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In Vitro Kinetics of Two Human *CYP1A1* Variant Enzymes Suggested to Be Associated with Interindividual Differences in Cancer Susceptibility

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A genetic polymorphism (A^{4889}→G) in the human *CYP1A1* gene which creates an Ile^{462}→Val amino acid substitution has been suggested to cause altered enzymatic properties of CYP1A1. Since several epidemiological studies have shown an association between the *CYP1A1-Val* allele and lung cancer, we considered it of importance to evaluate the *in vitro* kinetic properties of the two CYP1A1 variants after expression of each cDNA in yeast. No differences were found in K_m or V_{max} for CYP1A1 dependent *O*-dealkylation of ethoxyresorufin and 3-hydroxylation of benzo(a)pyrene between the two variants. The data indicate that the Ile/Val polymorphism in human CYP1A1 is not functionally important.

Exposure to polycyclic aromatic hydrocarbons (PAHs) increases the risk for cancer in lung, bladder, breast and colon. PAHs are efficiently metabolised and activated by CYP1A1, a cytochrome P450 enzyme expressed in extrahepatic tissues. It has been shown that cigarette smoke induces both CYP1A1 and benzo(a)-pyrene (BaP) hydroxylase activity in the lung (1). Two weeks after cessation of smoking the CYP1A1 mRNA levels are reduced (2). Lung cancer patients who smoke have significantly elevated AHH activity in lung tissue, compared to smoking controls (3, 4). There are pronounced interindividual differences in susceptibility to lung cancer and a genetic factor taken into consideration for this is a polymorphic distribution of enzymes activating or deactivating lung carcinogens, among them PAHs.

A polymorphism in the open reading frame of the *CYP1A1* gene, where a point mutation in exon 7 causes a Ile^{462}→Val amino acid substitution, was described by Hayashi *et al.,* (5,6). This substitution is most likely within the heme binding region of the protein. Several studies have provided evidence for an overrepresentation of the *Val* allele among lung cancer cases in Japan (7-12). However, in studies carried out on Caucasian populations, the results have been in line with (13,14) or opposite to the results from the Oriental investigations (15,16). Conflicting data have also been presented regarding the association of the *Val* allele to colorectal cancer (17) and breast cancer (18,19).

Functional differences between the two CYP1A1 variants *in vitro,* have been reported. Kawajiri *et al.,* (8) have given preliminary evidence for a higher BaP hydroxylase activity in CYP1A1-Val as compared to CYP1A1-Ile. Higher Aryl Hydrocarbon Hydroxylase (AHH) activity in noninduced mitogen stimulated lymphocytes from subjects homozygous for the *Val* allele as compared to the *Ile* allele was recently reported by Kiyohara *et al.* (20).

In view of the conflicting results regarding the association of the Ile/Val polymorphism to lung cancer incidence and the effect of this polymorphism on the catalytic activities of the corresponding enzymes, we considered it of importance to study the kinetic properties of the two variant enzymes *in vitro.* Our data indicate that the two forms do not differ significantly in their properties and that this polymorphism is of limited importance for interindividual differences in the rate of CYP1A1 dependent carcinogen activation *in vivo.*

MATERIALS AND METHODS

Chemicals and reagents. Pfu and *Vent* DNA polymerase were purchased from Stratagene (La Jolla, CA) and New England Biolabs Inc. (Beverly, MA) and restriction enzymes from Boehringer Mannheim (Mannheim, Germany). cDNA was prepared with Gene Amp RNA PCR Kit, Perkin Elmer (Foster City, CA). DNA fragments were purified with Wizard PCR Preps, Promega (Madison, WI). Site directed mutagenesis was performed with U.S.E. Mutagenesis Kit, Pharmacia Biotech (Uppsala, Sweden). Ethoxyresorufin, resorufin and benzo(a)pyrene were purchased from Sigma (St Lois, MO). All chemicals were of highest quality and used according to manufacturers recommendations.

