Korean J Physiol Pharmacol
Vol 3: 207−213, April, 1999

Assessment of Flavin-containing Monooxygenase (FMO) Activity by Determining Urinary Ratio of Theobromine and Caffeine in a Korean Population after Drinking a Cup of Coffee

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To examine individual variation in drug metabolism catalyzed by flavin-containing monooxygenase (FMO), 179 Korean volunteers' urinary molar concentration ratio of theobromine (TB) and caffeine (CA) was determined. Their urine was collected for 1 hr (between 4 and 5 hrs) after they drank a cup of coffee containing 115 mg CA and analyzed by an HPLC system. The lowest TB/CA ratio obtained was 0.40, the highest ratio was 15.17 (38-fold difference), and the median ratio for all subjects was 1.87. The mean was 2.66 with 2.36 S.D.. In 134 nonsmokers, the mean ratio was 2.35±1.93, that of 51 males was 2.30±2.26 and 83 females was 2.37±1.85, respectively. There was no significant gender difference in the obtained TB/CA ratio (Mann-Whitney test; p=0.518). There were no smokers among the 83 female volunteers. In the remaining 96 male subjects, the ratio obtained in 51 nonsmokers was 2.30±2.06 and that of 45 smokers was 3.62±3.19. This indicated that the TB/CA ratio was increased significantly in smokers (p=0.007). However, when the TB/CA ratios (FMO activity) obtained in all 179 Korean volunteers are compared with the urinary concentration ratios of paraxanthine (PX) plus 1,7-dimethylurate (17U) to CA (CYP1A2 activity), there was a weak but significant correlation (Pearson's correlation coefficient test; r^2=0.28, p<0.0001). This indicates that, although the urinary TB/CA ratio mostly represents FMO activity, minor contribution by CYP1A2 activity cannot be ignored. In conclusion, the FMO activity measured by taking the urinary TB/CA ratio from normal healthy Korean volunteers shows marked individual variations without significant gender differences and the increased TB/CA ratio observed in cigarette smokers may have been caused by the increased CYP1A2 activity.

Key Words: FMO activity, Caffeine, Theobromine, Koreans

INTRODUCTION

Cytochrome P450 (CYP) and flavin-containing monooxygenase (FMO) are the two main enzymes contained in liver microsomes which use molecular oxygen and NADPH for the initial oxidation of various endogenous and exogenous chemicals. While FMO is known to share the substrate specificity with CYP, FMO is also known to catalyze preferentially the oxidation of chemicals containing nucleophilic heteroatom bearing nitrogen- and sulfur-centers and to produce the respective *N*-oxide and *S*-oxide metabolites (Ziegler, 1988).

While some compounds like nicotine contained in cigarette are available for a non-invasive determination of FMO activity in human (Cashman et al, 1992), non-toxic probe drugs which are used commonly by human and whose metabolites are easily detected in urine have not been available.

The rate of producing urinary paraxanthine (PX) and its metabolites arising from caffeine (CA) in

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coffee, has been used widely to determine the CYP1A2 activity in human (Butler et al, 1989; Berthou et al, 1991). CA, an alkaloid contained in beverages such as coffee, tea and cola, undergoes extensive metabolism in human and produces at least 17 easily detectable urinary metabolites including PX, theobromine (TB) and theophylline (TP). In previous studies using rat and human liver microsomes, FMO was the major enzyme responsible for producing particularly the TB and TP from CA (Chung & Cha, 1997; Chung et al, 1998). This indicates that CA metabolism is useful not only for the determination of CYP1A2 activity, but also for the determination of FMO activity in human subjects.

FMO in human is known to be responsible for the oxidation of trimethylamine, and subjects who lack FMO may suffer from trimethylaminuria and "fish-odor" syndrome (Al-Waiz et al, 1987). Individuals with trimethylaminuria have been reported to be unable to metabolize medicines such as nicotine, nicotinamide, guanethidine and metyrapone, the drugs metabolized by FMO (Ayesh et al, 1988). Therefore, it was of immense interest to determine the FMO activity in human using the widely consumed CA whose metabolites are easily identified in urine.

