Effect of cytochrome P450 (CYP) inducers on caffeine metabolism in the rat

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Abstract:
Our previous studies, carried out using rat cDNA-expressed cytochrome P450 (CYP) isoforms, liver microsomes and specific CYP inhibitors, showed that the 1-N- and 3-N-demethylation of caffeine at a therapeutic concentration was predominantly catalyzed by CYP1A2 and CYP2C, its 7-N-demethylation was governed by P450s of the CYP2C subfamily, while its 8-hydroxylation was specifically mediated by CYP1A2. The present study was aimed at corroborating the above-described results using another experimental model, i.e. a study of caffeine metabolism in the liver microsomes and specific CYP inducers. Animals received one of the following inducers: β-naphthoflavone (100 mg/kg ip for 4 days), phenobarbital (10 mg/kg for 6 days or 100 mg/kg ip for 4 days), pregnenolone 16α-carbonitrile (100 mg/kg ip for 4 days) or 15% ethanol (± 11 g/kg in drinking water for 6 days). Sixteen hours after the last dose of an inducer liver microsomes were prepared and the caffeine metabolism and CYP isoform activities (testosterone 2β-, 2α-, 6β-, 7α-, 16β-hydroxylation and warfarin 7-hydroxylation) were investigated. β-Naphthoflavone (mainly a CYP1A inducer and CYP2C11 inhibitor) potently accelerated the metabolism of caffeine, the effect on 7-N-demethylation being the weakest. Moreover, the influence of β-naphthoflavone on caffeine metabolism was more potent at the substrate concentration of 100 μM than 800 μM, in particular in the case of 7-N-demethylation and 8-hydroxylation. Pregnenolone-16α-carbonitrile (mainly a CYP3A inducer and CYP2C11 inhibitor) moderately induced 8-hydroxylation only. Phenobarbital (an inducer of CYP2B and other CYPs and a CYP2C11 inhibitor) moderately stimulated the metabolism of caffeine, but practically did not affect 7-N-demethylation. Ethanol (mainly a CYP2E1 inducer) modestly increased the rates of the N-demethylation reactions. The presently obtained data confirm the pivotal role of CYP1A2 in the metabolism of caffeine, as well as the involvement of CYP3A in the 8-hydroxylation of caffeine and that of CYP2C11 in its 7-N-demethylation.

Key words: rat, liver microsomes, cytochrome P450 induction, β-naphthoflavone, phenobarbital, pregnenolone 16α-carbonitrile, ethanol, testosterone hydroxylation, warfarin 7-hydroxylation, caffeine metabolism

Introduction

Caffeine (1,3,7-trimethylxanthine), a purine alkaloid is oxidized in a few positions of its structure. The compound undergoes 1-N-demethylation to theobromine, 3-N-demethylation to paraxanthine, 7-N-demethylation to theophylline and 8-hydroxylation to 1,3,7-trimethyluric acid (Fig. 1). While in man 3-N-demethylation is quantitatively the main oxidation pathway, in the rat 8-hydroxylation seems to be a dominant metabolic reaction [4, 6, 20]. 3-N-demethylation to paraxanthine is regarded as a reaction specifically catalyzed by CYP1A2 [2, 4, 6, 10, 24, 27]. Therefore, caffeine is often used for phenotyping of human cytochrome P450, being applied as the selecti-
ve CYP-specific method or in the “cocktail approach” [5, 12, 26]. On the other hand, the CYP3A subfamily seems to contain the main isoenzyme catalyzing the 8-hydroxylation of caffeine to 1,3,7-trimethyluric acid [20, 27], though other data also suggest the contribution of CYP2B1 and CYP2E1 to the catalyzing of this reaction in the rat [6]. It has also been suggested that 1-N-demethylation and mainly 7-N-demethylation, are likely to engage CYP2B isoforms in rats [3], or CYP2E1 isoform in humans [27]. However, the most of the studies on caffeine metabolism carried out so far have been conducted at high (10 mM) caffeine concentrations [6, 11, 20], often using unspecific P450 inducers or inhibitors [6] or not including all CYP isoforms [11, 20].

