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FORMATION OF TWO MAJOR NICOTINE METABOLITES IN LIVERS OF GUINEA PIGS

HITOSHI NAKAYAMA,* SHINSUKE FUJIHARA, TOSHIKATSU NAKASHIMA and YUTAKA KUROGOCHI

Department of Pharmacology, Nara Medical University, Kashihara, Nara 634, Japan

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Abstract—Using antibody against NADPH–cytochrome P-450 reductase and several effectors of cytochrome P-450 and FAD-containing monooxygenase, we investigated nicotine metabolites formed by these two enzymes. When [^{3}H]nicotine was metabolized by the combination of liver microsomes of guinea pigs and partially purified aldehyde oxidase, three distinct spots corresponding to nicotine, cotinine and nicotine-1′-oxide were observed on fluorograms of thin-layer chromatography. Antibody against NADPH–cytochrome P-450 reductase inhibited the formation of cotinine but not nicotine-1′-oxide. Metyrapone and *n*-octylamine inhibited the cotinine formation, while methimazole prevented the formation of nicotine-1′-oxide. These results show that microsomal electron transport systems participate in the formation of nicotine-1′-oxide and strongly suggest the involvement of FAD-containing monooxygenase in the formation of nicotine-1′-oxide.

The physiological and pharmacological effects of nicotine in animals and humans have been investigated extensively. Nicotine has been reported to be metabolized predominantly in liver microsomes, and two major metabolites, cotinine and nicotine-1′-oxide, have been found in urine, blood and tissues of animals [1–8]. Hucker *et al.* presented the first study on enzymatic conversion of nicotine to cotinine and postulated the following pathway: hydroxylation of nicotine by microsomal enzymes and then nonenzymatic conversion to the corresponding aldehyde and, finally, production of cotinine by cytosolic aldehyde oxidase [3, 9]. Hill *et al.* [8] proposed that cytochrome P-450 and FAD-containing monooxygenase (EC 1.14.13.8) convert nicotine to hydroxynicotine and nicotine-1′-oxide respectively. However, since inhibitors that distinguish between cytochrome P-450 and FAD-containing monooxygenase were not found at that time and since inhibition of cytochrome P-450 by effectors varied with species of cytochrome P-450, their proposal has not been confirmed. Indeed, effects of SKF-525A, an inhibitor of cytochrome P-450, on cotinine formation have been found to vary with species [10, 11]. We showed that phenobarbital (PB)-inducible cytochrome P-450 catalyzes nicotine oxidation in a reconstituted system and in microsomes [12, 13]. FAD-containing monooxygenase isolated from pig livers was reported to catalyze nicotine oxidation [14]. More recently, the rates of FAD-containing monooxygenase-catalyzed nicotine metabolism have been reported with liver, lung and kidney microsomes from mouse, rat and rabbit [15]. In these reports, however, disappearance of NADPH or nicotine was monitored and the metabolites of nicotine oxidation were not identified. Since many kinds of substrates oxidized by cytochrome P-450 are reported to be also metabolized by FAD-containing monooxygenase [16], it is necessary to identify each reaction product of the metabolism of common substrates. In this report, we demonstrate the participation of microsomal electron transport systems in the conversion of nicotine to cotinine by using inhibitors of cytochrome P-450 and specific antibody against NADPH–cytochrome P-450 reductase which is known to be a component of microsomal electron transport systems. In addition, the inhibition of nicotine-1′-oxide formation by methimazole supports the view that FAD-containing monooxygenase catalyzes the converson of nicotine to nicotine-1′-oxide.

* Author to whom all correspondence should be addressed.

