	6480 (100)	676 (100)	206 (100)	14.6 (100)	19.8 (100)
	35	8165 (126)	744 (110)	202 (98)	13.4 (92)	32.9 (166)
	70	9850 (152)	846 (125)	228 (111)	12.0 (82)	54.6 (276)
CYP3A4	–	78 (100)	22.2 (100)	ND	ND	ND
	35	82 (105)	22.0 (99)	ND	ND	ND
	70	104 (133)	21.6 (97)	ND	ND	ND

P450 enzymes were used at 0.04, 0.08, and 0.20 μM concentrations for 3′-HPPH, 4′-HPPH, and phenytoin oxidations, respectively. Each substrate (described in the upper line of the table) was used at 100 μM. Results are the means of duplicate determinations. Values in parentheses indicate the percent of control activities (in the absence of catalase). ND, not determined.

of 7,12-dimethylbenz[*a*]anthracene and arylamines have been reported to be further activated by sulfotransferase [33] and acetyltransferase [34], respectively. However, the cytosolic factor(s) involved in the activation of other mutagens such as *N*-nitrosamines remains to be clarified. In contrast to these findings, there have been few reports of cytosolic enhancing effects on P450-mediated oxidative metabolism of drugs in liver microsomes [14,15].

In this study, catalase in human livers was identified as a factor that enhances the liver microsomal oxidative activities of phenytoin. First, it was confirmed that the addition of a purified protein fraction identified as catalase and commercially available catalase enhanced the 3′,4′-diHPPH formation catalyzed by human liver microsomes and in P450 reconstituted systems. Second, chemical inhibition with 3-amino-1,2,4-triazole and phenylhydrazine of the cytosolic enhancing effects also supported the role of catalase. This is the first report that catalase enhances drug oxidation activities catalyzed by P450s.

The enhancing effects of the HA fraction and catalase on 3′,4′-diHPPH formation from phenytoin seemed to be a little different (Fig. 5). This may be explained by the differences in the origin of the catalase, whether it is HA fraction-containing catalase purified from human livers or catalase from human erythrocytes. In the course of purification of a cytosolic enhancing factor, increased rates of microsomal 3′,4′-diHPPH formation were seen by the addition of some other fractions eluted from the DEAE-Sephacel column (Fig. 2A). Catalase activities were also found in some but not all of these fractions (data not shown). There was no significant correlation between catalase activity of the cytosols from the individual livers (results not shown) and their ability to activate phenytoin conversion to 3′,4′-diHPPH [11]. These findings suggest that there is another factor(s) in the liver cytosol that enhances microsomal 3′,4′-diHPPH formation.

Catalase (EC 1.11.1.6) is an iron-containing enzyme, a tetramer composed of four identical subunits with an apparent molecular mass of ∼55 kDa [35]. It has been suggested that the N-terminus of catalase is blocked by tetramer formation [36], consistent with our present analysis. Catalase catalyzes the conversion of H_2O_2 to H_2O and O_2 and is most abundant in human liver, kidneys, and erythrocytes [35]. The role of catalase in the conversion of hydrogen peroxide to water has been considered one of the defense mechanisms that can prevent the damage caused by reactive oxygen species. Reactive oxygen species are generated in cellular activities such as in mitochondrial respiration and endogenous enzyme systems and have been reported to contribute to several diseases [37]. It has been reported that there are 3-fold interindividual differences of catalase activities in human blood [38] and that Japanese-type acatalasemia patients with a deficiency of catalase have a G to A mutation at the fifth position of intron 4, resulting in alternative splicing [39].

There are some reports concerning phenytoin and catalase and/or hydrogen peroxide *in vivo*. Winn and Wells [40] have shown that the addition of catalase to a murine embryo culture system blocks phenytoin-initiated DNA oxidation and decreases embryotoxicity. Furthermore, they have reported that maternal administration of catalase conjugated with polyethylene glycol enhances embryonic catalase activity and decreases *in vivo* phenytoin teratogenicity [37]. In our previous study [27], the rates of phenytoin oxidation catalyzed by induced P450s in rat liver microsomes were decreased. Hydrogen peroxide formation was increased by chronic phenytoin administration [27]. Oxidative destruction of P450 systems by their own hydrogen peroxide generation has been established [41]. In the present study, since heat-inactivated catalase could not enhance microsomal 3′,4′-diHPPH formation, it is suggested that the enzymatic function of catalase, not the structure of the protein, is involved in the enhancement of microsomal 3′,4′-diHPPH formation. The detailed mechanism of the enhancing effects of catalase on phenytoin metabolism *in vitro* is not clear. However, taking these results into consideration, the increased rates of human liver microsomal 3′,4′-diHPPH formation by the addition of catalase might be accounted for by the scavenging of hydrogen peroxide formed in P450-mediated phenytoin metabolism.

Although catalase is found predominantly in peroxisomes, catalase has been reported to be found in cytosol [42–44]. It has been shown that 60% of the total catalase activity is of cytosolic origin in hepatocytes [43], and amino acid analysis revealed a slight difference between the cytoplasmic and the peroxisomal catalases in guinea pig [42,43] and rat [44] liver. Under the experimental conditions used in this study, it is not clear whether the catalase found in human liver cytosolic fractions is in the original cytoplasmic form, existing in peroxisomal form, and/or the result of leakage from peroxisomes by homogenization. In any case, catalase in human livers was identified as an enhancing factor of phenytoin oxidation activity. It is generally considered that any form of catalase, which is a scavenger of hydrogen peroxide, can participate in the enhancement of microsomal phenytoin oxidation *in vivo*. On the other hand, the addition of commercial glutathione peroxidase with glutathione, another hydrogen peroxide scavenger system, did not enhance the human liver microsomal 3′,4′-diHPPH formation in our preliminary experiments (results not shown). It would be interesting to determine whether catalase can enhance other drug oxidation activities catalyzed by P450s, because enhanced activities by catalase of *S*-warfarin 7-hydroxylation by CYP2C9 and *S*-mephenytoin 4′-hydroxylation by CYP2C19 also were observed in our preliminary studies.

In conclusion, we found that catalase could enhance the rates of phenytoin oxidative metabolism catalyzed by liver microsomal P450. It will be necessary to study further the role of catalase in phenytoin metabolism and the P450-mediated oxidation of other drugs.

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