Our earlier results on the influence of psychotropic drugs on the rate of caffeine metabolism showed intra- and inter-drug differences in the inhibitory effects on the four oxidation pathways of caffeine in rat liver microsomes [8, 9]. The observed reaction-dependent effects of psychotropics on caffeine metabolism provided an indirect evidence that CYP1A2 was not the only isoenzyme important for catalyzing the biotransformation of caffeine, which incited further pharmacological investigations. Our recent results obtained using rat cDNA-expressed P450s and liver microsomes (correlation and inhibition studies) indicate that the main oxidation pathway of caffeine in the rat is 8-hydroxylation, which is specifically catalyzed by CYP1A2 at caffeine therapeutic (up to 100 μM) concentration, and may be used as a marker reaction for this enzyme in the above-mentioned species [16]. 1-N- and 3-N-demethylations are also preferentially catalyzed by CYP1A2, but to a lesser degree than 8-hydroxylation, while 7-N-demethylation is governed by isoforms of the subfamily CYP2C, mainly CYP2C6 and CYP2C11. At higher substrate concentrations, the contribution of CYP1A2 to the metabolism of caffeine decreases in favor of the other P450s: CYP2C11 (N-demethylation reactions) and CYP3A2 (mainly 8-hydroxylation).

In the present study, we attempted to support the results of our recent in vitro study, using another experimental model, i.e. a study of caffeine metabolism in the liver microsomes of rats treated with some selected cytochrome P450 inducers at a lower (100 μM) and a higher (800 μM) concentration of the substrate.
Materials and Methods

Drugs and Chemicals

Caffeine and its metabolites – theobromine, paraxanthine, theophylline and 1,3,7-trimethyluric acid, as well as β-naphthoflavone, pregnenolone 16α-carbonitrite, NADP, DL-isocitric acid (trisodium salt), isocitric dehydrogenase and corn oil were purchased from Sigma (St. Louis, USA). Phenobarbital (Luminalum) was provided by Pliva (Kraków, Poland), ethanol was obtained from Polmos (Kraków, Poland). All organic solvents with HPLC purity were supplied by Merck (Darmstadt, Germany).

Animals

All the experiments with animals were performed in accordance with the Polish governmental regulations (Decree on Animal Protection DZ.U. 97.111.724, 1997). The experiments were carried out on male Wistar rats (230–260 g) kept under standard laboratory conditions. Animals received one of the following inducers: β-naphthoflavone (100 mg/kg ip for 4 days), phenobarbital (PB; 10 mg/kg for 6 days or 100 mg/kg ip for 4 days), pregnenolone 16α-carbonitride (100 mg/kg ip for 4 days) or 15% ethanol (≈ 11 g/kg in drinking water for 6 days) [1, 17, 19, 21]. The control animals received an appropriate volume of solvent, i.e. water (experiment with phenobarbital and ethanol) or corn oil (experiment with β-naphthoflavone and pregnenolone 16α-carbonitride). Sixteen hours after the last dose of an inducer rats were decapitated and their livers were immediately removed, weighed, and placed into ice-cold 20 mM Tris/KCl buffer (pH = 7.4). Liver microsomes were prepared by differential centrifugation in 20 mM Tris/KCl buffer (pH = 7.4), including washing with 0.15 M KCl, according to a conventional method. The metabolism of caffeine and CYP isoform activities were studied in the liver microsomes of control and inducer-treated rats as described below.

Caffeine metabolism in liver microsomes

Studies into caffeine metabolism in liver microsomes were carried out at the linear dependence of product formation on time, protein and substrate concentration. Incubation mixture contained the liver microsomes (ca. 1 mg of protein/ml), a phosphate buffer (0.15 M, pH = 7.4), MgCl₂ × 6H₂O (6 mM), and NADPH generating system: NADP (1.2 mM), DL-isocitric acid (6 mM) and isocitric dehydrogenase (1.2 U/ml). The final incubation volume was 1 ml. The reaction was initiated by adding caffeine (100 or 800 μM). After a 50-min incubation, the reaction was terminated by adding 700 μl of a 2% ZnSO₄ and 50 μl of 2 M HCl. Caffeine and its metabolites were analyzed by a high-performance liquid chromatography method (HPLC) described below.