MATERIALS AND METHODS

Preparation of anti-NADPH–cytochrome P-450 reductase. NADPH–cytochrome P-450 reductase was purified from liver microsomes of PB-treated guinea pigs by the method of Yasukochi and Masters [17] with some modifications. The purified enzyme showed a single protein band when submitted to sodium dodacylsulfate (SDS)-polyacrylamide gel electrophoresis. Antibody against NADPH–cytochrome P-450 reductase was produced in rabbits as described previously [13]. The sera with high titers were pooled, and the immunoglobulin G fractions were prepared by fractionation with ammonium sulfate and chromatography with DE-52 according to the method of Thomas *et al.* [18]. The immunoglobulin G fractions formed single precipitation lines with both the purified reductase and microsomal components in livers of PB-treated guinea pigs, and both precipitation lines fused. The anti-NADPH–cytochrome P-450 reductase immunoglobulin G fractions were designated as anti-NADPH–cytochrome P-450.

In vitro *metabolism of nicotine*. Guinea pigs weighing 300–350 g were fasted overnight and killed by decapitation. Their liver microsomes were prepared as described previously [13]. The standard reaction mixture of nicotine metabolism contained 0.1 M potassium phosphate buffer (pH 7.4), 0.6 mM $MgCl_2$, 0.5 mM NADPH, 0.5 mM nicotine, 0.2 μCi (−)-[*N-methyl*]-^{3}H]nicotine (60.0 Ci/mmol), 40–60 units of partially purified aldehyde oxidase and microsomes in a total volume of 0.2 ml. When inhibition of microsomal nicotine oxidation by anti-NADPH–cytochrome P-450 reductase was studied, microsomes were first mixed with the antibody in 20 mM potassium phosphate buffer (pH 7.4) for 10 min at room temperature, and then $MgCl_2$, NADPH, phosphate buffer and aldehyde oxidase were added, followed by an additional 2-min incubation at 37°. After this preincubation, reactions were started by the addition of the mixture of unlabeled and [^{3}H]nicotine. The reactions were carried out for 1 hr at 37° and terminated by the addition of 0.8 ml of 5% perchloric acid. About 50 nmol of cotinine and nicotine-1′-oxide was added to the terminated mixture as a carrier. A blank was run in the same manner except for the omission of microsomes. Aldehyde oxidase in 100,000 *g* supernatant of guinea pig livers was partially purified by fractionation of ammonium sulfate and heat treatment as described by Felsted *et al.* with some modifications [19].

Extraction of nicotine, cotinine and nicotine-1′-oxide. After the terminated reaction mixtures obtained as above were centrifuged at 3000 rpm for 10 min, the precipitates were suspended in 2 ml of 2% perchloric acid and recentrifuged. The combined aqueous extracts were applied to a column (0.5 × 1 cm) of Dowex 50W–X8 (H^+ form, 100–200 mesh), and then the columns were washed with about 20 ml of 0.5 N HCl-methanol. Nicotine, cotinine and nicotine-1′-oxide were eluted with about 4 ml of 5 N HCl-methanol. The elutes were evaporated under reduced pressure below 40° to dryness, and the residues were dissolved in methanol. About 80–85% of the original radioactivity was recovered in the methanol fraction.

Analysis of nicotine metabolites. The labeled-nicotine and its metabolites obtained in the methanol solution were divided into two portions, and each portion was chromatographed on silica gel plates (Kieselgel 60F_{254}, Merck). The compounds were separated by one-dimension thin-layer chromatography using two different solvent systems, *n*-butanol–ethanol–2.5 M NH_3 (4:1:1, solvent A) (TLC-1) and benzene–ethanol–acetone–aq. NH_3 (50:5:40:5, solvent B) (TLC-2) [6]. After development, thin-layer chromatograms were dried and sprayed with 7% 2.5-diphenyloxazole (PPO) in ethyl ether and then exposed to Kodak X-Omat R film at −70° for 5 days [20]. After the structures of nicotine metabolites were determined by GC–MS, the separated compounds on the silica gels were detected directly under u.v. light and scraped into the test tubes followed by extraction with methanol. GC–MS analysis was performed with an Hitachi M-80 gas chromatograph mass spectrometer equipped with a 3% OV-1 column (2 m), using an ionizing voltage of 20 eV and a helium carrier gas flow rate of 50 ml/min. Nicotine-1′-oxide was examined by the use of the direct inlet system. Koenig-positive zones of nicotine and its metabolites were revealed by spraying the chromatograms with ethanolic 1% *p*-aminobenzoic acid followed by exposure to BrCN [9] and scraped into the scintillation vials, and their radioactivities were determined by liquid scintillation counting.

