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Article

CYP2A6 activity in a healthy Spanish population: effect of age, sex, smoking, and oral contraceptives

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This study was performed to assess the influence of age, sex, smoking, and contraceptive use on CYP2A6 activity. In the metabolism of caffeine, the conversion of 1,7 dimethylxanthine (17X) to 1,7 dimethiylurate (17U) is catalyzed primarily by CYP2A6. CYP2A6 phenotype was determined by the urinary ratio 17U:17X in the interval of 4–5 h after caffeine intake in 179 healthy white Spaniards (102 women and 76 men). There were 99 non-smokers and 80 smokers. Among women, 26 were taking oral contraceptives. The age was the most important predictive factor of CYP2A6 activity ($P < 0.001$) with older subjects having higher activity. The influence of the gender was more modest ($P = 0.07$) with women exhibiting borderline increased values of the CYP2A6 marker than men. Tobacco smoking did not affect CYP2A6 activity. However, the CYP2A6 marker resulted to be strongly related to the use of oral contraceptives. The women users of oral contraceptives had higher values of CYP2A6 marker than both women not taking oral contraceptives and men ($P < 0.001$ in both comparisons). The results indicate that age, oral contraceptive use, and possibly gender should be controlled in epidemiological studies dealing with CYP2A6 activity and its relationship with xenobiotics exposure and genetic or pathological factor.

Key words: age; caffeine phenotyping; CYP2A6 activity; oral contraceptives; sex; smoking

Introduction

The CYP2A6 protein is a member of the cytochrome P450 superfamily of monooxygenases. CYP2A6 is expressed mainly in the liver and, at a lower extent, in extrahepatic tissues.[1] The CYP2A6 hepatic content accounts for 1–10% of the total liver P450 proteins.[2–5]

CYP2A6 is the high-affinity metabolizer of both coumarin,[6] nicotine, and its oxidized metabolite cotinine.[7,8] It also participates in the metabolism of cyclophosphamide, disulfiram, fradrozole, halothane, losigamone, tegafur, and valproic acid.[5,9,10] Importantly, CYP2A6 catalyses the metabolic activation of several promutagens and procarcinogens including aflatoxin B1[11,12] and dietary and tobacco-specific nitrosamines such as NNK (4-methylnitrosoamino-1-(3-pyridyl)-1-butanone)[13,14] and NDEA (*N*-nitrosodiethylamine). High CYP2A6 expression has been associated to increased susceptibility to nicotine addiction and to several types of cancer.[15–18]

In spite of the great interindividual variability (up to 300-fold) in the metabolism of coumarin, a CYP2A6-specific probe drug,[5,19] the CYP2A6 allelic variants have a low frequency in Caucasians populations.[10] Therefore, because interindividual variation in CYP2A6 phenotype is not well explained by CYP2A6 polymorphisms identified thus far, measurement of phenotype remains important in assessing individual CYP2A6 activity.

In the complex metabolism of caffeine (137X), CYP2A6 is a principal enzyme involved in the hydroxylation of methylxanthine (17X) to generate 1,7-dimethylurate (17U).[20] On this basis, the urinary metabolite ratio (MR) 17U:17X has been used to indicate CYP2A6 activity in several studies.[21–24]

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In this population-based study, we evaluate the potential influence of age, sex, smoking, and contraceptive use on CYP2A6 activity to characterize the predictors of this enzyme activity in our Spanish population.

Material and methods

Subjects

In this study, 178 healthy volunteers aged between 18 to 57 years were included among medical students, University and Hospital staff. A medical examination was made to identify subjects in good health. All individuals filled in a detailed questionnaire on smoking habits, diet, and drug consumption. Subjects with idiosyncratic reactions to caffeine or histories of chronic diseases or recent illnesses were excluded from the study, as well as those who were taking any medication known to alter drug metabolism. Other exclusion criteria were: pregnancy, presence of irregularities of the menstrual cycle, obesity, and regular alcohol drinking. All of them followed a controlled Mediterranean diet. Subjects on special diets we excluded. Smokers and non-smokers were recruited as well as women using oral contraceptives (25.49% of women). Combined estrogen-progestin pills were the oral contraceptive used in all cases. Demographic characteristics and smoking status of the participants are detailed in Table 1.

All participants in the study were white Caucasian living in the same geographical area of the North of Spain (Zaragoza and surrounding area). Each subject gave their written informed consent to participate in the study, which was approved by the Ethic Committee of Clinical Research of the University Hospital "Lozano Blesa" of Zaragoza, Spain.

