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SHORT COMMUNICATIONS

Microsomal nicotine metabolism: A comparison of relative activities of six purified rabbit cytochrome P-450 isozymes

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The *in vitro* microsomal metabolism of nicotine results in the formation of two major metabolites: nicotine *N'*-oxide and nicotine-1',5'-iminium ion which is oxidized to cotinine by a soluble aldehyde oxidase [1]. Previous studies have concluded, on the basis of differential response to inhibitors, that N'-oxidation of nicotine to nicotine *N'*-oxide is catalyzed by the microsomal flavin monooxygenase, while C-oxidation of nicotine is associated with the mixed-function oxygenase system [2, 3]. Murphy [4] first demonstrated that the initial microsomal 5'-metabolite could be trapped as 5'-cyanonicotine by the inclusion of KCN in the reaction mixture. The subsequent studies of Nguyen *et al.* [5] demonstrated that a second cyano compound, *N*-(cyanomethyl)-nornicotine, could be detected. This second cyano adduct is formed by trapping of the methyl iminium ion, thought to be an intermediate in the formation of nornicotine. In addition, this group demonstrated that microsomes isolated from phenobarbital pretreated rabbits made both the 5'-cyano and *N*-(cyanomethyl) adducts of nicotine although similar levels of the cyanomethyl adduct could also be formed nonenzymatically [5]. Our finding that, as the nornicotine contaminant of the substrate nicotine is decreased, the rate of *N*-(cyanomethyl)nornicotine decreases led us to conclude that little if any nicotine is metabolized by this pathway when isolated hamster microsomes are employed [6]. Incubation of post mitochondrial supernatant fractions in the absence of added KCN results in the formation of both nicotine *N'*-oxide and cotinine but not nornicotine [6]. Our previous work demonstrated that the *in vitro* rates of C and N'-oxidation of nicotine can be readily quantitated and that phenobarbital pretreatment of hamsters causes a 2-fold induction of 5'-hydroxylation of nicotine while N'-oxidation remains unchanged [6, 7]. The earlier studies of Nakayama and associates demonstrated that phenobarbital pretreatment of rats [8] and guinea pigs [9] increases microsomal rates of nicotine oxidase activity 62 and 42% respectively. Antibodies against either NADPH–cytochrome P-450 reductase or the phenobarbital inducible form of cytochrome P-450, purified from guinea pig, are only partially effective in inhibiting nicotine oxidase activity [9, 10]. This is not unexpected since the nicotine oxidase assay quantitates substrate disappearance and will therefore be a measure of the sum of all C- and N'-oxidation pathways. Recently, Rudell *et al.* [11] reported an 8-fold induction of cotinine formation in perfused rat liver following phenobarbital pretreatment.

The purpose of this study was to determine the effect of phenobarbital pretreatment on rabbits and the cytochrome P-450 isozyme specificity for nicotine metabolism. Phenobarbital pretreatment resulted in a 2-fold induction of both the 5'-hydroxylation and the N-methyl hydroxylation of nicotine, but a 30% reduction in the rate of N'-oxidation of nicotine. Reconstitution of nicotine metabolism using cytochrome P-450 isozymes purified from rabbit liver demonstrated that the highest rates for both 5'-hydroxylation and methyl hydroxylation of nicotine were associated with a phenobarbital inducible isozyme (form 2) as well as a constitutive isozyme (form 3b). Isozymes 6 and 3a were 25 and 14% as active, whereas isozymes 3c and 4 had less than 10% of the activity of isozyme 3b. In contrast, none of the isozymes tested exhibited nicotine *N'*-oxidase activity.

Materials and methods

Animal treatments, microsomal preparation, and enzyme purification. New Zealand White male rabbits (2.0 to 2.5 kg) with either untreated or given 0.1% (w/v) sodium phenobarbital for 7 days in place of drinking water. Animals were fasted for 12–14 hr prior to being killed by pentobarbital overdose. Hepatic microsomes were isolated as described previously and stored at −70° [12]. The specific contents of the microsomal preparations for total cytochrome P-450 were 1.77 and 2.54 nmol/mg protein for untreated and phenobarbital-pretreated rabbit preparations. The isozymes of cytochrome P-450 were purified to electrophoretic homogeneity by published procedures [13–15] and had specific contents of 15–20 nmol P-450/mg protein. NADPH–cytochrome P-450 reductase was purified according to the method of French and Coon [16] and catalyzed the reduction of 45–60 μmol of cytochrome *c*/min/mg protein. Protein was determined by the method of Lowry *et al.* [17], and cytochrome P-450 was measured as previously described [13].