Enzyme assays. NADPH–cytochrome P-450 reductase was assayed by its ability to catalyze cytochrome *c* reduction at 30° [21]. Aminopyrine demethylation and dimethylaniline N-oxidation were assayed at 37° by the methods of Nash [22] and Ziegler and Pettit [23] respectively. Aldehyde oxidase was assayed at 37° by the method of Felsted *et al.* [19]. SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli [24]. A unit of the enzyme activity is defined as the appearance of one nmol of product (or disappearance of one nmol of substrate) per min.

RESULTS

Figure 1 shows fluorograms of thin-layer chromatography of [^{3}H]nicotine and its metabolites after nicotine oxidation. When [^{3}H]nicotine was incubated with microsomes and aldehyde oxidase in the presence of NADPH and $MgCl_2$, three major spots were detected on the fluorograms in both solvents A and B (lane 3). These spots gave the positive Koenig reaction which was reported to be indicative of a pyridine ring [25]. Spots a, b and c coincided in position with those of authentic nicotine, cotinine and nicotine-1′-oxide, respectively, judging from Koenig-positive spots of these authentic compounds. However, spot b was found to contain cotinine and another minor Koenig-positive compound when the materials corresponding to spot b were cochromatographed in solvent B. The minor Koenig-positive compound was not detected on the fluorogram in solvent B. In solvent B, spts a′, b′ and c′ coincided in position with those of authentic nicotine, cotinine and nicotine-1′-oxide respectively. Since spot c′ remained at the origin in this solvent, the materials corresponding to spot c′ were cochromatographed in solvent A. The materials corresponding to spot c′ were separated to nicotine-1′-oxide (spot c) and another minor compound (spot x). The compounds corresponding to spots a, b′ and c were further confirmed to be nicotine, cotinine and nicotine-1′-oxide, respectively, based on GC–MS analysis. Therefore, spots a, b′ and c were scraped into the scintillation vials, and their radioactivities were determined. Omission of microsomes resulted in disappearance of cotinine and nicotine-1′-oxide (Fig. 1, lane 1). When partially purified aldehyde oxidase fraction was omitted from the reaction mixture, cotinine was not detected on the fluorograms (Fig. 1, lane 2), showing that partially purified aldehyde oxidase fraction was necessary to form cotinine. Figure 2 shows the formation of cotinine and nicotine-1′-oxide as a function of microsomal protein concentration. To obtain clear spots of these compounds on thin-layer plates, prolonged incubation was allowed to proceed until migration deviated from linearity. Decrease of nicotine and increase of cotinine and nicotine-1′-oxide were dependent on the