In the present study, we have assessed human FMO activity by determining the urinary molar concentration ratio of TB/CA in 179 Korean subjects after administration of a cup of coffee. Then, we have compared the FMO activity between male and female as well as smoking and nonsmoking volunteers.

METHODS

Study protocol

179 Korean healthy subjects who were all unrelated medical and nursing students as well as some personnel at Inha University took part in this study after giving written consents. They were healthy and normal based on their history. They were all native Korean by birth and lineage. None had received any medication (including contraceptives for female volunteers) for at least 1 week before the study. They were informed of the purpose and the procedure of this study before enrollment and provided with an institutionally approved written consent form.

The volunteers were asked not to take any of the methylxanthine containing foods or drugs such as coffee, tea, cola and chocolates from midnight before the previous day of participation. The subjects were composed of 96 males (45 smokers and 51 nonsmokers) and 83 females (nonsmokers). Their ages ranged from 18 to 33 (mean±S.D.; 21.8±2.5), and their body weights ranged from 42 to 95 kg (mean±S.D.; 60.2±11.1). We did not control the menstrual cycle of the female volunteers.

In the morning of the test day, after blank urine was collected, the subjects took a cup of coffee which was prepared with 2 packs of instant coffee (Taster's Choice; 3.6 g) containing 115 mg of caffeine *in toto*. The one hour urine between 4 and 5 hours after the coffee intake was collected and its volume was measured. Immediately thereafter, the pH of all urine samples was adjusted to 3.5 with HCl as was done by Nakajima et al (1994), and then a 10 ml aliquot was stored at −80°C for analysis within two months.

Analysis of caffeine and its metabolites in urine

After the frozen aliquot has been thawed, 500 μl urine was taken and mixed with 250 mg ammonium sulfate for 2 minutes to precipitate proteins which might be present in the urine. Subsequently, 24 μg of 4-acetaminophenol (internal standard) dissolved in 6 ml of solvent mixture composed of chloroform and isopropanol (4 : 1; v : v) was added to the urine. The solution was mixed for 1 min and centrifuged for 5 min at 1400×g. The organic solvent layer (5.5 ml) was collected by suction and was concentrated by vacuum centrifugation for 90 min at 45°C. The concentrated residue was then reconstituted to 800 μl with 0.05% acetic acid and mixed with vortex. After filtering the sample through a filter (0.45 μm pore), 100 μl of the filtrate was injected into an HPLC column. The caffeine and its metabolites were analyzed by using an HPLC system according to the method described previously by Chung & Cha (1997).

Data analysis

FMO activity was assessed by using the molar concentration ratio of TB over CA (TB/CA). The blank urine contained negligible amount of CA and no measurable TB and thus, was used in the assessment of FMO activity. As the frequency distribution plot of TB/CA ratio obtained from 179 urine samples was fairly skewed, a logarithmic scale of the ratio was used for statistical analysis. To determine

whether the FMO activity is affected by factors such as smoking behavior or the gender, statistical parameters were compared between these groups using Mann-Whitney test. To assess whether the frequency distribution shows bimodality, the normal test variable (NTV) plot was used (Endrenyi & Patel, 1991).

RESULTS

The molar concentration ratio of urinary TB over CA (TB/CA) was calculated from each of the HPLC chromatograms obtained from 179 volunteer subjects and was used to assess individual FMO activity in human. The lowest TB/CA ratio observed was 0.40 and the highest ratio was 15.17, showing 37.9-fold difference. The median ratio for the total volunteers was 1.87 and the statistical mean was 2.66 with S.D. of 2.36.