CYP2A6 activity marker

For 48 h before and during the 5 h of the caffeine test, the volunteers refrained from consuming alcoholic drinks and foods or beverages containing methylxanthines. After 48 h, each subject discarded the first morning urine and drank a glass of instant coffee containing 200 mg of caffeine in 125 mL of water. During the entire study, subjects followed a controlled diet. All women performed the caffeine test in the luteal phase of the menstrual cycle.

The urine of 4–5 h after caffeine intake was collected in bottles preloaded with 1 mol/L citric acid phosphate buffer, pH = 3. To analyze caffeine and its metabolites, the HPLC method described by Grant *et al.*,[25] with some modifications, was used. Caffeine and their metabolites were separated by a Waters Novapak C18 reverse-phase column (4 μm particle size, 25 cm × 4.6 mm internal diameter) (Millipore Ibérica S.A.), which was isocratically eluted with a mobile phase containing acetic acid/methanol/water (0.5:90:905.5 vol/vol/vol) at a flow rate of 1 mL/min and a pressure of 1500 psi. The compounds were detected by UV absorbance at 280 nm. The chromatograph was an LC Module I Plus equipped with a Millenimum 2010 software (Waters Corp., Madrid, Spain). Calibration curves were elaborated with known amounts of metabolites in a range from 5 to 80 μg/mL added to blank urine samples, and then processed as described above. For data analysis, caffeine and its metabolite were expressed as mmol/L. The MR used to assess CYP2A6 activity was 17U/17X.[21] To assess whether or not any urinary constituent could co-migrate with caffeine or caffeine metabolites used in the ratio, a Water 996 photodiode array detector was used to compare the peaks with the spectral libraries of standards. Within and between run coefficients of variation were <5.2% and <6.4%, respectively. The intraindividual coefficient of variation for the ratio 17U:17X was determined in six healthy individuals for 3 weeks, resulting in a range from 10% to 23%.

Statistical analysis

The characteristics of the distribution of the urinary caffeine MR 17U:17X were investigated using the

Table 1 Demographic characteristics and smoking behavior

	Men		*Women*	
	(n = 77)	*Total (n = 102)*	*–OC (n = 76)*	*+OC (n = 26)*
Age	26.52 ± 8.01	27.53 ± 8.21	27.11 ± 8.70	28.77 ± 6.50
Smoking status				
Non-smokers	43	56	43	13
Smokers	34	46	33	13
Cigarettes per day	20.88 ± 8.14	19.37 ± 7.88	18.21 ± 7.92	22.31 ± 7.25

+OC, oral contraceptive users; –OC, non-oral contraceptive users.

Kolmogorov–Smirnov test for normality. Variable (x) was transformed to natural log to obtain a well-modeled normal distribution. We evaluated the influence of subjects' characteristics (i.e., smoking status, amount smoked, sex, and oral contraceptives on CYP2A6 activity marker using multivariate linear regression. Non-parametric test were used for comparisons (Wilcoxon rank sum test or median test for two groups and non-parametric trend test for more than two groups). Correlation between CYP2A6 marker values and those of CYP1A2 was analyzed by Spearman rank correlation test. We used SPSS 12.0 statistical software for Windows.

Results

The caffeine MR showed a well-modeled normal distribution (data not shown) when data were logarithmically transformed. The ratio presented a range of 0.31–5.56, and a mean (SD) of 1.31 (0.85) (not including oral contraceptive users).

Age and smoking status for all subjects in this study are summarized in Table 1. Non-parametric tests for comparisons showed the absence of statistically significant differences in either age or tobacco consumption between the groups of men, all women, women users of oral contraceptives, or women who were not using oral contraceptives ($P > 0.05$ in all comparisons).

Table 2 shows the mean and the median values of CYP2A6 activity in subjects (excluding oral contraceptives users) according to subjects' characteristics. The most important predictive factor of CYP2A6 activity resulted to be the age of individuals ($P = 0.001$) indicating that older subjects have a higher enzyme activity. Sex presented a borderline influence on CYP2A6 activity with women having more elevated values of the CYP2A6 marker in relation to men ($P = 0.07$). Smoking neither did significantly affect CYP2A6 activity nor did tobacco consumption in cigarettes per day.

