Caffeine as a metabolic probe: Exploration of the enzyme-inducing effect of cigarette smoking

It has been realized recently that the primary metabolism of caffeine in humans is catalyzed by P-450IA2 and that the rate of caffeine metabolism can be estimated from a metabolic ratio in a single urine sample. A population of 178 students including 19 smokers were subjected to this caffeine test to establish their P-450IA2 index. Both stated numbers of cigarettes smoked per day and urinary cotinine levels as a confirmatory measure correlated significantly with enzyme activity showing dose-effect relationships (r = 0.62 and 0.89, respectively). Nevertheless, more nonsmokers than smokers had the highest enzyme indexes, suggesting that dietary elements or other factors may determine P-450IA2 activities in populations. Because P-450IA2 is a monooxygenase that may be confined to the liver, caffeine reveals directly the Ah-receptor–dependent enzyme induction only in the liver, but it may also be a signal of induction elsewhere. (CLIN PHARMACOL THER 1991;49:44-8.)

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The past few years have brought remarkable progress in the understanding of the cytochromes P-450 as a genetic superfamily, many of its individual components, and their regulation.[1] The particular cytochrome targeted in this report is P450IA2.[2] It is also known as phenacetin-*O*-deethylase, cytochrome $P\text{-}450_{PA}$, P_3-450 in humans and mice, P-450d in rats, and form 4 in rabbits. It belongs to the IA family, which consists of the cytochromes P-450IA1 and P-450IA2.

Both IA1 and IA2 are inducible by polycyclic aromatic hydrocarbons (methylcholanthrene-type inducers) as in cigarette smoke and by chlorinated agents, of which 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most potent.[3] The two cytochromes differ[1,3] in that IA1 is virtually absent unless induced, whereas IA2 is normally present but substantially augmented by these inducers. For unknown reasons, induction of IA2 often takes less inducer than does induction of IA1. The two cytochromes differ in their tissue distribution: IA1 is widely distributed when induced, whereas IA2 locations are restricted; human liver seems to contain normally only IA2, whereas human placenta contains only IA1. A prototype substrate of IA1 (the aryl hydrocarbon hydroxylase) is benzo[*a*]pyrene; IA2 catalyzes the *N*-oxidation of numerous arylamines.[4] Thus both cytochromes convert precarcinogens into carcinogens.

Because the known substrates of P-450IA1 are toxic chemicals or carcinogens that cannot be given deliberately to human subjects, there are no systematic data on the population distribution of this enzyme's activity. However, functional in vivo studies that are impossible for IA1 can be performed for IA2. It was recently demonstrated that caffeine is demethylated in humans by cytochrome P-450IA2.[5] Tests, designed on the basis of enzyme kinetics,[6] have shown empirically in volunteer subjects that the proportion of certain caffeine metabolites in urine indicates the rate of caffeine metabolism.[7] In short, urinalysis after a cup of coffee can be used to derive the activity of cytochrome P-450IA2 in human subjects; some precautions are necessary because enzyme inhibition[8] and liver disease[9] can reduce enzyme activity.

The purposes of this article were, first, to establish the variation of cytochrome P-450IA2 activities as determined by means of caffeine in a population of healthy volunteers and, second, to see how the variation in smokers compared with that of the nonsmoking majority of this population.

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METHODS

The biochemical basis of the test. Caffeine is 1,3,7-trimethylxanthine. Its main and primary metabolite in humans[10] is paraxanthine; this is formed by 3-demethylation by P-450IA2.[5] Paraxanthine is further metabolized in two parallel reactions that are essentially independent of each other[6,10]: One reaction is the formation of 8-hydroxyparaxanthine, which involves an unidentified, apparently noninducible cytochrome P-450. The other reaction is the 7-demethylation of paraxanthine, which leads to three products, namely 1-methylxanthine, 1-methylurate, and 5-acetylamino-6-formylamino-3-methyluracil (AFMU); the latter is a labile acetylated uracil that, by deformation, decomposes to, or is converted to, the stable and reliably measurable 5-acetyl-6-amino-3-methyluracil (AAMU). All four metabolites originating from paraxanthine appear in urine about as fast as they are formed because of active renal tubular secretion. The sum of the urinary concentrations of the three end products of the paraxanthine demethylation, divided by the concentration of 8-hydroxyparaxanthine, yields a caffeine metabolic ratio (CMR = [AAMU + 1-methylxanthine + 1-methylurate]/8-hydroxyparaxanthine) that correlates with the directly measured caffeine demethylation rate, with $r = 0.91$.[7] The measurements of CMR will be referred to as the P-450IA2 index. The merit of the CMR is that a single urine specimen after a commonly used beverage leads to the estimation of an enzymatic reaction rate, a distinct advantage for population studies.[11]

