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Molecular Cloning and Expression of Fusion Proteins Containing Human Cytochrome P450 3As and Rat NADPH-P450 Reductase in *Escherichia coli*

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ABSTRACT : *Cytochrome P450 3As such as 3A4 and 3A5 metabolize a wide range of pharmaceutical compounds. The vectors for the expression of fusion proteins containing an N-terminal human P450 3A4 or P450 3A5 sequences and a C-terminal rat NADPH-cytochrome P450 reductase moiety were constructed. These plasmids were used to express the fusion proteins in Escherichia coli DH5α cells. High levels of expression were achieved (100~200 nmol/liter) and the expressed fusion proteins in E. coli membranes were catalytically active for nifedipine oxidation, a typical enzymatic activity of P450 3A4. The NADPH-P450 reductase activities of these fusion proteins were also determined by measuring reduction of cytochrome c. To find a specific inhibitor of P450 3A4 from naturally occurring chemicals, a series of isothiocyanate compounds were evaluated for the inhibitory activity of P450 using the fusion proteins in E. coli membranes. Of the five isothiocyanates (phenethyl isothiocyanate, phenyl isothiocyanate, benzyl isothiocyanate, benzoyl isothiocyanate and cyclohexyl isothiocyanate) tested, benzoyl isothiocyanate showed a strong inhibition of P450 3A4 with an IC_{50} value of 2.8 μM. Our results indicate that the self-sufficient fusion proteins will be very useful tool to study the drug metabolism and benzyl isothiocyanate may be valuable for characterizing the enzymatic properties of P450 3A4.*

Key Words : *Cytochrome P450 3A4, Cytochrome P450 3A5, NADPH-P450 reductase, Heterologous expression, Fusion protein, Benzoyl isothiocyanate*

I. INTRODUCTION

Human cytochrome P450 (P450) 3As such as 3A4, 3A5 and 3A7 are abundant cytochrome P450 expressed in human liver and intestines and are reported to be responsible for the metabolism of many therapeutic drugs (Maurel, 1996). The amounts of P450 3A4 and P450 3A5 represent together about 35% of total hepatic P450s (Wrighton *et al.*, 1990). P450 3A5 was usually found at levels less than P450 3A4 between 6% and 60% of total 3A family proteins. A point mutation resulting in the synthesis of an unstable protein may account for 3A5 polymorphisms.

A very large number of structurally divergent therapeutic drugs are metabolized by P450 3A4 and approximately 40~50% of drugs used in human may be metabolized by P450 3A-mediated drug oxidation. Because P450 3A proteins play a major role in drug metabolism, these enzymes have attracted considerable interest.

Catalytically active fusion proteins containing P450 sequence and NADPH-P450 reductase sequence are quite useful. The *Bacillus megaterium* P450 BM3 is a natural form of P450 fusion protein in which a fatty acid hydroxylase P450 is fused to a NADPH-P450 reductase (Munro *et al.*, 2002). Nitric oxide synthase is also a fusion protein composed of P450 domain and NADPH-P450 reductase domain (Alderton *et al.*, 2001). Several artificial fusion proteins containing P450s fused with NADPH-P450 reductase were expressed in *E. coli* to use these enzymes to study of structure-function relationships (Chun *et al.*, 1996; Deeni *et al.*, 2001; Parikh and Guengerich, 1997; Shet *et al.*, 1993; Shet *et al.*, 1996).

Several dietary constituents and phytochemicals are identified as important factors affecting drug

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metabolism and an increasing number of these chemicals have been characterized as chemopreventive agents. Particular interests have been given to isothiocyanate compounds from the cruciferous vegetables. For example, phenethyl isothiocyanate and benzyl isothiocyanate have been shown to inhibit chemically induced carcinogenesis in animals (Chung *et al.*, 1992; Hecht *et al.*, 2000; Morse *et al.*, 1992; Wattenberg, 1987). The mechanism of chemopreventive effects by these isothiocyanates may be considered to be the inhibition of P450s involved in the metabolic activation of carcinogens. Recently, it has been reported that phenethyl isothiocyanate is a relatively strong inhibitor of human P450 1A2 and 2B6 (Nakajima *et al.*, 2001). Benzyl isothiocyanate is also shown to act as a mechanism-based inactivator of human P450 2B6 and 2D6 (Goosen *et al.*, 2001).

In the current report, we investigated the effect of five structurally related isothiocyanates on human P450 3A4 activity by using *E. coli* membranes expressing the fusion protein containing P450 3A4 and NADPH-P450 reductase.