Isozyme reconstitution. Purified isozymes of cytochrome P-450 were reconstituted by mixing the isozyme of cytochrome P-450 with cytochrome P-450 reductase and letting the concentrated enzyme mixture (usually 10–20 μM) stand on ice for a minimum of 5 min. Sonicated dilauroyl glyceryl 3-phosphorylcholine (1 mg/ml stock solution) was added to the enzyme mixture, and aliquots were added to the reaction tubes so that the final phospholipid concentration was 30 μg/ml and cytochrome P-450 and cytochrome P-450 reductase concentrations were 0.1 and 0.3 μM respectively.

In vitro *nicotine metabolism.* The standard assay for determination of the rates of nicotine N'- and C-oxidation consisted of: 20 μmol nicotinamide, 1 μmol $NADP^+$, 5 μmol glucose-6-phosphate, 4 units glucose-6-phosphate dehydrogenase, 6 μmol $MgCl_2$, 12 or 30 μmol nicotine, 1 μmol potassium cyanide, and 40 μmol Tricine, pH 7.4, in a final volume of 1.0 ml as described previously [6]. Reactions were initiated by the addition of either microsomes (1 mg protein) or reconstituted systems (0.1 nmol cytochrome P-450).

Results

The effects of variations in nicotine concentration on the rates of C- and N'-oxidation by microsomes from control and phenobarbital-pretreated rabbits are presented in Figs. 1 and 2. Phenobarbital pretreatment results in a 2-fold increase in the rates of both 5'-hydroxylation and N-methyl hydroxylation, whereas the rates of N'-oxidation were decreased 30%. Apparent K_m values for 5'-cyano, *N*-cyanomethyl, and *N'*-oxide formation, as determined by Lineweaver–Burk analysis, demonstrated that the apparent K_m values for nicotine were in the range of 5–8 mM for *N'*-oxidase, 5', and *N'*-methyl hydroxylase activities for both control and phenobarbital preparations. However, due to

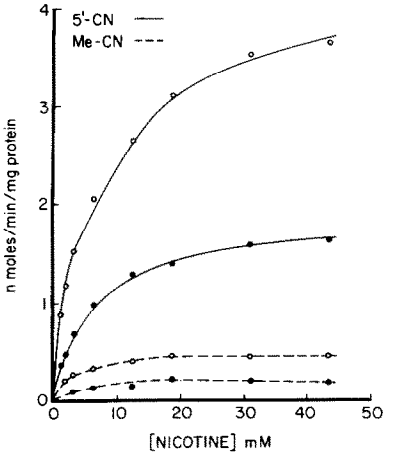

Fig. 1. Effect of variation in nicotine concentration on the rates of 5'-cyanonicotine and *N*-(cyanomethyl)nornicotine formation. Microsomes from untreated (●) or phenobarbital-pretreated (○) rabbits were incubated with the indicated concentrations of nicotine as described under Materials and methods, and the rates of 5'-cyanonicotine (———) and *N*-(cyanomethyl)nornicotine (---) were determined for duplicate determinations.

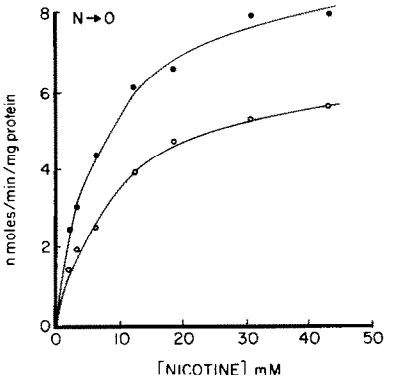

Fig. 2. Effect of variations in nicotine concentration on the rate of nicotine-*N'*-oxide formation. Microsomes from untreated (●) or phenobarbital pretreated (○) rabbits were assayed for nicotine *N'*-oxide formation as described under Materials and methods.

the non-linearity of the double-reciprocal plots exact determinations were not possible.

The ability of purified cytochrome P-450 isozymes to catalyze nicotine metabolism in a standard reconstitution assay is shown in Table 1. Isozymes 2 and 3b were the most effective with forms 3a and 6 also exhibiting activity. The appearance of *N*-(cyanomethyl)nornicotine paralleled 5'-cyanonicotine formation. When the rates for 5'- and methylhydroxylation for untreated microsomes are expressed per nanomole of cytochrome P-450, the reconstituted rates of 5'-hydroxylation were 30-fold higher for isozymes 2 and 3b and 8-fold higher for isozyme 6. The increases observed for rates of formation of *N*-(cyanomethyl)nornicotine were 62-, 78- and 28-fold, respectively, but probably represent an overestimation due to the low rates obtained with control microsomes. *N*-(Cyanomethyl)nornicotine formation was 12.5% of 5'-cyano formation in microsomes, but was increased to 25–33% in reconstitution experiments with isozymes 2 and 3b, respectively, and was 40% of that seen with isozymes 3a and 6. All of the isozymes used in this study catalyzed the oxidation of marker substrates, indicating that each isozyme was catalytically active. Nicotine *N'*-oxide formation was not observed with any of the reconstituted cytochrome P-450 isozymes. Under our current assay conditions, we feel confident that we could have detected as little as 80 pmol nicotine *N'*-oxide.