Determination of P450 isoform activities

The activity of CYP1A2 was studied by measuring the rate of caffeine 8-hydroxylation at the substrate concentration of 100 μM as described above. The activity of CYP2C6 was studied by measuring the rate of warfarin 7-hydroxylation at the substrate concentration of 60 μM as described previously [7]. Incubations were carried out in a system containing liver microsomes (1 mg of protein in 1 ml), Tris/KCl buffer (0.1 M, pH = 7.4), MgCl₂ (2.5 mM), NADP (0.33 mM), glucose 6-phosphate (7.85 mM) and glucose-6-phosphate-dehydrogenase (5.6 U in 1 ml). The final incubation volume was 0.5 ml. After a 15-min incubation at 37°C, the reaction was stopped by adding 10 μl of 70% perchloric acid and then by cooling it down in ice.

The activities of CYP2A, CYP2B, CYP2C11 and CYP3A2 were studied by measuring the rate of P450-specific reactions: the 7α-, 16β-, 2α-, 2β- and 6β-hydroxylation of testosterone (respectively) at the substrate concentration of 100 μM as described previously [13]. Incubations were carried out in a system containing liver microsomes (1 mg of protein in 1 ml), Tris/KCl buffer (50 mM, pH = 7.4), MgCl₂ (3.0 mM), EDTA (1 mM), NADP (1.0 mM), glucose 6-phosphate (5 mM) and glucose-6-phosphate-dehydrogenase (1.7 U in 1 ml). The final incubation volume was 1 ml. After a 15-min incubation, the reaction was stopped by adding 200 μl of methanol and then by cooling it down in ice.

Determination of caffeine and its metabolites

Caffeine and its four primary metabolites were assessed using the HPLC method based on Rasmussen.
et al. [23] as previously described [8]. Briefly, after incubation, samples were centrifuged and the water phase containing caffeine and its metabolites was extracted with 6 ml of an organic solvent mixture consisting of ethyl acetate and 2-propanol (8:1, v/v). The residue obtained after evaporation of the microsomal extract was dissolved in 100 μl of the mobile phase described below. An aliquot of 20 μl was injected into the HPLC system. The Merck-Hitachi chromatograph, “LaChrom” (Darmstadt, Germany), equipped with a L-7100 pump, an UV detector and a D-7000 System Manager was used. The analytical column (Supelcosil LC-18, 15 cm × 4.6 mm, 5μm) was from Supelco (Bellefonte, USA). The mobile phase consisted of 0.01 M acetate buffer (pH = 3.5) and methanol (91:9, v/v). The flow rate was 1 ml/min (0–26.5 min), followed by 3 ml/min (26.6–35 min). The column temperature was maintained at 30°C. The absorbance of caffeine and its metabolites was measured at a wavelength of 254 nm. The compounds were eluted in the following order: theobromine (9.7 min), paraxanthine (15.8 min), theophylline (16.9 min), 1,3,7-trimethyluric acid (23.4 min), caffeine (30.5 min). The sensitivity of the method allowed for quantification of theobromine content of 0.001 nmol, paraxanthine 0.004 nmol, theophylline 0.005 nmol, 1,3,7-trimethyluric acid 0.01 nmol and caffeine 0.005 nmol in one sample. The accuracy of the method was 1.2–2.3%. The intra- and inter-assay coefficients of variance were about 4% and 6%, respectively for all metabolites.