It deserves to be emphasized that caffeine is not inducing its own metabolism[12]; an earlier specific investigation has shown that neither single metabolites nor groups or ratios of metabolites showed consistent, significant correlations with habitual coffee consumption.[13] However, this observation cannot be expected to hold at very high levels of caffeine intake because caffeine demethylation is saturable.[14]

Subjects of study. The study was conducted with healthy volunteers as approved by the Human Experimentation Committee of the University. The subjects responded to advertising posters displayed in the Medical Sciences Building of the University of Toronto and gave informed consent. They were asked to refrain from chocolate, alcohol, and drug consumption for 1 day before the test, to consume on the test day between one and four cups of coffee at noon or afternoon, and to bring their first-void (morning) urine of the following day to the laboratory. The only exclusion criterion was any chronic medication. Most of the volunteers were students, with men and women in comparable numbers. The volunteers were asked about their habitual caffeine intake and their smoking habits; 86% of the subjects consumed daily coffee, tea, or soft drinks at at a rate averaging two drinks a day; 19 stated that they were smokers, with consumption averaging 9.3 ± 1.7 (SE) cigarettes per day, with a range from 1 to 24.

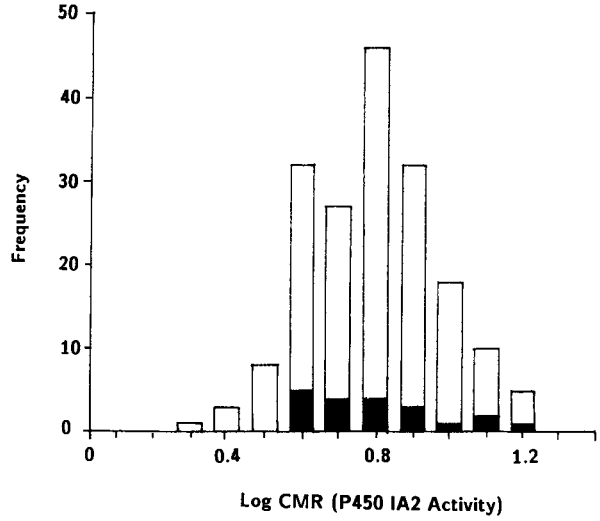

Fig. 1. Frequency distribution of the caffeine metabolic ratio indicative of cytochrome P-450IA2 activity in a population of 178 healthy volunteers. The *black bars* indicate the number of subjects who stated that they were cigarette smokers.

Laboratory procedures. The HPLC methods for measuring urates and 1-methylxanthine were as described by Campbell et al.[7] The measurements of AAMU were performed according to the method of Tang et al.[15] Cotinine was determined by the same procedure with the TSK-20 column without any modification.[16] Under these conditions, the retention times were 5.3, 11.6, and 22.0 minutes for cotinine, AAMU, and benzoxyurea (internal standard), respectively. An enumeration of potential pitfalls in measuring caffeine metabolites is contained in a parallel publication.[16]

RESULTS

The mean and SE of the CMR–P-450IA2 index was 5.96 ± 0.18. Fig. 1 shows the frequency distribution in the 178 volunteers; the scale of the abscissa is logarithmic. There appears to be a log-normal distribution of the P-450IA2 index over an almost tenfold range of values.

