Effects of *CYP1A2* Gene Polymorphisms on Antipyrine *CYP1A2*-Dependent Metabolism

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The distribution of two *CYP1A2* gene polymorphisms, *CYP1A2*F* and *CYP1A2*D*, was studied in a group of 38 men. Antipyrine elimination test was carried out and urinary cotinine was measured. The contribution of these *CYP1A2* gene polymorphisms, age, and tobacco smoking to accumulation of three main antipyrine metabolites in the urine was evaluated by regression analysis. The impact of both studied polymorphisms was essential for urinary levels of 3-hydroxymethylantipyrine (metabolite most dependent on cytochrome P-4501A2) and hence, for functional activity of this isoform of the enzyme.

Key Words: *genetic polymorphism; cytochrome P-4501A2; metabolism; antipyrine; cotinine*

Cytochrome P-4501A2 (CYP1A2) is one of the most important liver cytochromes involved in metabolism of numerous drugs, some procarcinogenes, and endogenous substrates [12,15]. It is hypothesized that some of the detected polymorphic variants of *CYP1A2* gene modulate functional activity of the encoded enzyme [9,15]. It was shown that *CYP1A2*1F* (-163C>A) AA genotype is associated with high activity of the enzyme in tobacco smokers [14]. The data on other polymorphisms are contradictory. Some authors indicate that *CYP1A2*1D* polymorphism (-2467delT) is inessential for activity of CYP1A2 [9], while others noted its increase in carriers of -2467delT allele [12].

The expression of CYP1A2 is most pronounced in the liver, due to which its activity is indirectly measured by monitoring of test drugs in the urine [13]. Caffeine is often used for this purpose [5] and antipyrine less often [2]. Antipyrine has not been used as a drug for a long time and in many cases is preferable for use in phenotyping than more specific CYP1A2 substrates due to absence of natural sources of antipyrine consumption. Many studies of genetic polymorphism of xenobiotic biotransformation enzymes did not include studies of gene-regulated activities of the corresponding enzymes. At such an approach, genotyping fails to evaluate the contribution of the studied gene polymorphisms and various modulatory factors to enzymatic activity.

We evaluated the effects of *CYP1A2* gene polymorphisms, age, and tobacco smoking on cytochrome P-4501A2-dependent metabolism of antipyrine.

MATERIALS AND METHODS

A group of men (N=38) participating in the study included 28 firemen and 10 representatives of other professions. The mean age of the volunteers was 44.4±0.87 years, the tobacco smokers to nonsmokers proportion was 1:1. Each volunteer gave written informed consent to participation in the study. The protocol of the study was approved by Biomedical Ethics Committee of East Siberian Research Center.

Venous blood was collected for studies of *CYP1A2* gene polymorphisms. DNA was isolated using DNA-sorb-B commercial kits. PCR was carried out in a Tertsik amplifier (DNA Technologies) and restriction fragment length polymorphisms were analyzed. The mixture for amplification (25 μl) contained 10 pM each primer, 2.5 μl 10-fold buffer (Sigma-Aldrich), 2.5 mM $MgCl_2$ (Sigma-Aldrich), 0.2 mM each dNTP,

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and 1 Unit Taq polymerase. The following primers (Medigene) were used: for *CYP1A2*F*: 5'-CCC AGA AGT GGA AAC TGA GA-3' and 5'-GGG TTG AGA TGG AGA CAT TC-3'; for *CYP1A2*D*: 5'-TGA GCC ATG ATT GTG GCA TA-3' and 5'-AGG AGT CTT TAA TAT GGA CCC AG-2' [6]. Restriction endonuclease *Apa*I (Fermentas) was used for *CYP1A2*F* (-163C>A) genotyping. Electrophoresis was carried out in 1.5% agarose gel. For genotyping of *CYP1A2*D* (-2467delT) *Nde*I restrictase (Fermentas) was used, electrophoresis was carried out in 10% polyacrylamide gel. The results were evaluated in transmitting UV light after ethidium bromide staining.