Determination of the concentration of testosterone and its metabolites in liver microsomes

Testosterone and its metabolites were determined as described previously [13]. Briefly, they were extracted from the microsomal suspension with dichloromethane and assessed by the HPLC method based on Sonderfan et al. [25]. The residue obtained after evaporation of the extracts was dissolved in 100 μl of 50% methanol. An aliquot (20 μl) was injected into the HPLC system (LaChrom, Merck-Hitachi), equipped with UV detector, L-7100 pump and D-7000 System Manager. The analytical column (Supelcosil™ LC-18, 5 μM, 4.6 × 150 mm) was purchased from Supelco (Bellefonte PA, USA). The mobile phase consisted of 0.25% H₃PO₄ (pH = 2.2) and acetonitrile in proportion 62:38 (v/v). The flow rate was 2.0 ml/min. The column temperature was 25°C. The fluorescence was measured at a wavelength of 320 nm (excitation) and 415 nm (emission). The compounds were eluted in the following order: 7-hydroxywarfarin 4.6 min and warfarin 9.6 min. The sensitivity of the method allowed for quantification of 7-hydroxywarfarin content of 0.001 nmol in one sample. The accuracy of the method amounted to 2%. The intra- and inter-assay coefficients of variance were below 5% and 7%, respectively.

Calculations and statistics

Statistical significance was assessed using an analysis of variance followed by Duncan’s test or Student’s t-test.
Results

The effect of cytochrome P450 inducers on the activities of CYP2A, CYP2B, CYP2C and CYP3A

Pregnenolone-16α-carbonitrile elevated the activity of CYP3A measured as the rate of 2β- and 6β-hydroxylation of testosterone, diminishing at the same time the activity of CYP2C11 (Fig. 2). Phenobarbital potently stimulated the activity of CYP2B both at a dose of 10 and 100 mg/kg (to about 2600 and 4000% of the control value, respectively), raising also the activity of CYP3A (to 300–500% of the control value), and CYP2A (to nearly 300% of the control value after a higher dose) and CYP2C6 (to 150% of the control value after a lower dose) (Fig. 2, 3). At the same time, phenobarbital markedly decreased the activity of CYP2C11, especially after a higher dose (to 5% of the control value). Ethanol only slightly diminished the activity of CYP2A (Fig. 2).

β-Naphthoflavone potently increased the activity of CYP1A2 measured as the rate of caffeine 8-hydroxylation at the substrate concentration of 100 μM (to about 1800% of the control value) (Fig. 4) and moderately stimulated the CYP2A activity, measured as the rate of testosterone 7α-hydroxylation (to 240% of the control value) (Fig. 2). At the same time, β-naphthoflavone decreased the activity of CYP2C11 (the rate of 2β-hydroxylation), CYP2B (the rate of 16β-hydroxylation) (Fig. 2) and CYP2C6 (the rate of 7-hydroxylation of warfarin) (Fig. 3).

The effect of cytochrome P450-inducers on caffeine metabolism

β-Naphthoflavone (mainly a CYP1A inducer and CYP2C11 inhibitor) potently accelerated all the reactions tested, the effect on 7-N-demethylation being
the weakest (Fig. 4). Moreover, the influence of β-naphthoflavone on caffeine metabolism was more potent at the substrate concentration of 100 μM than 800 μM, in particular in the case of 7-N-demethylation and 8-hydroxylation.

Pregnenolone-16α-carbonitrile (mainly a CYP3A inducer and CYP2C11 inhibitor) moderately induced 8-hydroxylation only (Fig. 5).
Phenobarbital (an inducer of CYP2B and other CYPs and a CYP2C11 inhibitor) moderately stimulated the oxidation pathways of caffeine both at a dose of 10 mg/kg (a small decrease in CYP2C11 activity) and 100 mg/kg (a great decrease in CYP2C11 activity) (Fig. 6A, B). An exception was 7-N-demethylation, which was gently stimulated only after a phenobarbital dose of 10 mg/kg and at caffeine concentration of 100 μM. Moreover, the rate of 1-N-demethylation increased only at a caffeine concentration of 800 μM.

Ethanol (mainly a CYP2E1 inducer) modestly increased the rate of the studied reactions, except for 8-hydroxylation (Fig. 7). As previously observed for phenobarbital, 1-N-demethylation was stimulated only at a caffeine concentration of 800 μM.