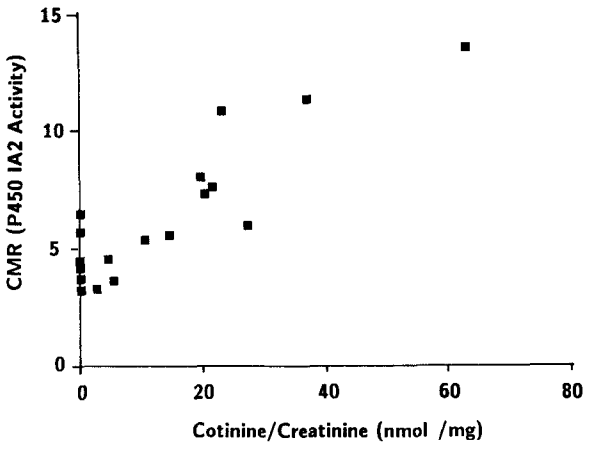

Fig. 2. Correlation between the cytochrome P-450IA2 index and cotinine excretion in urine: $y = 0.153x + 4.26$; $r = 0.89$. Cotinine is a metabolite of nicotine and was measured routinely in all subjects participating in this study; no one claiming to be a nonsmoker had cotinine in their urine. Cotinine data of only those who had stated that they were cigarette smokers are included. The graph illustrates the enzyme-inducing effect of cigarette smoke as a dose-effect relationship. The ordinate intercept can be interpreted as indicating the constitutive level of P-450IA2 in human liver.

The black part of many of the bars (Fig. 1) indicates the number of volunteers who stated that they were cigarette smokers. None of the volunteers who claimed to be nonsmokers had cotinine in their urine. The personal statements of cigarette consumption, the cotinine measurements in urine, and the CMR index of P-450IA2 activity have significant correlation coefficients with each other (Table I). One volunteer claimed to smoke 15 cigarettes a day, but his urine did not contain detectable levels of cotinine; this discrepancy did not undermine the significance of the correlations.

Fig. 2 illustrates the relationship between P-450IA2 activity as measured by CMR and cotinine excretion by the same subjects. It appears that the induction is dose dependent. Furthermore, cotinine excretion and enzyme index have an ordinate intercept at 4.26, which indicates that there is a level of IA2 activity that is independent of smoking. If the enzyme index is plotted against the number of cigarettes smoked, the ordinate intercept is 4.00. The values 4.26 and 4.00 must be indicative of the average constitutive level of enzyme activity; these values are only slightly below the average activity index in the nonsmoking population, which is 5.94 ± 0.19 (SE).

Table I. Correlation coefficients between stated daily cigarette consumption, cotinine excretion in urine, and the caffeine metabolic ratio indicative of the P-450IA2 index ($n = 19$)

	*Correlation matrix**	
	No. of cigarettes	*Cotinine excretion (μmol/mg creatinine)*
Cotinine excretion	0.76	1.00
P-450IA2 index	0.62	0.89

*$p < 0.01$ for each of the correlation coefficients.

DISCUSSION

The log-normal population distribution of the enzyme activity suggests multifactorial variation; without further specific tests, it is impossible to tell whether, or to what extent, the causative factors are environmental or genetic. The magnitude of the interindividual variation exceeds substantially the intraindividual variation, which was found to have a coefficient of variation of 16% (Kalow W, Tang BK. Unpublished data, July 1990.).

Because cytochrome P-4501A2 is identical with phenacetin-*O*-deethylase,[5,17] it is appropriate to relate the observations made with caffeine with older data on phenacetin (i.e., data collected when phenacetin was a widely used drug).

An apparent absence of this enzyme was observed by Shahidi[18] in 1968 in two sisters. Thus genetic deficiency of this enzyme may occur but obviously too rarely to show up in a population of the size tested here.

The classic studies of Pantuck et al.[19] have shown that the phenacetin plasma levels are reduced drastically by smoking and also by certain foods, including charcoal-broiled beef,[20] suggesting an enhanced first-pass metabolism of phenacetin. In these studies with phenacetin, smokers and nonsmokers were compared whereby smokers were defined as persons smoking 15 or more cigarettes per day; in this study everyone who stated he or she smoked regularly was classified as a smoker. The smokers presented a range of smoke exposures, as illustrated in Fig. 2. The correlations illustrated in Table I and Fig. 2 strongly suggest a dose-response relationship between smoke exposure and enzyme induction. The fact that caffeine clearance is accelarated in smokers is not a new observation.[13]

Human variability in response to methylcholanthrene-type inducers has been observed repeatedly,[21,22] a variability that might obscure any dose-response relationship. Perhaps for that reason there does not seem

to be any previous evidence for a relationship between dose of this type of inducer and response in humans. Because cotinine formation from nicotine shows person-to-person variability,[23] the display of the dose-response relationship shown in Fig. 2 could be fallacious. However, this seems unlikely because each individual's statement of smoke exposure also correlates with the P-4501A2 index. Furthermore, although there is evidence for interindividual differences in inducibility from studies with human lymphocytes[21,22] and observations with placenta,[24] years of search have not produced evidence of human variability of the TCDD receptor,[3] although the Ah-receptor differences between strains of mice[3] have been a stimulus for such searches.