II. MATERIALS AND METHODS

1. Materials

Restriction enzymes were purchased from Promega (Madison, WI). Ethoxyresorufin, resorufin, DMSO, thiamine, isopropyl-1-thio-β-D-galactopyranoside (IPTG), δ-aminolevulinic acid and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO). Bactopeptone, yeast extract and bacto-agar were obtained from Difco Lab. (Detroit, MI). Other chemicals were of highest grade commercially available.

2. Plasmid construction

The expression vectors of P450 3A4 and P450 3A5 (Gillam *et al.*, 1993, 1995) were used for mutagenesis to modify the coding region of the carboxyl terminus of P450 3A4 or 3A5 and the coding region for the

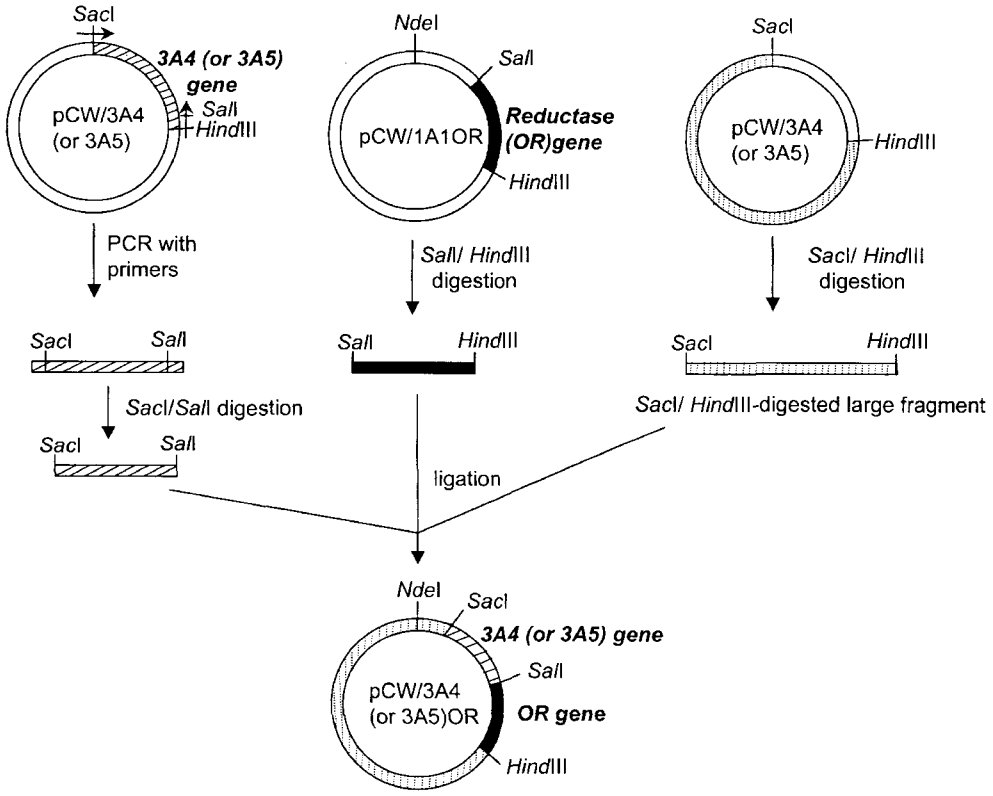

Fig. 1. Construction of the fusion plasmids containing human P450 3A (P450 3A4 or P450 3A5) and rat NADPH-P450 reductase genes. The arrows and crossed arrows depict PCR primers containing additional sequence used to insert appropriate restriction sites.

amino terminus of NADPH-P450 reductase in order to allow fusion of these sequences with the dipeptide linker Ser-Thr (Fig. 1). After the digestion of pCW/3A4 (or pCW/3A5) with *Sac*I and *Hin*dIII, the DNA fragment of *Sac*I and *Hin*dIII was used for cDNA cloning. The 5-PCR primer (5'-GCTCTGTCCGATCTGGAGCT-CGTGGCCCAATC-3') and 3'-PCR primer (5'-CGTC-CCATGGTCTAGAGTCGACGCTCCACTTACGGTGCC-3') were designed to introduce a *Sal*I site in the PCR product for P450 3A4 gene and the 5'-PCR primer (5'-CACAAAGCTCTGTCTGATCTGGAGCTCGCAGCCCAG-3') and 3'-PCR primer (5'-CGTCCCATGGTCTAGAGT-CGACTCTCCACTTAGGGTTCC-3') were designed to introduce a *Sal*I site in the PCR product for P450 3A5 gene. The PCR products were digested with *Sal*I and *Sac*I and *Sal*I and *Sac*I fragments were purified by the GeneClean II method (Qbiogene, La Jolla, CA). The pCW/1A1OR plasmid (Chun *et al.*, 1996) was digested with *Sal*I and *Hin*dIII and the DNA fragment were isolated to obtain truncated rat NADPH-P450 reductase cDNA. The 3-way unidirectional ligation with *Sac*I and *Hin*dIII-digested pCW/3A4 (or pCW/3A5), *Sac*I and *Sal*I-digested PCR product, and *Sal*I and *Hin*dIII-digested pCW/1A1OR plasmid of truncated NADPH-P450 reductase was performed. Ampicillin-resistant colonies of *E. coli* DH5α cells transformed with fusion plasmids were selected. The nucleotide sequences of the coding region of the constructs were confirmed by the restriction enzyme digestion or the dideoxy DNA sequencing method with isolated fusion plasmids.