Discussion

Our previous studies, carried out using rat cDNA-expressed CYP isoforms, liver microsomes and specific CYP inhibitors, showed that the 1-N- and 3-N-demethylation of caffeine at a therapeutic concentration was predominantly catalyzed by CYP1A2 and CYP2C, its 7-N-demethylation was governed by P450s of the CYP2C subfamily, while its 8-hydroxylation (the main oxidative pathway) was specifically mediated by CYP1A2 [16]. Hence, in the rat caffeine can be applied as a marker substance for assessing the activity of CYP1A2 (using 8-hydroxylation), as well as for a preliminary estimation of CYP2C activity (using 7-N-demethylation). The above-mentioned study also indicated that at higher substrate concentrations, the contribution of CYP1A2 to the metabolism of caffeine decreased in favor of the other P450s: CYP2C11 (N-demethylation reactions) and CYP3A2 (mainly 8-hydroxylation).

Therefore, our present study was aimed at corroborating the above-described results using another experimental model, i.e. a study of caffeine metabolism in the liver microsomes of rats treated with selected cytochrome P450 inducers at a lower (therapeutic; 100 μM) and a higher (800 μM) concentration of the substrate. Moreover, using the same animal liver samples, we measured the direction and degree of changes in CYP isoform activities. The obtained results showed that it was difficult to precisely estimate the qualitative contribution of CYP isoforms to the metabolism of caffeine, since the inducers turned out not to be sufficiently selective, and some of them even displayed a fairly wide spectrum of changes in CYP isoform activities. The latter observation is consistent with the results of other authors indicating non-specificity of some of the CYP inducers used in our study [1, 21]. However, the effect of these inducers on CYP2C6 and that of pregnenolone-16α-carbonitrile on CYP1A and CYP2C11 have not been studied so far. The cause of the above-mentioned non-specificity may lie in partially overlapping regulatory mechanisms of some CYP isoforms (mainly 2B, 3A, 2C), which makes finding a specific inducer difficult or even impossible [14, 22, 28]. Therefore, the use of induced liver microsomes, though theoretically appropriate, may be recommended and regarded only as complementary to other metabolic tests (e.g. cDNA-expressed CYPs, control microsomes + a correlation or an inhibition study); moreover, it only suggests that involvement of an enzyme in the catalysis of the metabolic pathways of caffeine is feasible. It seems, however, that when such a test is applied, identification of a CYP isoform engaged in a specific metabolic reaction of caffeine should be carried out by comparing the percentage of change in the rate of this reaction with that in the rate of CYP isoform marker reactions caused by the inducer under study. It is not only the direction of changes in CYP activities that should be taken into account.
Thus β-naphthoflavone (mainly a CYP1A inducer and a CYP2C11 inhibitor), which potently accelerated all the four oxidation pathways of caffeine (exerting the weakest effect on 7-N-demethylation), provides evidence of the essential contribution of CYP1A2 to caffeine metabolism and indicates a minor role of this isoform in the 7-N-demethylation of the drug. The relatively smaller influence of β-naphthoflavone on caffeine metabolism at the substrate concentration of 800 µM than that at its concentration of 100 µM (in particular in the case of 7-N-demethylation and 8-hydroxylation) corresponds well with the previously observed diminished contribution of CYP1A2 and the increased participation of other CYP isoforms (CYP2C11 or CYP3A2) in caffeine metabolism at a higher substrate concentration.

Pregnenolone-16α-carbonitrile (mainly a CYP3A inducer and a moderate CYP2C11 inhibitor), which induced similarly potently 8-hydroxylation of caffeine as did the CYP3A-governed 2β- and 6β-hydroxylation of testosterone, suggests a role of CYP3A in catalyzing this reaction, especially at a higher concentration of caffeine.