Discussion

The data presented here demonstrate that C-oxidation of nicotine is a mixed-function oxygenase activity while N'-oxidation is not associated with any of the cytochrome P-450 isozymes tested and is likely catalyzed by microsomal flavin monooxygenase. At the present time, however, we canot exclude the possibility that an isozyme of cytochrome P-450 not tested (for example from 1 or 5) may be responsible for N'-oxidation of nicotine. Phenobarbital pretreatment of rabbits induces isozymes 2 and 5 [18]. Our reconstitution experiments demonstrate that isozyme 2 was responsible for significant amounts of nicotine C-oxidation. We have not as yet tested the ability of form 5 to catalyze either C- or N'-oxidation nicotine. Additionally, isozyme 3b, a constitutive hepatic isozyme in rabbits [19], catalyzed the C-oxidation of nicotine at a rate comparable to form 2. The fact that significant, albeit lower, turnover was observed for forms 6 and 3a may be important in that form 6 is inducible by polycyclic xenobioties [2] and form 3a is inducible by ethanol [21]. Thus, the microsomal C-oxidation of nicotine has the potential of being modulated by different types of inducers of microsomal cytochrome P-450.

Microsomal N-demethylation reactions are thought to proceed via hydroxyl-methyl intermediates [22]. Subsequent oxidation results in demethylated (nor) products with the release of formaldehyde. Nguyen *et al.* [5] and Peterson *et al.* [23], demonstrated that microsomes from phenobarbital-pretreated rabbits can catalyze the formation of both 5'-cyanonicotine and *N*-(cyanomethyl)-nornicotine. We have been unable to detect the formation of nornicotine under conditions which allow for the formation of cotinine with either hamster [6] or rabbit post-mitochondrial supernatant fractions (unpublished observations). Additionally, Hucker *et al.* [24] were unable to detect release of formaldehyde when nicotine was incubated with rabbit post-mitochondrial supernatant fractions under conditions which readily catalyzed cotinine formation. The possibility of nonenzymatic formation of *N*-(cyanomethyl)nornicotine needs to be considered since a preferred method of synthesis for the *N*-cyanomethyl derivative involves the incubation of nornicotine, formaldehyde and potassium cyanide under mild conditions [5].

The lack of formation of nornicotine under conditions which allow for the formation of cotinine would seem to argue against the enzymatic formation of a hydroxy methyl intermediate under our current assay conditions. It is interesting to note that, relative to the 5'-cyanonicotine, purified forms 2 and 3b produced greater amounts of the *N*-(cyanomethyl)nornicotine then isolated microsomes. The fact that *N*-(cyanomethyl)nornicotine was formed with purified isozymes and that the rates of formation were not uniform but distributed, as were the rates of 5'-oxidation, would argue against facile chemical formation since all reconstitution assays were performed concurrently under identical incubation conditions. It is possible that the initial microsomal metabolite shares a common intermediate with both the 1',5' and the methyl iminium ion. This would help to explain the similar distribution of the rate of formation of the 5' and the methyl cyano adducts.

In summary, the *in vitro* rates of N' and C-oxidation of nicotine by rabbit liver microsomes and purified cyto-

Table 1. Reconstitution of nicotine C oxidation by purified rabbit liver cytochrome P-450 isozymes

Cytochrome P-450 isozyme	Product formed (nmol/min/mol cytochrome P-450) 5′-Cyanonicotine	*N*-(Cyanomethyl)nornicotine
2	28.5	6.8
3a	4.1	1.8
3b	29.4	8.6
3c	2.3	1.3
4	1.4	1.0
6	7.4	3.1

Assays were performed as described under Materials and methods. The nicotine concentration was 30 mM, and the final assay volume was 0.5 ml. Values are averages of duplicate incubations.

chrome P-450 isozymes were determined. In the presence of 1 mM potassium cyanide, microsomes from untreated rabbits catalyzed the formation of 5′-cyanonicotine, *N*-(cyanomethyl)nornicotine, and nicotine *N*′-oxide. Pretreatment of rabbits with 0.1% (w/v) phenobarbital in the drinking water for 7 days caused a greater than 2-fold induction in the rates of formation of both 5′-cyanonicotine and *N*-(cyanomethyl)nornicotine. In contrast, the rate of formation of nicotine *N*′-oxide was decreased 30%. Reconstitution studies with six purified rabbit cytochrome P-450 isozymes indicated that the C-oxidation of nicotine was greatest with isozymes 2 and 3b, a phenobarbital inducible and constitutive form, respectively. In addition, isozymes 6 and 3a were also capable of catalyzing the C-oxidation of nicotine. The parallel rates of formation of 5′-cyanonicotine and (*N*-cyanomethyl)nornicotine in microsomes and reconstituted systems suggest involvement of a common intermediate. None of the six isozymes tested was able to catalyze the N′-oxidation of micotine.