3. Expression of fusion proteins in *E. coli*

A selected ampicillin-resistant colony was grown overnight at 37°C in LB medium containing 100 μg ampicillin ml^{-1}. A 10-m*l* aliquot was used to inoculate each 1.0 liter of terrific broth (TB) containing 0.2% bactopeptone (w/v), ampicillin (100 μg ml^{-1}), 1.0 mM thiamine, trace elements, and 0.5 mM δ-ALA. Induction of the *tac* promoters was initiated by the addition of 1.0 mM IPTG and allowed to proceed to 4 days at 28°C and 125 rpm in a shaking incubator. After incubation, cells were harvested by centrifugation at 6,500 g for 20 min. Spheroplasts were prepared using lysozyme and disrupted by sonication. The cellular sonicates were centrifuged at 10,000 g for 20 min and the membranes were pelleted by centrifugation at 110,000 g for 90 min and were resuspended in 10 mM Tris-HCl buffer (pH 7.4) containing 1.0 mM EDTA and 20% glycerol (v/v) (Gillam *et al.*, 1993; Guengerich *et al.*, 1996). Protein concentrations were estimated using the bichinchoninic acid method according to the supplier's recommendations (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as a standard. The isolated microsomal fractions were stored at -80°C. P450 content of cells and membranes was quantitated by the spectral method of Omura and Sato (1964) using an extinction coefficient of 91 mM^{-1} cm^{-1} with a Shimazu UV-160A spectrophotometer at ambient temperature For immunoblot analysis, proteins separated by SDS-PAGE on 10% acrylamide gel were transferred onto nitrocellulose membrane filter and probed with rabbit anti-human P450 3A4 or 3A5 antibodies. The proteins were visualized by the ECL method (Amersham Bioscience Co., Piscataway, NJ).

4. Preparation of human liver microsomes

Frozen human liver samples were thawed in 0.1 M Tris acetate buffer (pH 7.4) containing 0.1 M KCl, 1.0 mM EDTA, and 20 μM BHT and homogenized in a Teflon-glass homogenizer. The homogenate was centrifuged at 10,000 g for 20 min at 4°C, and the resulting supernatant was centrifuged for 60 min at 100,000 g at 4°C. The microsomal pellets were resuspended in 10 mM Tris acetate buffer (pH 7.4) containing 1 mM EDTA and 20% (v/v) glycerol (Guengerich, 1994). The isolated microsomes were stored at -80°C.

5. Enzyme assays

Nifedipine oxidation was determined by HPLC as a measure of recombinant human P450 3As activity using previously described methodology (Guengerich *et al.*, 1996). Reaction mixtures contained 50 mM HEPES (pH 7.4), 30 mM $MgCl_2$, 0.2 mM nifedipine, *E. coli* membranes expressed a recombinant fusion protein of P450 3A4 (or 3A5), and 1 mM NADPH. Incubations were performed in a shaking water bath for 20 min at 37°C and terminated by addition of 2 m*l* of CH_2Cl_2 and 100 μ*l* of 1 M Na_2CO_3 (pH 10.5) containing 2 M NaCl. The samples were centrifuged at 3,000 g for 10 min and 1.4 m*l* of each lower organic

layer were dried under a N_2 stream. Determination of a nifedipine metabolite was performed using a 150-mm×4.6-mm steel C_{18} Nucleosil column with ultraviolet detection at 254 nm. The flow rate was 1.0 ml/min, and the mobile phase was 64% methanol. Under these conditions, retention times of nifedipine metabolite and nifedipine were 6.9 and 9.1 min, respectively. The detection limit of a nifedipine metabolite was ~50 pmol with a signal-to-noise ratio of 3 : 1.