In order to evaluate the effect of smoking on the FMO activity, the TB/CA ratio values obtained from 134 nonsmokers (composed of 51 males and 83 females) and 45 male smokers were compared first. The median ratio for nonsmokers was 1.68 and it was 2.33 for the smokers. The range of TB/CA ratio in the nonsmokers was 0.40 to 10.73 (26.8-fold difference) and 0.54 to 15.17 (28.1-fold difference) in the smokers. The statistical mean±S.D. for the nonsmokers was 2.35±1.93, and 3.62±3.19 for the smokers. The mean ratios between nonsmokers and smokers were significantly different (p=0.003). As there were no smokers among the female volunteers participating in this study, the TB/CA ratios obtained in smokers and nonsmokers among the 96 male subjects were compared. The mean ratio of 51 male nonsmokers (2.30±2.06) was also significantly different (p=0.007) from that of 45 male smokers (3.62 ±3.19). This indicated that the urinary TB/CA ratio (FMO activity) in the smokers was higher than that of the nonsmokers.

Table 1. Effect of smoking and gender on the Flavin-containing monooxygenase activity in 179 Korean subjects

	Median	Range	Mean±S.D.
Smoker (n=45)	2.33	0.54~15.17	3.62±3.19
Male (n=45)	2.33	0.54~15.17	3.62±3.19
Female (n=0)	–	–	–
Nonsmoker (n=134)	1.68	0.40~10.73	2.35±1.93**
Male (n=51)	1.64	0.40~10.73	2.30±2.06
Female (n=83)	1.68	0.40~8.93	2.37±1.85†
Total (n=179)	1.87	0.40~15.17	2.66±2.36

**p<0.01, compared to smoker using Mann-Whitney test, †No significant difference, compared to male nonsmoker.

The TB/CA ratios obtained in the 51 nonsmoking males and 83 nonsmoking females were compared to determine whether gender would affect the FMO activity. The median ratio in male subjects was 1.64 with a range of 0.40 to 10.73 (26.8-fold difference) and in female subjects, it was 1.68 with a range of 0.40 to 8.93 (22.3-fold difference). The statistical mean±S.D. for the male subjects was 2.30±2.26 and for the female subjects, it was 2.37±1.85. The mean ratios between male and female nonsmokers were not significantly different (p=0.518). This indicated that the urinary TB/CA ratio (FMO activity) was not affected by gender.

Using the log values of TB/CA ratios, frequency distribution plots of FMO activities for all test subjects, smokers and nonsmokers were obtained (Fig. 1a, b, c). The frequency distribution for the smokers was found to be shifted towards higher TB/CA ratio than that obtained in the nonsmokers (Mann-Whitney test; $p<0.01$), indicating that the FMO activity was increased in smokers (Fig. 1b).

In order to determine whether the higher TB/CA ratio observed in smokers was caused also by the induction of CYP1A2 which is known to produce paraxanthine (PX) and 1,7-dimethylurate (17U) from CA, the TB/CA and (PX+17U)/CA ratios obtained from all 179 volunteers were compared by Pearson's correlation coefficient test. As the results in Fig. 2 show, there was a weak but significant correlation (r^2=0.28, $p<0.0001$).

In order to detect deviation from the normal distribution, NTV plot was applied to all frequency distributions (Fig. 1a, b, c). The minimal NTV values were −0.04 for the total subjects, −0.05 for the nonsmokers, and −0.08 for the smokers. These results suggested the presence of bi-modal distribution since the minimal NTV values were all < −0.03. The assumed antimodes obtained from the NTV plots were 3.16 in total subjects, 3.16 in the nonsmokers and 4.50 in the smokers. The subjects with TB/CA