Smokers show a dramatic increase of benzo[*a*]pyrene hydroxylation by human placenta.[24] This hydroxylation is indicative of P-4501A1 activity[1,3] and it has its earmarks: The activity is nil or almost nil in nonsmokers but can rise to very high levels in smokers. Caffeine is not metabolized by human placenta in vitro, an observation consistent with it being demethylated by P-4501A2.[6]

Cytochrome P-4501A2 appears always to be present in human liver[1] (i.e., it is a constitutive enzyme, albeit an enzyme whose concentration can be enhanced by TCDD-type inducers).[3] We have therefore interpreted the ordinate intercept in Fig. 2 as indicative of the average level of constitutive P-450IA2. By plotting the number of cigarettes smoked per day against the P-450IA2 index, virtually the same ordinate intercept is obtained; this may be taken as support for our interpretation of the intercept in Fig. 2 as being indicative of the constitutive level of enzyme activity. It makes sense to find that the apparent constitutive level is similar to, or perhaps slightly lower than, the average enzyme index in the nonsmoking population.

What caffeine reveals is a smoking-caused enzyme induction in human liver; it cannot directly reveal enzyme induction in placenta and other tissues containing only P-450IA1. However, both P-450IA1 and IA2 are induced by the same inducers acting on the Ah receptor.[3] Thus it could be that a relatively minor increase of caffeine metabolism signals a proportionally much larger increase of P-450IA1 activity. Caution is necessary: There could be additional tissue specificity of induction that prevents a concurrent response of both enzymes, and there is some evidence of preferential induction within the P-450IA family; methylcholanthrene preferentially induces IA1, whereas isosafrol preferentially induces IA2.[17]

The lessons of Figs. 1 and 2 taken together demonstrate that, although smoking clearly enhances enzyme activity, it is still only one and perhaps only a minor determinant of the overall variation of enzyme activity. For instance, of the five subjects with the highest enzyme activity, only one is a smoker, albeit one with a consumption of 24 cigarettes a day. Some other factor that is more common than cigarette smoke must be at least as powerful a determinant of enzyme activity as is smoking; charcoal-broiled beef is another such powerful inducer,[18] probably like other products of burning or of food and drink. Under such conditions, the extent of variation of the constitutive enzyme level is difficult to determine, but it would be wrong to dismiss out of hand inborn variations of P-450IA2 activity.

Enzyme induction is one of the biologic effects of cigarette smoke that are measurable with relative ease and that may be precursory to cancer formation[3] or other toxic effects.[25] It is important to note that even complete elimination of smoking would leave cases of high enzyme activity.

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References

1. Gonzalez FJ. Molecular genetics of the P-450 superfamily. Pharmacol Ther 1990;45:1-38.
2. Ikeya K, Jaiswal AK, Owens RA, Jones JE, Nebert DW, Kimura S. Human CYP1A2: sequence, gene structure, comparison with the mouse and rat homologous gene, and differences in liver mRNA expression. Mol Endocrinol 1989;3:1399-408.
3. Okey AB. Enzyme induction in the cytochrome P-450 system. Pharmacol Ther 1990;45:241-98.
4. Shimada T, Iwasaki M, Martin MW, Guengerich FP. Human liver microsomal cytochrome P-450 enzymes involved in the bioactivation of procarcinogens detected by *umu* gene response in *Salmonella typhimurium* TA 1535/pSK1002[1]. Cancer Res 1989;49:3218-28.
5. Butler MA, Iwasaki M, Guengerich FP, Kadlubar FF. Human cytochrome $P\text{-}450_{PA}$ (P-450IA2), the phenacetin-*O*-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and *N*-oxidation of carcinogenic arylamines. Proc Natl Acad Sci USA 1989;86:7696-700.
6. Kalow W, Campbell M. Biotransformation of caffeine by microsomes. ISI Atlas of Science. 1988:890-983.
7. Campbell ME, Spielberg SP, Kalow W. A urinary me-