Ethoxycoumarin *O*-deethylation (ECOD) activity was determined as previously described (Greenlee and Poland, 1978). The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.4), *E. coli* membranes, and 2 mM 7-ethoxycoumarin. The reaction mixtures were preincubated at 37°C for 3 min, and the reaction was initiated by addition of 1 mM NADPH. Incubations were performed in a shaking water bath for 15 min at 37°C and terminated by addition of 25 μl of 10 % trichloroacetic acid and 1 ml of CH_2Cl_2. The samples were centrifuged at 3,000 g for 10 min and 3 ml of 30 mM sodium borate (pH 9.0) was added to 0.8 ml of each lower organic layer. After centrifugation at 3,000 g for 10 min, the formation of hydroxycoumarin was determined fluorometrically with a Perkin-Elmer LS 5 spectrofluorometer (with excitation and emission wavelengths of 370 nm and 450 nm).

NADPH-P450 reductase activity was estimated by measuring cytochrome *c* reduction rates in 0.3 M potassium phosphate buffer, pH 7.7 at ambient temperature as described (Guengerich, 1994).

III. RESULTS

Expression DNA constructs made in this study was designed using the approaches found in successful in previous studies (Chun *et al.*, 1996; Fisher *et al.*, 1992; Parikh and Guengerich, 1997). A Ser-Thr di-

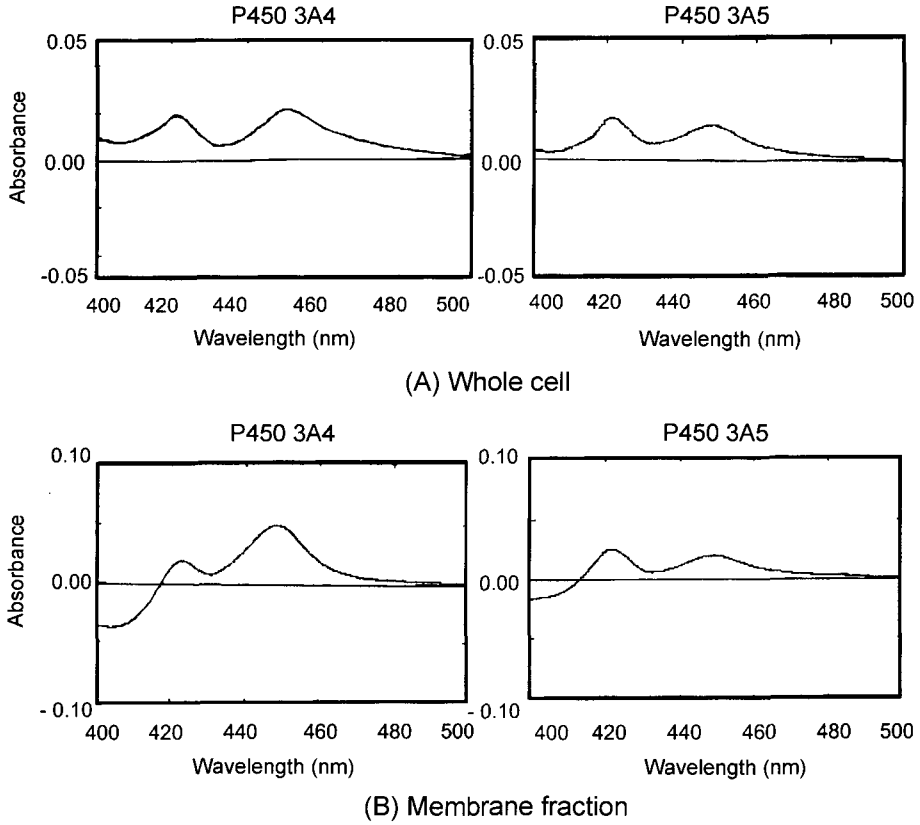

Fig. 2. Expression levels of P450 3A by recombinant fusion proteins in *E. coli*. (A) whole cells, (B) membrane fractions. *E. coli* cells were transformed with the fusion plasmids and incubated for 48 h after seeding in TB medium containing IPTG and other supplements for P450 expression. Bacterial membranes were prepared and absorbance spectra were recorded with whole cells or bacterial membranes in 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol (v/v).

peptide linker has been successfully employed to bridge the P450 and reductase domains of the fusion proteins.