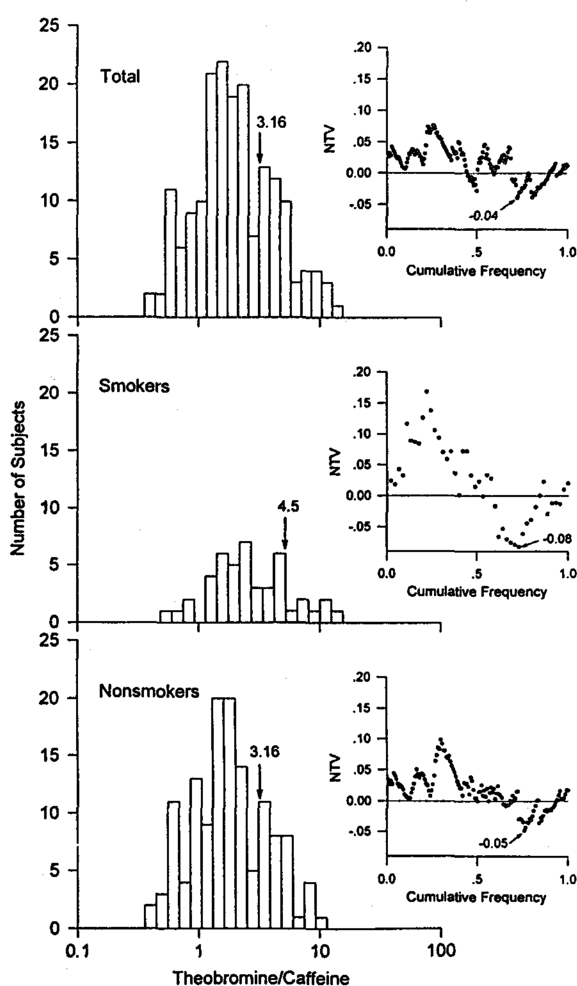

Fig. 1. Frequency distribution and NTV plots of FMO activity for 179 Korean volunteers grouped by total subjects, smokers and nonsmokers. The top, middle and bottom plots indicate the frequency distribution of TB/CA ratios obtained from total volunteers, smokers and nonsmokers, respectively. While the arrows in frequency distribution plots show the assumed antimode values, the arrows in NTV plots show the minimal NTV values. All of the minimal NTV values in each of the NTV plot are < -0.03 and this suggests a bimodal distribution of FMO activity in Korean population. The frequency distribution plot for smokers is shifted towards higher TB/CA ratio than that of the nonsmokers and this suggests that the FMO activity may be increased in smokers.

ratio over the assumed antimode values are considered to be extensive metabolizers (EM) and there were 50 EMs among the total of 179 volunteers (27.9%), 12 in 45 smokers (26.7%) and 32 in 134 nonsmokers (23.9%).

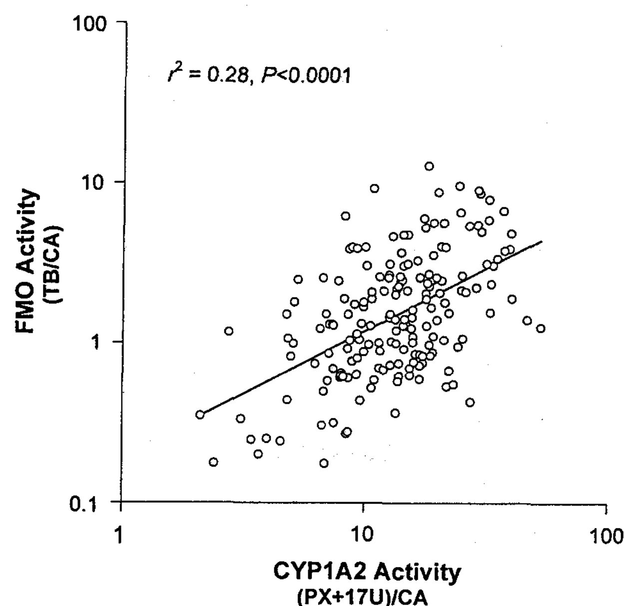

Fig. 2. Pearson's correlation coefficient plot comparing the TB/CA (FMO activity) and (PX+17U)/CA (CYP1A2 activity) ratios. The urinary TB/CA (FMO activity) ratios obtained from 179 volunteers are compared with that of the urinary (PX+17U)/CA (CYP1A2 activity) ratios obtained from the same volunteers after their log transformation to determine whether there is any correlation between the two enzyme activities. The obtained r^2 value was 0.28 ($p < 0.0001$) and indicated that there was a weak but significant correlation.