In the total group of women (including oral contraceptive users), linear regression of natural log of 17U/17X showed a similar predictive profile on CYP2A6 marker of the age ($P < 0.001$), smoking ($P = 0.32$), and tobacco consumption ($P = 0.23$) than that observed in the sample when oral contraceptive users were excluded of the analysis (Tables 2 and 3). However, the use of oral contraceptives resulted to have a marked predictive value for CYP2A6 activity ($P < 0.001$). In this regard, non-parametric comparisons between men, women users of oral contraceptives and women who did not use oral contraceptives showed that those women using oral contraceptives presented a strikingly higher CYP2A6 activity than either those women who were not using oral contraceptives or men (Figure 1).

Table 2 Caffeine metabolite ratio, as marker of CYP2A6 activity, by subject characteristics, excluding oral contraceptive users

	n	*Mean*	*Median (95% CI)*	P[a]	P[b]
All subjects	153	1.31	1.05 (1.18–1.45)		
Sex					
Males	77	1.16	1.04 (1.03–1.29)		
Females	76	1.47	1.15 (1.23–1.71)	0.14	0.07
Age					
<25	82	1.12	0.98 (1.00–1.23)		
25–40	53	1.43	1.03 (1.13–1.74)		
≥40	18	1.86	1.76 (1.38–2.34)	0.002	0.001
Smoking status					
Non-smoker	86	1.35	1.09 (1.15–1.55)		
Smoker	67	1.27	1.03 (1.08–1.45)	0.74	0.76
Cigarettes per day					
<25	24	1.09	0.89 (0.82–1.36)		
25–40	28	1.39	1.06 (1.03–1.75)		
≥40	15	1.33	1.24 (1.07–1.58)	0.13	0.23

[a]*P* is from non-parametric tests between groups.
[b]*P* is from linear regression of natural log of the caffeine metabolite ratio including all covariates shown in this table.

Table 3 Caffeine metabolite ratio, as markers of CYP2A6 activity, in all women and women stratified by oral contraceptive use

	n	*Mean*	*Median (95% CI)*	P
All women	102	1.63	1.41 (1.43–1.82)	
–OC	76	1.47	1.15 (1.23–1.71)	
+OC	26	2.09	1.94 (1.83–2.36)	0.001

P from Wilcoxon rank sum test for comparison between two groups.
+OC, oral contraceptive users; –OC, non-oral contraceptive users.

Discussion

In this study we used the caffeine urinary MR 17U/17X as marker of CYP2A6 activity because there exist evidence to support its use to measure CYP2A6 activity. Even though in early studies both CYP2A6 and CYP1A2 were shown to catalyze the conversion of 17X to 17U,[26] others have reported the specificity of the isoform CYP2A6 in this reaction. In this regard, it has been found that for low substrate concentrations, those reflecting in-vivo conditions, CYP2A6 selectively catalyzes the hydroxylation of 17X to 17U.[24] Moreover, in liver microsomes, CYP2A6 has been found to be the principal enzyme involved in hydroxylation of 17X; this activity being highly associated with coumarin 7-hydroxylase activity[20] and coumarin is the most used probe drug for CYP2A6 because of its hydroxylation is specifically catalyzed by

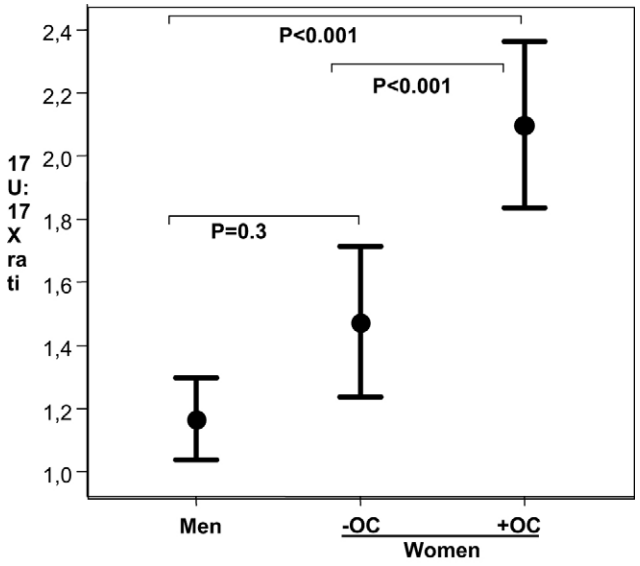

Figure 1 The points and error bars represent median and 95% confidence intervals, respectively. *P* is from comparisons of medians. –OC, oral contraceptive users; +OC, non-oral contraceptive users.