Optimal expression appeared to occur at 28°C and 48 h. The typical levels of expression were approximately 190 nmol of spectrally detectable P450 3A4 (liter culture)$^{-1}$ and 140 nmol of P450 3A5 in bacterial whole cell lysates (Fig. 2). The expression level of P450 3A4 was relatively higher than that of P450 3A5 (Fig. 3). Because the fusion proteins have higher molecular weight (≈130 kDa) and seem to be more sensitive to protease digestion, the optimization of culture conditions may be important for high level expression of the fusion proteins in *E. coli*. Changes in the incubation and shaking condition (e.g. 30°C, 250 rpm for 24 h) significantly enhanced the denatured proteins.

To determine the expression of the fusion proteins in *E. coli*, individual fractions such as sonicated frac-

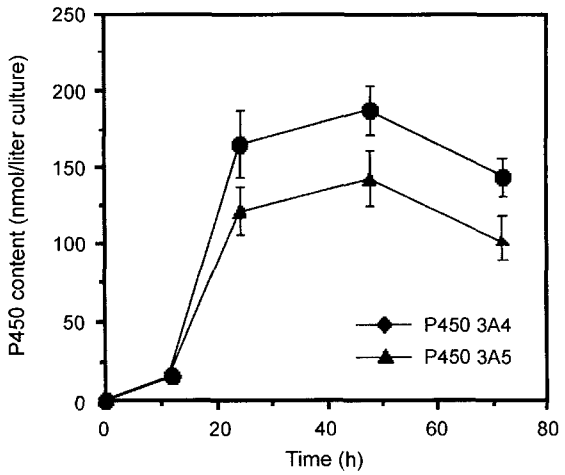

Fig. 3. Time-dependent expression of P450 3A fusion proteins in *E. coli*. *E. coli* cells were transformed with the fusion plasmids and incubated for 0, 12, 24, 48 and 72 h after seeding in TB medium containing IPTG and other supplements for P450 expression. Bacterial membranes were prepared and absorbance spectra were recorded with bacterial membranes in 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol (v/v).

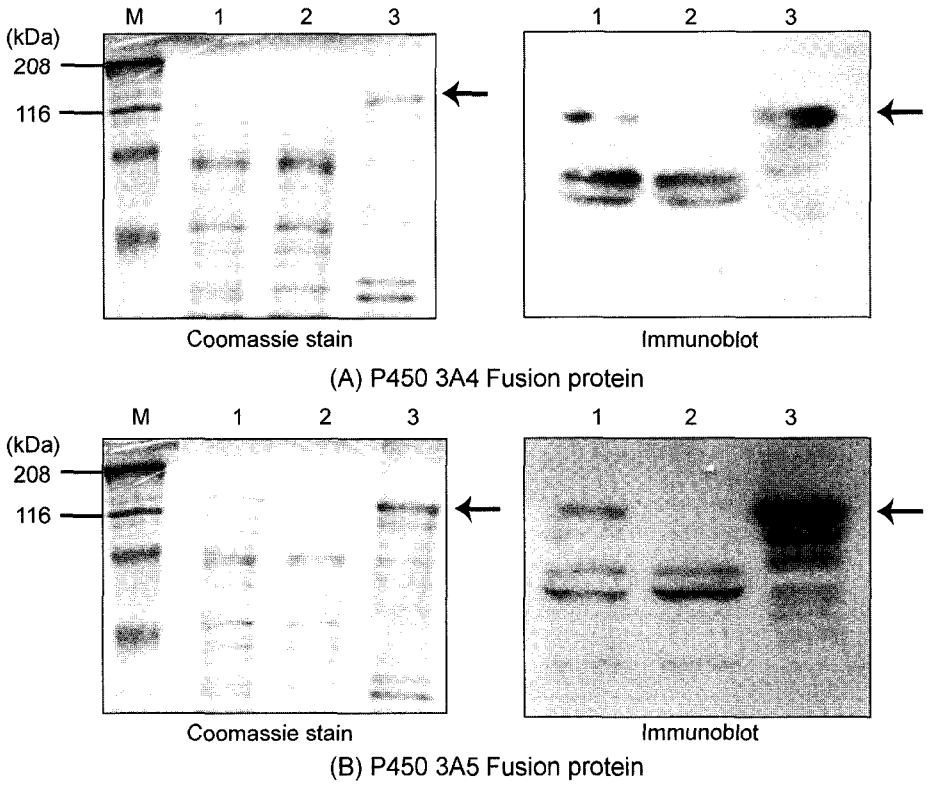

Fig. 4. Analyses of fusion protein expression. *E. coli* cells were transformed with the fusion plasmids and incubated for 48 after seeding in TB medium containing IPTG and other supplements for P450 expression. Cell sonicates, cytosolic and membrane fractions were prepared and 10 μg of protein from each fraction was separated by 10% SDS-PAGE and the gel was stained with Coomassie Blue dye. The gels were transferred into nitrocellulose filter and immunoblotted with anti-rat NADPH-P450 reductase antibody. M, protein size marker; 1, cell sonicates; 2, cytosolic fraction; 3, membrane fraction. (A) P450 3A4 fusion protein, (B) P450 3A5 fusion protein. The arrows indicate the size of the expressed fusion proteins (approximately 130 kDa).