Phenobarbital (an inducer of CYP2B and other CYPs and an inhibitor of CYP2C11), which only moderately stimulated the 1-N- and 3-N-demethylation and 8-hydroxylation of caffeine (compared to strong CYP2B induction) and practically did not affect 7-N-demethylation, indicates lack of CYP2B involvement in caffeine metabolism and suggests CYP2C11 contribution to the 7-N-demethylation of the drug. While the rate of 16β-hydroxylation, a CYP2B-specific reaction, increased 40 times after a higher dose of phenobarbital, the rate of phenobarbital-induced caffeine metabolism was raised 2–4 times only. On the other hand, the observed dramatic decrease in CYP2C11 activity seemed to counteract the stimulating effect of phenobarbital on other CYP isoforms involved in the 7-N-demethylation of caffeine (mainly 1A and 3A), and to prevent the increase in the rate of that reaction.

Finally, our study has shown that ethanol (mainly a CYP2E1 inducer) only modestly raises the rate of caffeine N-demethylation reactions (twice) compared to the strong increase (16 times) in the rate of p-nitrophenol hydroxylation (a CYP2E1 specific reaction), observed by other researchers [15]. The latter findings indicate a non-significant role of this enzyme in the metabolism of caffeine, which is consistent with our previous studies using cDNA-expressed CYPs [16].

Hence, the results presented above confirm the pivotal role of CYP1A2 in the metabolism of caffeine, as well as the involvement of CYP3A in the 8-hydroxylation of caffeine and that of CYP2C11 in its 7-N-demethylation. The obtained results are only partly in line with those of Chung et al. [6] who observed – using less selective CYP inducers and controlling CYP activity – that acetone (which induced 2E1, 3A and inhibited CYP2C11), dexamethasone (which induced 3A and inhibited 2A, 2C11) and phenobarbital (which induced 2B, 3A and inhibited 2C11) increased 8-hydroxylation only, whereas 3-methylcholanthrene (which induced 1A, 2A, 3A and inhibited CYP2C11) increased all the caffeine reactions in liver microsomes at a very high substrate concentration of 10 mM. On the basis of the obtained results, the latter authors proposed that at the increased levels of CYP2B, CYP3A and 2E1, liver microsomes could catalyze 8-hydroxylation at an elevated rate, while microsomes with the enhanced activity of CYP1A2 catalyzed N-demethylation reactions at an increased rate. The discrepancy between our present results and those reported by Chung et al. [6] may arise from the use of less selective inducers by the latter authors (and relating the rates of caffeine reactions to the activity of the main induced enzyme only), the choice of a very high non-therapeutic concentration of caffeine, as well as from disregarding CYP2C11 in data interpretation. As has been mentioned elsewhere, our recent studies show that 8-hydroxylation is mainly catalyzed by CYP1A2 at a therapeutic concentration of caffeine; however, at a higher level of caffeine, CYP3A contribution to 8-hydroxylation markedly increases and may even prevail over that of CYP1A2. At the same time, CYP2C11 involvement in N-demethylation processes is on the rise [16]. It is possible, therefore, that Chung et al. [6] observed a decrease after dexamethasone and no change after acetone or phenobarbital (i.e. after inducers which decrease CYP2C11 activity) in the rates of N-demethylation reactions as a result of a very high (10 mM) caffeine concentration. The only exception was 3-methylcholanthrene, which – apart from decreasing CYP2C11 activity – increased the rate of caffeine N-demethylation, the latter effect being probably caused by the concurrent positive regulation of CYP1A2 contribution to those reactions. However, the authors disregarded the relationship between CYP2C11 activity and caffeine metabolism in the interpretation of their results.
In summary, the results of our present study are in line with our previous data, obtained in other experimental models using recombinant enzymes and specific inhibitors. The presently obtained data confirm the pivotal role of CYP1A2 in the metabolism of caffeine, as well as the involvement of CYP3A in the 8-hydroxylation of caffeine and that of CYP2C11 in its 7-N-demethylation. On the other hand, however, our study gives no support to the contribution of CYP2B and CYP2E1 to caffeine metabolism, as suggested by an analogous experiment conducted by other authors.

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transcriptase-polymerase chain reaction (RT-PCR).


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