Antipyrine of pharmacopoiean purity (Fluka) served as the substrate for cytochrome P-4501A2 enzymatic activity. It was used in a dose of 18 mg/kg orally after overnight fasting, after which urinary specimens were collected throughout 24 h into a flask with 200 mg $Na_2S_2O_5$ for stabilization of metabolites. Samples for liquid chromatography were prepared using Touret's H-3 β-glucuronidase from *Helix pomatia* (Sigma) for enzymatic hydrolysis of conjugated metabolites. The metabolite extraction procedure was carried out by two steps. Step 1 consisted in extraction of 4-hydroxyantipyrine (4-HAP) and norantipyrine (NAP), step 2 was extraction of 3-hydroxymethylantipyrine (3-HMAP) and antipyrine (AP) [3]. Phenacetin (Aldrich) served as the internal reference sample. Urinary samples were analyzed for AP, its metabolites, and phenacetin by HPLC on a Milichrome-A02 liquid chromatographer (EcoNova; 2×75 mm column, Siladsorb SPH C18, 5 μ). Detection was carried out at λ=244 nm. The gradient was prepared by mixing two solutions: eluent A, a mixture of methanol and 0.05 M phosphate buffer (pH 6.7; 10:90), and eluent B, 90% methanol; flow velocity 200 μl/min, column temperature 45°C. In order to attain complete separation of the analyzed components as rapidly as possible, isocratic (7% B, 10 min) elution was followed by gradient elution (from 7 to 100% B in 4.5 min). A signal from unidentified admixture, poorly separated under those conditions from the phenacetin peak, was observed in some specimens at the site of phenacetin peak. In these cases the sample was repeatedly subjected to chromatography in an isocratic mode (20% B, 10 min), this leading to complete resolution of phenacetin and the impurity component peaks. Calibration curves for AP and its metabolites, plotted in the "peak area/phenacetin peak area–concentration" coordinates, were presented by straight lines with coefficients of correlation R^2>0.98. Each specimen was analyzed twice, the means were statistically processed.

The intensity of tobacco smoking, including passive one, was evaluated by cotinine content in the urine, measured by Cotinine Direct ELISA Kit (Bio-Quant, Inc.) on an ELx800 universal reader for plates (Bio Tek). Urinary specimens for these measurements were collected directly before blood collection and oral intake of AP.

Statistical analysis was carried out using Statistica 6.1 software.

RESULTS

The distribution of genotype frequencies by *CYP1A2*F* polymorphism in the studied sample corresponded to Hardy–Weinberg's equilibrium (Table 1). A shift from the balance was observed for *CYP1A2*D* locus; it was presumably caused by the presence of an individual with del/del genotype in the small sample.

The content of AP metabolites in the urine (Fig. 1) decreased in the following order: 4-HAP, 3-HMAP, NAP, their proportions and levels being in line with the previous data [4]. The sums of AP metabolites (NAP+4-HAP+3-HMAP) and nonmetabolized AP were also within the reference range of values.

Measurements of cotinine in the urine showed great variability of its levels: the mean level for tobacco smokers was 15,090 ng/ml (2235-51,154), for nonsmokers 30 ng/ml (0.9-158). These results attested to possible contribution of passive tobacco smoking, the absence of correlation with the number of cigarettes smoked, and by the level of cotinine in the urine were in good agreement with published data [11].

TABLE 1. Distribution of *CYP1A2* Gene Genotypes and Alleles in Volunteers

Polymorphism	Genotype	Actual number	Expected number	Allele incidence	χ^2
CYP1A2*F	A/A	18	17.11	A 0.67	0.43
(-163C>A)	A/C	15	16.78		d.f.=1
	C/C	5	4.11	C 0.33	p>0.05
CYP1A2*D	T/T	34	33.16	T 0.93	4.86
(-2467delT)	T/del	3	4.67		d.f.=1
	del/del	1	0.16	del 0.07	p<0.05

The contribution of the studied *CYP1A2* gene polymorphisms to functional activity of the enzyme was evaluated by regression analysis. AP metabolites were related signs, the prognostic signs were age (AGE), cotinine level (COT), and *CYP1A2*F* and *CYP1A2*D* genotypes ranked from 1 to 3.