tions, cytosolic fractions, and membrane fractions during membrane preparation were separated by 10% SDS-PAGE (Fig. 4). In sonicated fractions and membrane fractions, a new protein band having a molecular weight of approximately 130 kDa was detected. Using anti-rat NADPH-P450 reductase antibody, the immunoblot analyses were performed. The results showed that a protein band of 130 kDa contains part of rat NADPH-P450 reductase protein detected by antibody. Because the fusion proteins have a NADPH-P450 reductase truncated N-terminal membrane-anchoring region, membrane-bound region of P450 may involve in incorporation into *E. coli* plasma membrane and a NADPH-P450 reductase domain may be exposed to cytosol. The topology of fusion proteins in *E. coli* membranes needs to be determined.

The fusion proteins catalyzed nifedipine oxidation and the formation of the pyridine metabolite, a major oxidized metabolite of nifedipine were detected by HPLC (Fig. 5). This metabolite was also detected in the reaction of human liver microsomes. The results showed that the fusion proteins expressed in *E. coli* are enzymatically active and may be useful tools to mimic P450 3A-mediated oxidations in human liver.

The NADPH-P450 reductase domain of the fusion proteins was catalytically active, as judged by the capacity to reduce cytochrome *c* (Fig. 6). The respective V_{max} and K_m values measured by 3A4 and 3A5 fusion protein were 2,300 and 2,600 nmol (cytochrome *c* reduced) min^{-1} (mg protein)$^{-1}$ and 7.1 and 10.7 μM. The V_{max} and K_m values of purified rabbit NADPH-P450 reductase which is usually used for the

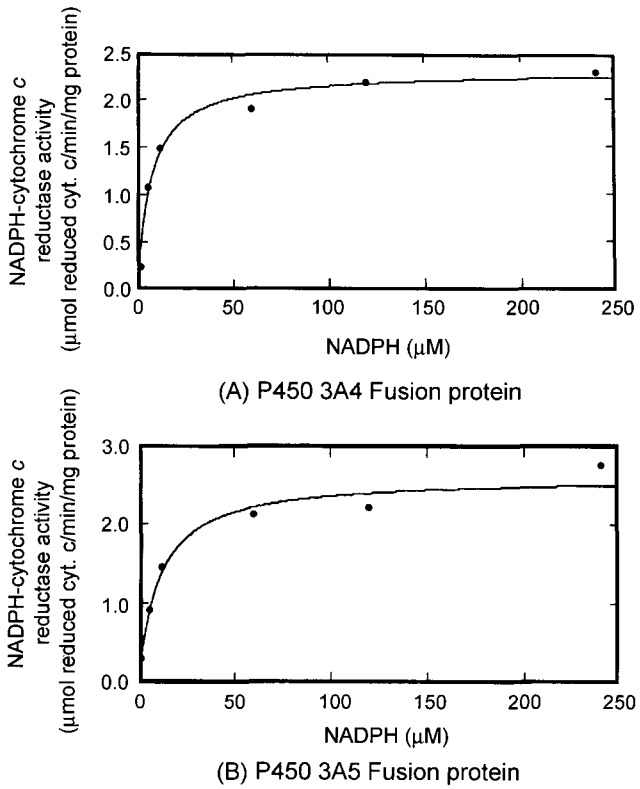

Fig. 6. Michaelis-Menten plots of cytochrome *c* reduction by fusion proteins expressed in *E. coli*. Membranes were incubated with 40 μM of cytochrome *c* in 0.3 M potassium phosphate buffer (pH 7.7) for 3 min at ambient temperature. NADPH was added and the absorbance at 550 nm was measured by time. (A) P450 3A4 fusion protein, (B) P450 3A5 fusion protein.