tabolite ratio that reflects systemic caffeine clearance. CLIN PHARMACOL THER 1987;42:157-65.
8. Tarrus E, Cami J, Roberts DJ, Spickett RGW, Celdran E, Segura J. Accumulation of caffeine in healthy volunteers treated with furafylline. Br J Clin Pharmacol 1987;23:9-18.
9. Varagnolo M, Plebani M, Mussap M, Nemetz L, Paleari CD, Burlina A. Caffeine as indicator of metabolic functions of microsomal liver enzymes. Clin Chim Acta 1989;183:91-4.
10. Grant DM, Tang BK, Kalow W. Variability in caffeine metabolism. CLIN PHARMACOL THER 1983;33:591-602.
11. Kalow W, Tang BK. Caffeine as a metabolic probe: exploration of P450IA2 and xanthine oxidase activities [In press].
12. Arnaud MJ. The pharmacology of caffeine. Prog Drug Res 1937;31:273-313.
13. Kalow W. Variability of caffeine metabolism in humans. Arzneimittelforschung 1985;35:319-24.
14. Cheng WSC, Murphy TL, Smith MT, Cooksley WGE, Halliday JW, Powell LW. Dose-dependent pharmacokinetics of caffeine in humans: relevance as a test of quantitative liver function. CLIN PHARMACOL THER 1990;47:516-24.
15. Tang BK, Kadar D, Kalow W. An alternative test for acetylator phenotyping with caffeine. CLIN PHARMACOL THER 1987;42:509-13.
16. Tang BK, Kadar D, Qian L, Iriah J, Yip J, Kalow W. Caffeine as a metabolic probe: validation of its use for acetylator phenotyping [In press].
17. Sesardic D, Edwards RJ, Davies DS, Thomas PE, Levin W, Boobis AR. High-affinity phenacetin *O*-deethylase is catalysed specifically by cytochrome P-450d (P-4501A2) in the liver of the rat. Biochem Pharmacol 1990;39:489-98.
18. Shahidi NT. Acetophenetidin-induced methemoglobinemia. Ann NY Acad Sci 1968;151:822-32.
19. Pantuck EJ, Hsiao KC, Maggio A, Nakamura K, Kuntzman R, Conney AH. Effect of cigarette smoking on phenacetin metabolism. CLIN PHARMACOL THER 1974;15:9-17.
20. Conney AH, Pantuck EJ, Hsiao K-C, et al. Enhanced phenacetin metabolism in human subjects fed charcoal-broiled beef. CLIN PHARMACOL THER 1976;20:633-42.
21. Kellermann G, Luyten-Kellermann M, Horning MG, Stafford M. Correlation of aryl hydrocarbon hydroxylase activity of human lymphocyte cultures and plasma elimination rates for antipyrine and phenylbutazone. Drug Metab Dispos 1975;3:47-50.
22. Kouri RE, McLemore T, Jaiswal AK, Nebert DW. Current cellular assays for measuring clinical drug metabolizing capacity: impact of new molecular biologic techniques. In: Kalow W, Goedde HW, Agarwal DP, eds. Ethnic differences in reactions to drugs and xenobiotics. New York: Alan R. Liss, 1986:453-69.
23. Haley NJ, Sepkovic DW, Hoffman D. Elimination of cotinine from body fluids: disposition in smokers and nonsmokers. Am J Public Health 1989;79:1046-8.
24. Welch RM, Harrison YE, Gommi BW, Poppers PJ, Finster M, Conney AH. Stimulatory effect of cigarette smoking on the hydroxylation of 3,4-benzpyrene and the *N*-demethylation of 3-methyl-4-monomethylaminoazobenzene by enzymes in human placenta. CLIN PHARMACOL THER 1969;10:100-9.
25. Raucy JL, Lasker JM, Lieber CS, Black M. Acetaminophen activation by human liver cytochromes P-450IIE1 and P-450IA2. Arch Biochem Biophys 1989;271:270-83.