CYP2A6,[27] The ratio 17U/17X has been used to assess CYP2A6 in other studies.[21–24] Another argument supporting that the ratio 17U/17X really measures CYP2A6 activity comes from the lack of influence of tobacco smoking on 17U/17X values found in this study. It is well known that tobacco smoking increases CYP1A2 activity.[28] In this regard, we investigated the association between the marker of CYP2A6 activity used here and the MR AFMU + 1X + 1U/17U [(5-acetylamino-6-formylamino-3-methyl uracil + 1-methylxanthine + 1-methyluric acid)/1,7-dimethylurate] as indicative of CYP1A2 activity and no significant relationship was detected in the correlation study involving all subjects ($r = -0.08$, $P = 0.13$), this suggesting a negligible in-vivo role of CYP1A2 in the conversion of 17X to 17U.

Age in this study resulted to be an important factor affecting CYP2A6 activity with a positive association with the age of individuals. This finding is consistent with that found in a previous work[24] in which a weaker association between CYP2A6 activity marker and age was detected. The stronger influence of age in our study could be due to the differences in the age range of individuals. In the present work, the population was younger in relation to that of the above-refereed study in which most people were older than 60 years. In fact, clearance of nicotine, a specific CYP2A6 substrate, has been reported to be decreased in elderly (age > 65) compared with adults.[29] In-vitro data on microsomes also indicate that CYP2A6 activity increases with age.[30]

Even though men had lower marker values of CYP2A6 marker than women, the difference was limited ($P = 0.14$). In previous phenotyping studies using coumarin or caffeine to measure CYP2A6, a tendency for faster metabolism in women compared with men has been reported[24,27,31] and significant gender differences have been noted in several studies using nicotine or cotinine as markers of CYP2A6 activity.[32,33] Interestingly, the results of a recent study[34] on CYP2A6 genotypes/phenotypes, devoted to analyze the interindividual and interethnic differences in nicotine metabolism, are also compatible with a higher CYP2A6 activity in women than in men.

In this study, women taking contraceptives pills were 25.49% of the total women. This percentage is similar to that reported for Spanish women.[35] We have found that women taking oral contraceptives presented higher CYP2A6 activity than women not taking oral contraceptives (28.57% over the median values). The mechanism underlying this result could be related to female sex hormones. Some previous results appear to reinforce this hypothesis. Thus, pregnancy has been reported to increase nicotine and cotinine metabolism by 60% and 140%, respectively, in relation to postpartum.[36] In addition, a recent study supports the involvement of estrogen in the faster metabolism of nicotine because users of estrogen-alone contraceptives had higher nicotine clearance than both those who took no hormones and women who used progesterone-only contraceptives.[37] Previous studies indicate that oral contraceptives inhibit oxidative metabolism mediated by CYP1A2, CYP3A4, and CYP2C19.[38–40] This is in contrast with our finding. It is possible that P450 responses to oral contraceptives are variable and dependent on particular isoforms. Nevertheless, an inhibitory effect of estrogen on CYP2A6 has been observed *in vitro*.[41] In this regard, it is possible that the higher complexity of the regulation of CYP2A6 by estrogen *in vivo* could account for this discrepancy. However, increased metabolism by oral contraceptives has been described for phase II conjugating enzymes, such as glucuronidation and glycine conjugation.[42,43]

Neither smoking behavior nor tobacco consumption has resulted to be predictors of CYP2A6 activity. The association between tobacco smoking and cigarette consumption with CYP2A6 polymorphisms has been studied in several works and no clear report of interdependency has been found.[44–46]

A possible limitation of the present study is that we did not determine *CYP2A6* genotypes. Therefore, differences in the proportion of carriers of the allelic

variants linked to abnormal activity in subgroups of subjects could presumably account for the differences found in the caffeine MR. However, the low prevalence of these allelic variants in white Caucasian people[10] makes this possibility remote.

In summary, we have found that CYP2A6 activity measured by caffeine phenotyping was significantly influenced by age, oral contraceptive use, and possibly by gender. Although further research is clearly needed using a larger sample size, the data of the present study could indicate the need for controlling these three characteristics in epidemiological studies dealing with CYP2A6 activity and its relationship with xenobiotics exposure and genetic or pathological factors.

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