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TABLE 1

Primers Used for Genotyping, Cloning, Mutagenesis, and Sequence Analysis of the *CYP1A1* Gene

CYP1A1-F	5′-GAC GGA TCC ATG CTT TTC CCA ATC-3′
CYP1A1-R	5′-GAC GGT ACC CTA AGA GCG CAG CTG CAT-3′
CYP1A1-2B	5′-AAG ACC TCC CAG CGG GCA AC-3′
CYP1A1-2A	5′-AAG ACC TCC CAG CGG GCA AT-3′
Target mutagenic primer	5′-GGT GAG ACC GTT GCC CGCT-3′

Yeast strain and vectors. *Saccaromyces cerevisae* strain W303-1B modified by insertion of the GAL10-CYC1 galactose-inducible promoter immediately upstream of the 5′ end of the yeast reductase open reading frame designated W(R) was used (21). The expression vector pYeDP60 (22) (pV60) was used to construct pV60-1A1wt and pV60-1A1mut by inserting the open reading frame of the two CYP1A1 variants.

Cloning and expression of CYP1A1 variants. RNA was purified from HepG2 cells treated for 12h with TCDD as described by Okoyama *et al.*, (23). cDNA was prepared and amplified with PCR using two primers CYP1A1F and CYP1A1R (Table 1) flanking the open reading frame of *CYP1A1*. The primers incorporated a *BamH*I recognition site in the 5′-end and *Kpn*I in the 3′-end of the double stranded cDNA produced in the reaction. The 1547 bp PCR product was digested with *BamH*I and *Kpn*I over night and ligated into the pBluscript KS vector (Stratagene, La Jolla, CA.). The cloned cDNA was found to be of the wild-type genotype (*CYP1A1-Ile*). Site directed mutagenesis was performed to create the *CYP1A1-Val* variant. For genotyping, primer CYP1A1F was used together with Primer CYP1A12A (wildtype) and CYP1A12B (mutant) (Table 1) in an allele specific PCR. The genotypes of the clones were confirmed by sequencing.

The cloned *CYP1A1* cDNA variants were inserted into the pV60 vector. For culture and expression of the CYP1A1 variants the W(R) yeast strain was transformed with the pV60-1A1wt and pV60-1A1mut plasmids using methods described by Cullin *et al.* (24). After mechanical fractionating of the yeast cells, microsomes were isolated by precipitation of the 20 000 × g supernatant with polyethylene glycol (PEG-4000).

Characterisation of subcellular fractions. Protein content was measured by method of Lowry (25). Cytochrome P450 content was determined by the reduced carbon monoxide difference spectra (26). NADPH-cytochrome P450 reductase activity was determined by monitoring reduction of cytochrome c at 550 nm. Antibodies against rat CYP1A1 (Daiichi Pure Chemicals LTD, Tokyo) was used in Western Blot analysis of 5 μg of microsomal protein and detected by ECL (Amersham, Bristol, England).

Enzymatic assays. Resorufin formation by P450 dependent *O*-dealkylation of ethoxyresorufin (EROD activity) and formation of 3-OH-BaP from BaP was measured fluorimetrically at 28°C essentially as described by Cullin *et al.*, (24). Incubations were carried out using equivalent amounts of P450 and microsomal protein by adjusting protein levels with microsomal proteins from yeast transformed with pV60. Microsomes prepared from two (EROD) and four (3-OH-BaP) clones of each genotype were assessed.

RESULTS

Expression of CYP1A1 in yeast. Expression of the CYP1A1-Val and CYP1A1-Ile cDNA in yeast revealed similar amount of spectrally determined cytochrome P450, similar rate of NADPH-cytochrome P450 reductase activity and similar levels of apo CYP1A1 protein in the two types of microsomes isolated (Table 2 and Figure 1). The Western blotting analysis revealed a single protein product with an apparent *Mr* of 58,000 (Figure 1).