The following models were obtained:

NAP=-0.353 AGE+0.109 COT+0.072 CYP1A2F+ 0.004 CYP1A2D
(R^2=0.131, F=1.24 p<0.310, SEE 4.98, p_1=0.049, p_2=0.528, p_3=0.671, p_4=0.982);

4-HAP=-0.191 AGE+0.348 COT+0.110 CYP1A2F+ 0.169 CYP1A2D
(R^2=0.206, F=2.14 p<0.098, SEE 8.99, p_1=0.257, p_2=0.041, p_3=0.494, p_4=0.329);

3-HMAP=-0.099 AGE+0.574 COT+ 0.266 CYP1A2F+0.321 CYP1A2D
(R^2=0.536, F=9.53 p<0.00003, SEE 6.07, p_1=0.438, p_2=0.000, p_3=0.036, p_4=0.019),

where R^2 is multiple determination coefficient, F are values of Fisher's test and p for regression equation, SEE is standard error of the model evaluation, and p_n are p values for standardized regression coefficients.

All the resultant models indicate (with a different level of significance) a feedback between the level of the main AP metabolites and volunteers' ages, which is in line with available data on age-associated reduction of functional activity of the majority of cytochrome P-450 isoforms, specifically, of CYP1A2 [8]. The results of analysis indicate that the model obtained for 3-HMAP adequately describes the relationship between the signs. The p_2-p_4 level of significance of regression coefficients was below 0.04, while multiple determination coefficient was the highest in comparison with other metabolites. Measurement of cotinine level demonstrated the contribution of tobacco smoking; its level was also the maximum in the 3-HMAP model (0.574, p=0.000). High contribution of cotinine to stimulation of reactions determining the formation of 3-HMAP and 4-HAP was in line with the data indicating CYP1A2 induction in tobacco smoking [7].

The model obtained for 3-HMAP indicates the impact of both studied polymorphisms of *CYP1A2* gene for its functional activity, which was demonstrated by regression coefficients and their levels of significance (p_3=0.36 and p_4=0.19 for *CYP1A2*F* and *CYP1A2*D*, respectively). The following facts confirm that 3-HMAP is the metabolite most dependent on CYP1A2. It has been shown that the contribution of CYP1A2 to the formation of the main metabolites in AP N-demethylation, 4-hydroxylation, and 3-methylhydroxylation reactions could be evaluated as 20-25, 30, and 50%, respectively [13]. In addition, urinary levels of 3-HMAP in experimental rats correlated with CYP1A2 activity measured by methoxyresorufine O-dealkylation rate in the liver homogenate microsomal fraction [1]. As CYP1A2 is one of the dioxy-induced isoforms of the enzyme [10], an additional evidence in favor of 3-HMAP as the metabolite most linked with cytochrome P4501A2 is its significant correlation with the dioxine levels in the volunteers.

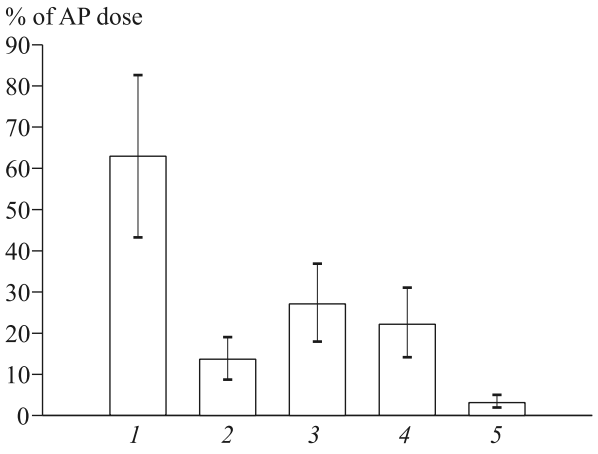

Fig. 1. Antipyrine metabolism in volunteers. *1*) sum of antipyrine metabolites; *2*) norantipyrine; *3*) 4-hydroxyantipyrine; *4*) 3-hydroxymethylantipyrine; *5*) antipyrine.

The minimum levels of these metabolites were found in the urine of the individual with homozygotic deletion genotype of *CYP1A2*1D* polymorphism. *CYP1A2*1F* genotyping revealed AA genotype associated with high CYP1A2 activity in this individual.

Hence, the impact of both studied polymorphisms (*CYP1A2*F*, *CYP1A2*D*) of *CYP1A2* gene for AP metabolism dependent on this isoform of the enzyme has been revealed. The model derived for 3-HMAP demonstrated the contribution of genetically determined component to the functional activity of cytochrome P-4501A2 in the presence of factors modifying it – age and tobacco smoking.

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