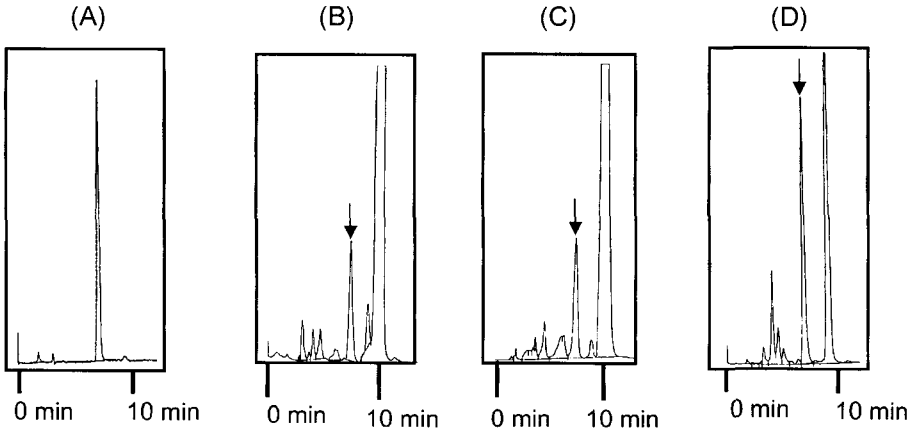

Fig. 5. Nifepidine oxidation by *E. coli* membranes expressing the P450 3A4 or P450 3A5 fusion proteins and human liver microsomes. (A) Authentic nifepidine metabolite (B) P450 3A4 fusion protein, (C) P450 3A5 fusion protein, (D) human liver microsomes. Nifedipine metabolite produced was analyzed using a 150-mm×4.6-mm steel C_{18} Nucleosil column with ultraviolet detection at 254 nm with 64% methanol. The retention times of nifedipine metabolite and nifedipine were 6.9 and 9.1 min, respectively.

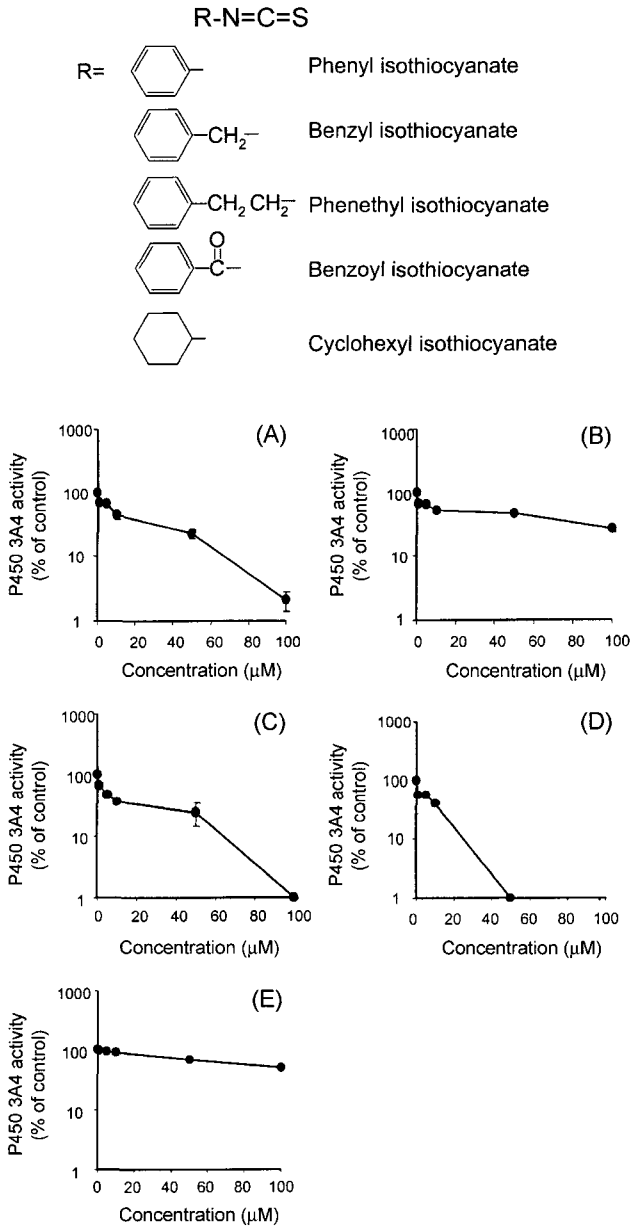

Fig. 7. Inhibition of P450 3A4 activity by isothiocyanates. ECOD activities were determined fluorometrically with bacterial membranes in 0.1 M potassium phosphate buffer (pH 7.4) containing 2 mM ethoxycoumarin and 1 mM NADPH. Results are expressed as a percentage of control activity determined in the absence of isothiocyanates. Control activity in the absence of chemical was 1.1 nmol of 7-hydroxycoumarin formed min^{-1} (mg of protein)$^{-1}$. (A) Phenethyl isothiocyanate, (B) phenyl isothiocyanate, (C) benzyl isothiocyanate, (D) benzoyl isothiocyanate, and (E) cyclohexyl isothiocyanate.

reconstitution assay was 4,000 nmol (cytochrome *c* reduced) min^{-1} (mg protein)$^{-1}$ and 7.5 μM. The result suggests that the NADPH-P450 reductase domain of the fusion proteins is active enough to transfer the electrons to P450 domain.