EROD activity. The rate of ethoxyresorufin *O*-deethylase (EROD) activity was monitored fluorimetrically and was found to be linear with time for at least three minutes at all substrate concentrations used. Microsomes from two different yeast preparations separately transformed with the two different plasmids were used and analysis was carried out in triplicate in each case. The microsomes were incubated with the substrate at 5 different concentrations. Linweaver-Burke plots for the EROD activity in the two types of microsomes are shown in Figure 2A. The K_m for the reaction was found to be the same, 0.12 μM, for both the CYP1A1-Val and the CYP1A1-Ile forms. The V_{max} differed insignificantly, 30.8 and 34.1 nmol × min^{-1} × nmol $P450^{-1}$, respectively (Table 2). No EROD activity was registered in microsomes isolated from yeast transformed with pV60 without insert.

Benzo(a)pyrene metabolism. Formation of 3-OH-BaP from BaP was linear with time for fifteen minutes and no production of 3-OH-BaP was detected in incubations with control microsomes (pV60). Four separately transformed batches of yeast were used for each variant enzyme and the microsomes were incubated with 5 different concentrations of BaP. Figure 2B shows the Linweaver-Burke plot for the BaP-3-hydroxylase using mean values from all experiments. The apparent K_m value for both the CYP1A1-Ile and CYP1A1-Val form was 1.44 μM, and the apparent V_{max} for the 3-hydroxylation of BaP was 44.6 nmol × nmol $P450^{-1}$ × min^{-1} for the CYP1A1-Ile form and 39.5 nmol × nmol $P450^{-1}$ × min^{-1} for the CYP1A1-Val form. The difference was not statistically significant.

DISCUSSION

A difference in AHH activity and lung cancer risk between individuals carrying the *CYP1A1-Ile* and *CYP1A1-Val* allelic variants has been observed (8,10,12, 27, 28). The mechanism suggested is altered enzyme activity due to the amino acid substitution, causing interindividual differences in the rate of metabolism and bioactivation of carcinogens. This was supported by Kawajiri *et al.* (8) who reported increased 3-OH-BaP activity for the CYP1A1-Val variant expressed in yeast. Kiyohara *et al.* (20) found increased AHH activities in lymphocytes from individuals homozygous for the *CYP1A1Val* allele. In several studies the Ile^{462}→Val substitution has been found to cosegregate with a point mutation in the 3′ flanking region, causing

TABLE 2

Characteristics of Microsomes Isolated from Yeast Expressing the CYP1A1-Ile and CYP1A1-Ile Forms

	CYP1A1-Ile[a]	CYP1A1-Val	pV60
Protein content (mg/ml)	21.4 ± 4.0	21.3 ± 1.3	19.3 ± 0.2
CYP450 content (pmol/mg protein)	86.4 ± 52	82.0 ± 50	
NADPH-cytochrome c reductase activity (nmole/min · mg)	517 ± 134	652 ± 217	932
EROD activity[b]			
K_m (μM)	0.12 ± 0.05	0.12 ± 0.03	—
V_{max} (nmol × min^{-1} × nmol $P450^{-1}$)	34.1 ± 4.1	30.8 ± 0.7	—
BaP 3-hydroxylase activity[c]			
K_m (μM)	1.44 ± 0.60	1.44 ± 0.23	—
V_{max} (nmol × min^{-1} × nmol $P450^{-1}$)	44.6 ± 2.5	39.5 ± 8.4	—

[a] Values were obtained using yeast microsomes from cells transfected with pV60-1A1wt (Ile), pV60-1A1mut (Val), and pV60.
[b] Mean values from triplicate experiments using two microsomal preparations for each CYP1A1 form.
[c] Mean values from duplicate experiments using four microsomal preparations for each CYP1A1 form.

a new *Msp*I restriction site (5,15,16). The *Msp*I- *m2* allele has been associated with high CYP1A1 inducibility (20, 29) and increased risk for lung cancer among Japanese (30-32). In another study, however, mRNA expression in lymphocytes was shown not to vary across the genotypes detected with *Msp*I (33), indicating that the polymorphism is not linked to mutations of importance for gene expression.