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Effect of inhibition of glutathione synthesis on the metabolism and protein conjugation of [^{14}C]captopril in the rat

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Captopril (CP)* is an orally active angiotensin-converting enzyme inhibitor used in the treatment of hypertension [1, 2]. The major *in vivo* biotransformations of CP involve mixed disulphide formation with endogenous thiols derived from cysteine [3]. A number of adverse reactions associated with CP are thought to have an underlying immunological mechanism [4], and it has been suggested that CP with its free sulphydryl moiety, or one of its metabolites, may act as a hapten. However, metabolic studies have shown that CP covalently bound to plasma proteins can be dissociated by a thiol disulphide interaction both *in vitro* and *in vivo* [3, 5], thus affecting the ability of CP to act as a hapten. Therefore, we have investigated the effect of glutathione (GSH) depletion on the formation of CP–plasma protein conjugates *in vivo*, in relation to the metabolism of CP. Depletion of GSH was by pre-treatment with DL-buthionine sulfoximine (BSO) which is a potent and specific inhibitor or γ-glutamylcysteine synthetase, the enzyme which catalyses the initial step of glutathione synthesis [6]. In this study, the covalent binding of [^{14}C]CP to tissue proteins was also determined.

Materials

[^{14}C]Captopril (4.66 μCi/mg) labelled in the amide carbonyl group, and authentic standards of captopril, captopril disulphide, captopril–cysteine mixed disulphide and captopril–glutathione mixed disulphide were supplied by the Squibb Institute (New Brunswick, NJ). DL-Buthionine sulfoximine was obtained from Chemical Dynamics Corporation (NJ, U.S.A.). Silica gel ($60\,F_{254}$) thin-layer chromatography plates (Merck, 20 × 20 × 0.02 cm) were obtained from British Drug Houses (Poole, U.K.). *N*-Ethylmaleimide, glyoxalase I (Grade IV), methylglyoxal, morpholino-propanesulfonic acid and other general reagents were obtained from Sigma Chemical Company (Poole, U.K.). Dialysis tubing was obtained from Medicell Ltd (London, U.K.). Scintillation fluid (NE260) was obtained from Nuclear Enterprises, (Edinburgh, U.K.). NCS tissue solubilizer solution was obtained from Amersham (Bucks, U.K.). All solvents were redistilled before use.

Methods

Depletion of glutathione by inhibition of synthesis. Male Wistar rats (250–300 g) were given free access to food and water. Glutathione synthesis was inhibited by the method of Griffith and Meister [6]. DL-Buthionine sulfoximine was administered intraperitoneally (8 mmol/kg), one half of the dose was given initially, and the remainder given 1.5 hr later. The control rats received saline only.

Determination of glutathione concentrations. The glutathione concentrations of the liver samples were determined on the same day by the glyoxalase I method [5]. This method is specific for reduced GSH and is not affected by the presence of sulphydryl compounds such as captopril.

Effect of glutathione depletion on the metabolism of [^{14}C]*captopril in the rat.* The metabolism of [^{14}C]CP was studied 3 hr after the initial buthionine sulfoximine (BSO) injection. The rats were anaesthetised with urethane (14% w/v in saline, 10 ml/kg, i.p.) and the trachea, carotid artery and jugular vein cannulated with polythene tubing of the appropriate sizes. [^{14}C]CP (4.66 μCi/mg, 4 mg/kg) in saline was administered via the jugular vein. Blood samples (0.5 ml) were collected at 5, 30, 60, 120 and 180 min. Plasma was obtained immediately by centrifugation (2000 *g*) in a Microfuge and *N*-ethylmaleimide (NEM, 4 mg/ml) was added to derivatise any free captopril present. The volume of blood taken out was replaced by an equal volume of saline. [^{14}C]captopril covalently bound to plasma proteins and [^{14}C]CP metabolites were determined by SDS-equilibrium dialysis and thin-layer radiochromatography, respectively, by methods previously described [3].

After 3 hr, the rat was killed and the liver, lungs, kidneys and spleen were removed and weighed. A section of the liver was taken for the determination of glutathione concentration by the glyoxalase I method. The tissues were

* Abbreviations used: BSO, buthionine sulfoximine; CP, captopril; CP-cys, captopril cysteine mixed disulphide; CPD, captopril disulphide; EDTA, ethylenediamine tetreacetic acid; GSH, reduced glutathione; NEM, *N*-ethylamaleimide; SDS, sodium dodecyl sulphate.