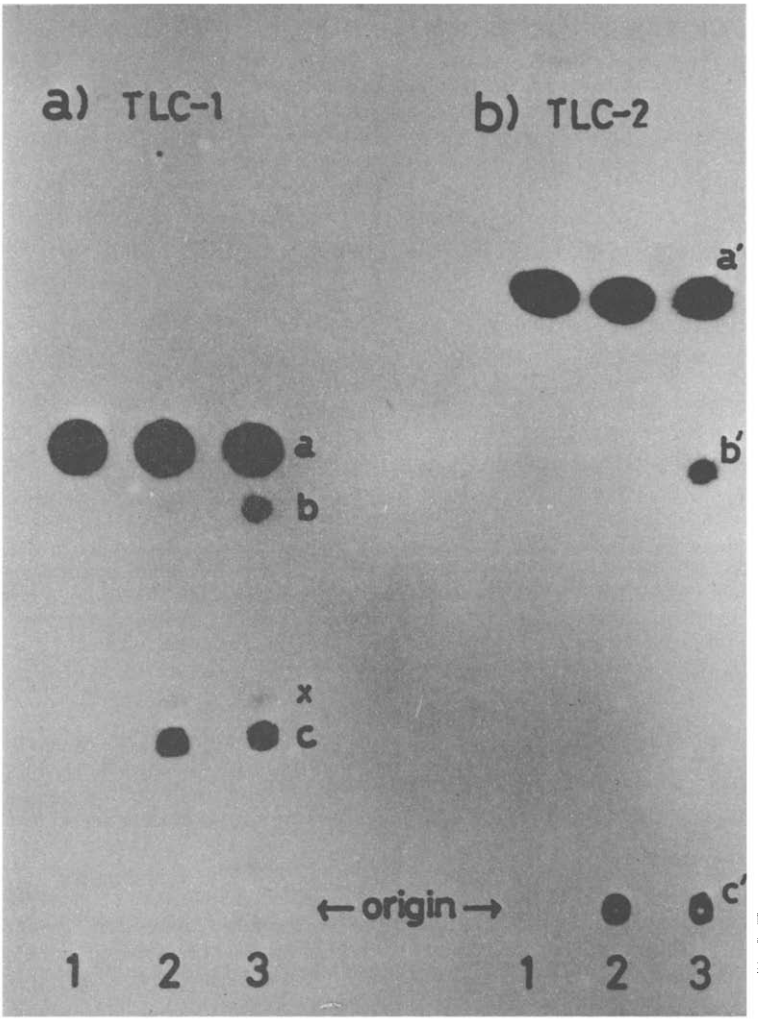

Fig. 1. Fluorograms of thin-layer chromatography of [^{3}H]nicotine and its metabolites extracted after *in vitro* metabolism of [^{3}H]nicotine. Metabolism of [^{3}H]nicotine was performed in the presence of aldehyde oxidase (lane 1), microsomes (lane 2), or microsomes plus aldehyde oxidase (lane 3). Reaction conditions and extraction of [^{3}H]nicotine and its metabolites were described in Materials and Methods. One-dimensional thin-layer chromatography was performed with two solvent systems: (a) *n*-butanol–ethanol–2.5 M NH_3 (4:1:1, solvent A, TLC-1), and (b) benzene–ethanol–acetone–aq. NH_3 (50:5:40:5, solvent B, TLC-2).

protein concentration. As shown in Fig. 3a, anti-NADPH–cytochrome P-450 reductase strongly inhibited NADPH-dependent cytochrome *c* reduction and aminopyrine demethylation but not dimethylaniline N-oxidation. It has been shown that aminopyrine demethylation is catalyzed exclusively by microsomal electron transport systems, whereas dimethylaniline N-oxidation is catalyzed by FAD-containing monooxygenase [26]. These results show that the antibody is useful to investigate the participation of microsomal electron transport systems in nicotine oxidation. Figure 3b shows the effects of anti-NADPH–cytochrome P-450 reductase on the formation of cotinine and nicotine-1′-oxide. The antibody inhibited more than 95% of the cotinine formation but did not inhibit the formation of nicotine-1′-oxide, showing that microsomal electron transport systems in livers of guinea pigs participate in the conversion of nicotine to cotinine. Table 1 shows the actions of several effectors of cytochrome

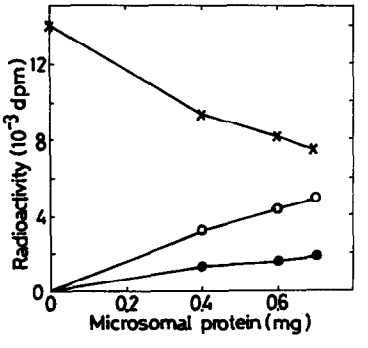

Fig. 2. Formation of cotinine and nicotine-1′-oxide as a function of microsomal protein concentration. Reaction conditions and extraction of [^{3}H]nicotine, cotinine and nicotine-1′-oxide were described in Materials and Methods except for variations in the microsomal protein concentration. Key: (×) nicotine, (●) cotinine, and (○) nicotine-1′-oxide.