By contrast, Zang *et al.* (34) reported, after this work was completed, an equal rate of 3-OH-BaP and EROD formation by the two CYP1A1 variants in a reconstituted system containing purified *E.Coli.* expressed enzymes. In our study, the kinetics of the two CYP1A1 substrates were assessed *in vitro* after expression in a eucaryotic system, and no differences in substrate affinity or V_{max} were obtained, for the two CYP1A1 variants. It might therefore be concluded that the substrate binding site probably is not affected by the amino acid substitution and that the kinetic properties of the enzyme are only insignificantly influenced. It is conceivable that the amino acid substitution from valine and isoleucine, both highly hydrophobic amino acids and structurally similar, does not cause any major influence on the enzyme conformation. The controversy of CYP1A1 inducibility with respect to the *CYP1A1-Val* and -*Msp1* polymorphisms has yet to be resolved but the current study indicates that the association between the *CYP1A1-Val* genotype and lung cancer, is not likely to be caused by any alterations in enzyme properties.

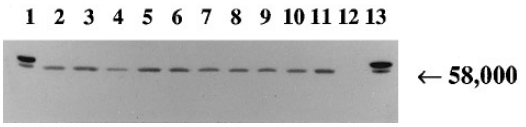

FIG. 1. Western blot analysis of microsomal protein isolated from yeast transformed with pV60-*1A1wt* and pV60-*1A1mut* recombinants. Microsomes corresponding to 5 μg were analyzed in each lane using rabbit antibodies raised against rat CYP1A1. Lanes 2-6 contain microsomal proteins from yeast with the CYP1A1-Ile variant and lanes 7-11 microsomes from the CYP1A1-Val variant. Lane 12 contains microsomal proteins from yeast transfected with the pV60 vector. In lanes 1 and 13, 200 ng of β-naftoflavone induced rat liver microsomes was loaded. The CYP1A1 reactive protein band has an apparent *Mr* of 58,000.

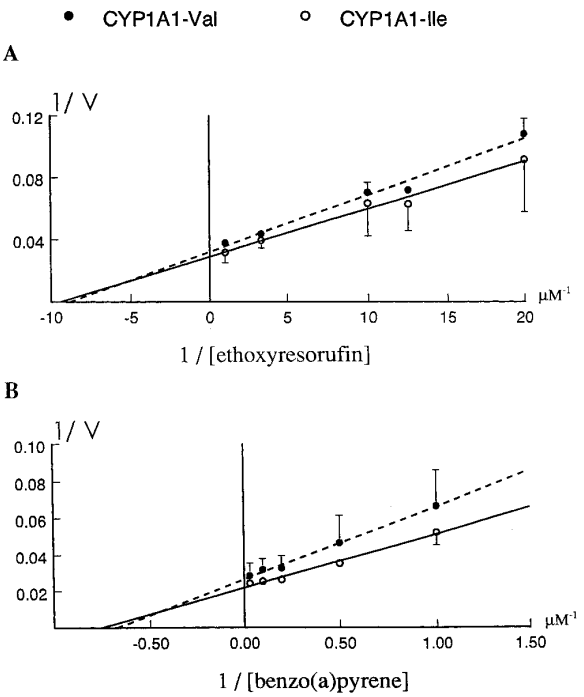

FIG. 2. Lineweaver-Burke plot of EROD and BaP 3-hydroxylase activity in microsomes from yeast expressing the CYP1A1-Ile and CYP1A1-Val variants. (A) EROD activity was measured at 0.05, 0.08, 0.1, 0.3, and 1.0 μM substrate concentration. V is expressed as nmol resorufin × min^{-1} × nmol $P450^{-1}$. (B) 3-OH-BaP formation was monitored using 1, 2, 5, 10, and 30 μM substrate concentration. V is expressed as nmol 3-OH-BaP × min^{-1} × nmole $P450^{-1}$.

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