DISCUSSION

While FMO is known to be present in human liver microsomes together with CYP and to share substrate specificity with CYP, it is known to catalyze preferentially the oxidation of nitrogen- and sulfur-containing medicines and alkaloids. However, unlike the CYP mediated reaction mechanism sequences which require initial binding of an acidic substrate to receive electron and oxygen input for eventual drug oxidation, the FMO is physiologically kept under reactive state, having the 4a-hydroperoxy flavin form at a high pH to oxidize the basic drugs with high pKa immediately upon contact (Ziegler, 1993). The hydroperoxy flavin moiety contained in FMO is known to make an electrophilic attack on the nitrogen atom and oxidize numerous tertiary amine type drugs to produce their *N*-oxide metabolites. In general, the *N*-oxide metabolites formed from tertiary amines are known to be relatively stable and thus, these have served as reliable markers for the FMO catalyzed

oxidation for many substrates. With regard to this, a study conducted by Kawaji et al (1993) on the FMO catalyzed *N*-oxidation of benzydamine has indicated that the initial *N*-oxide metabolite was formed enzymatically by FMO and then deformylated to the final *N*-demethylated product in a non-enzymatic manner. In support of this, preliminary results obtained in this laboratory showed that CA was *N*-demethylated to TB and TP, while producing formaldehyde when oxidized with human FMO expressed and purified from bacteria (Chung et al, manuscript in preparation).

Previous studies conducted to identify the specific enzymes involved in the production of the various CA metabolites have indicated that CYPs 1A2, 2A6, 2D6, 2E1, 3A4 and 3A5 as well as xanthine oxidase are involved (Rostami-Hodjegan et al, 1996). In an additional study conducted in this laboratory utilizing the rat and human liver microsomes as well as the respective inhibitors of CYPs and FMO, FMO has been identified to catalyze the production of TB and TP together with CYPs (Chung & Cha, 1997). Attempts to determine the contributions of CYP and FMO in liver microsomes of rat and human by inhibiting the CA metabolism with SKF525A (CYP inhibitor) and methimazole (FMO inhibitor) showed clearly that CYP played a primary role for the production of PX, together with FMO, which played a minor but significant role. Conversely, for the production of TB and TP, FMO played a major primary role and CYP played only a minor but significant role. This has provided a basis for the present study, which aims to determine whether the rates of TB and TP production from CA could be used to determine FMO activity in human.

The rates of TB and TP production from CA (FMO activity) were measured from the 1 hr urine collected at 5 hr after the CA administration as was done to determine the CYP1A2 activity (Butler et al, 1989). After several attempts to optimize the duration required for the abstention of methylxanthine containing foods or drinks, urine collection time as well as the dose of CA to be administered for specific measurement of TB/CA and TP/CA ratios, we arrived to a conclusion that it was not necessary to change either the dose of CA or the collection time and urine accumulation duration already optimized for the determination of CYP1A2 activity in human (Butler et al, 1989; Nakajima et al, 1994).