To determine whether various isothiocyanates affected P450 3A4-mediated drug oxidation, the abilities of five isothiocyanate compounds including phenethyl isothiocyanate, phenyl isothiocyanate, benzyl isothiocyanate, benzoyl isothiocyanate, and cyclohexyl isothiocyanate to inhibit P450 3A4 activity were assessed with *E. coli* membranes expressing P450 3A4 fusion protein (Fig. 7). Of the compounds tested, benzoyl isothiocyanate showed the most potent inhibition of P450 3A4 with IC_{50} value of 2.8 μM. Benzyl isothiocyanate and phenethyl isothiocyanate also showed strong inhibition with IC_{50} values of 3.8 and 5.4 μM. Phenyl isothiocyanate was a relatively weak inhibitor of P450 3A4 with IC_{50} value of 12 μM and cyclohexyl isothiocyanate did not show any P450 3A4 inhibition up to 1 mM.

IV. DISCUSSION

Because of the clinical importance of P450 enzymes and the difficulty of availability of suitable human tissues, many researchers have been interested in developing heterologous expression system of P450 in *E. coli*. In an effort to produce a catalytically self-sufficient P450, several fusion proteins containing clinically important P450s fused with NADPH-P450 reductase were constructed (Shet *et al.*, 1993; Parikh and Guengerich, 1997; Deeni *et al.*, 2001; Chun *et al.*, 1996; Shet *et al.*, 1996).

Because P450 3A4 and 3A5 are responsible for a number of drug metabolism and are regarded as the major enzymes for inactivation of anti-cancer agents, construction and high-level expression of self-sufficient fusion proteins of human P450 3A4 or 3A5 should facilitate the determination of metabolites produced by human liver tissues and the development of P450 3A4-targeted therapy against the cancer drug-resistant tumor cell lines.

In this study, the fusion proteins were expressed at a reasonably high level, ~190 nmol of spectrally detectable P450 3A4 (liter culture)$^{-1}$ and ~140 nmol of P450 3A5 (liter culture)$^{-1}$, in *E. coli* DH5α cells. SDS-PAGE analyses of fusion protein-expressed *E. coli* membranes revealed the presence of a distinct band, Mr≈130 kDa, not present in the membranes of cells transformed with the pCW vector alone (data not

shown). Immunoblot analyses with anti-rat NADPH-P450 reductase antibodies revealed the presence of immunoreactive protein bands in the fusion protein-expressed cells. The expressed fusion proteins catalyzed the nifedipine oxidation and cytochrome *c* reduction. The data suggest that the fusion proteins are catalytically active for P450 3A as well as NADPH-P450 reductase.

To evaluate the usefulness of fusion protein systems on high-throughput screening, the inhibition of P450 3A4 by five isothiocyanate compounds was investigated. Benzoyl isothiocyanate (IC_{50} = 2.8 μM) and benzyl isothiocyanate (IC_{50} = 3.8 μM) were shown to act as a strong inhibitor of human P450 3A4. The mechanism of inhibition by benzyl isothiocyanate of P450 was suggested (Goosen *et al.*, 2000, 2001). Benzyl isothiocyanate may be oxidatively desulfurated to the reactive benzyl isocyanate intermediate that covalently modified the P450 apoenzyme. The mechanism of inhibition by benzoyl isothiocyanate of P450 3A4 may be similar although we did not specifically test it. In future studies, it will be interesting to determine how benzoyl isothiocyanate inhibits P450 3A4 and other P450 enzymes.

In summary, we have demonstrated here the successful construction and heterologous expression of the fusion proteins containing human P450 3A4 (or 3A5) and rat NADPH-P450 reductase in *E. coli*. Expression levels of these proteins were relatively high and the expressed proteins are catalytically self-sufficient. These fusion proteins may be useful for the study of structure-function relationships and high-throughput screening of P450 modulators from natural and synthetic chemical sources.

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