It is known that FMO is not induced by the well known inducers of CYP like barbiturates and polycyclic hydrocarbons (Masters & Ziegler, 1971; Ziegler 1993). However, the FMO activity in several mammals has been reported to be modulated by developmental and hormonal changes (Osimitz & Kulkarni, 1982; Dixit & Roche, 1984; Williams et al, 1985; Dannan et al, 1986; Tynes & Philpot, 1987). With regard to this, Lee et al (1993; 1995) have shown that levels of rabbit liver FMO1 protein were enhanced during pregnancy and that the levels of FMO1 mRNA could be increased by the administration of steroids, progesterone or dexamethasone. Furthermore, it was demonstrated recently that the level of FMO1 mRNA in rat liver pretreated with 3-methylcholanthrene (MC) was increased by 3.5 fold along with 2.9 fold increase in the FMO activity (thiobenzamide S-oxidation) (Chung et al, 1997). MC is known to be present in cigarette smoke and is also a well known inducer of CYP1A2 and thus, rats pretreated with MC are used widely as an experimental model to determine the effects of smoking cigarettes on drug metabolism in human. The rate of CA metabolism in rats pretreated with MC has been reported to increase (Yabusaki et al, 1984; Komori et al, 1992; Soucek & Gut, 1992). In our hands, the rate of 7-hydroxytestosterone formation from testosterone (CYP1A2 specific) was found to increase by 3.7-fold and the rate of PX formation from CA (major contribution by CYP1A2) was also increased by 3.9-fold in the liver microsomes of MC pretreated rat. Furthermore, the rates of TB and TP production (major contribution by FMO) in these microsomes were increased also by 6.8- and 2.6-folds, respectively (Chung et al, 1998).

Although the production of both TB and TP has been demonstrated to be catalyzed by FMO (Chung & Cha, 1997) and the FMO activity has been shown to increase by the MC pretreatment in rats (Chung et al, 1997), for the calculation of the FMO activity in human, we have selected to use only the urinary molar concentration ratio of TB/CA instead of the (TB+TP)/CA for the following reasons. Firstly, TB and TP are produced from CA by undergoing N-demethylation at different sites and thus, different FMO isozymes could have been involved. Secondly, unlike that of rat urine, the molar concentration of TB in human urine is much higher than that of TP by more than 5 fold (data not shown). Thirdly, the rate of TB production by human liver microsomes is inhibited more extensively by methimazole, a well known competitive inhibitor of FMO activity, than

that of TP (Chung & Cha, 1997). These have provided the basis for selecting the urinary TB/CA ratio to estimate the FMO activity in human.

Cigarette smoke is known to contain many polyaromatic hydrocarbons and heterocyclic aromatic amines including MC and arylamines which are, if not already present in the fermented tobacco leaves, generated as the leaves are burned at high temperature. As mentioned above, the polyaromatic hydrocarbons and heterocyclic arylamines contained in cigarette smoke, as represented by MC, have been well known to enhance the rate of PX production from the administered CA and to enhance the CYP1-A2 activity in human (Kadlubar et al, 1990; Nakajima et al, 1994). Also, as was indicated by the results of *in vitro* CA metabolism using human liver microsomes (Chung & Cha, 1997), CYP (presumably CYP1A2) contributes approximately 30% of TB production from CA. Thus, in an effort to determine whether the increased TB production and increased urinary TB/CA ratio observed in smokers (Table 1) are caused at least in part by the induction of CYP1A2, Pearson's correlation coefficient test is conducted by comparing the TB/CA (FMO activity) and (PX+17U)/CA (CYP1A2 activity) ratios obtained from all 179 volunteers. As the result shown in Fig. 2, the obtained r^2 value is 0.28 ($p<0.0001$). Thus, there is a weak but significant correlation, and the increased TB/CA ratio observed in smokers (Table 1) may have been caused by the induction of CYP1A2 activity in smokers. This, in turn, may have been responsible for the right shift in frequency distribution plot for the smokers (Fig. 1b). However, the specific proportion contributed by the induced CYP1A2 activity with smoking on the increased TB/CA ratio (FMO activity) still remains elusive.

In conclusion, the FMO activity measured by taking the urinary TB/CA ratio in Korean volunteers showed marked individual variations without significant gender differences, and the increased TB/CA ratio observed in cigarette smokers may have been caused by the increased CYP1A2 activity.

ACKNOWLEDGEMENTS

We thank Mr. Sung-Wan Ahn for his superb technical assistance. This study was supported by the Research Fund from Korean Ministry of Education (1997).

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