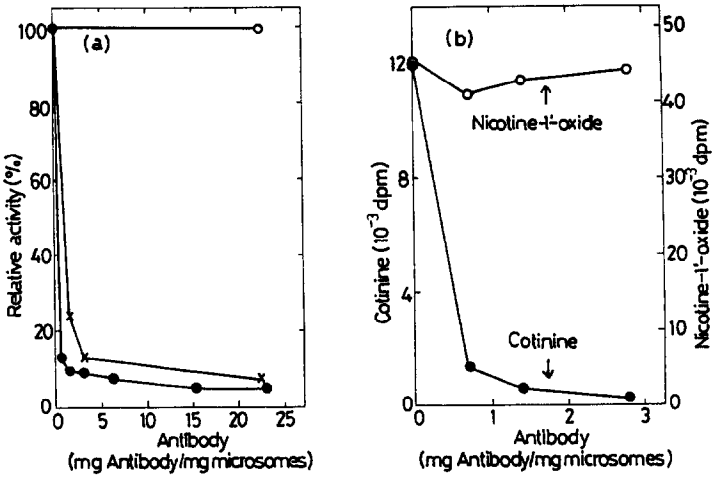

Fig. 3. Effects of anti-NADPH–cytochrome P-450 reductase on (a) the activities of NADPH-dependent cytochrome *c* reduction, aminopyrine demethylation and dimethylaniline N-oxidation and (b) the formation of cotinine and nicotine-1′-oxide. All experimental conditions were described in Materials and Methods. Control activities of NADPH-dependent cytochrome *c* reduction, aminopyrine demethylation and dimethylaniline N-oxidation were 3.4, 0.0082 and 2.8 nmol of product formed/min respectively. Key: (a) (●) NADPH-dependent cytochrome *c* reduction, (×) aminopyrine demethylation, and (○) dimethylaniline N-oxidation; (b) (●) cotinine, (○) nicotine-1′-oxide.

P-450 and FAD-containing monooxygenase on the formation of cotinine and nicotine-1′oxide. Metyrapone has been reported to inhibit cytochrome P-450-dependent reactions, while methimazole has been used as an inhibitor for FAD-containing monooxygenase reactions [27, 28]. *n*-Octylamine has been reported to inhibit cytochrome P-450 but to activate FAD-containing monooxygenase [26, 28]. Both metyrapone and *n*-octylamine inhibited the formation of cotinine. The formation of cotinine was also inhibited by KCN and α-naphthoflavone (data not shown) which are known to be inhibitors of aldehyde oxidase and cytochrome P-450 respectively [9, 29]. These results show that cotinine was formed by a combination of microsomal electron transport systems and aldehyde oxidase. Methimazole strongly inhibited the formation of nicotine-1′-oxide. A metabolite of methimazole which is formed in prolonged incubation has been reported to inhibit some P-450-dependent reactions [30]. When the incubation time of nicotine oxidation was shortened to 20 min, methimazole also completely inhibited the formation of nicotine-1′-oxide, supporting the view that FAD-containing monooxygenase catalyzes the conversion of nicotine to nicotine-1′-oxide [8].

Table 1. Effects of metyrapone, *n*-octylamine and methimazole on the formation of cotinine and nicotine-1′-oxide

Effectors	Cotinine (dpm)	Cotinine (%)	Nicotine-1′-oxide (dpm)	Nicotine-1′-oxide (%)
Control	12,200	100	43,900	100
Metyrapone (5 mM)	1,360	11	40,400	92
n-Octylamine (3 mM)	1,200	10	56,100	128
Methimazole (0.5 mM)	13,700	112	54	0.1

[^{3}H]Nicotine (0.2 μCi) was incubated for 1 hr at 37° with 100 mM potassium phosphate buffer (pH 7.4), 6 mM $MgCl_2$, 0.5 mM NADPH, 0.5 mM unlabeled nicotine, 0.58 mg of microsomes, and 50 units of partially purified aldehyde oxidase in the presence of effectors listed.

DISCUSSION

Fluorographic analysis of nicotine metabolism by the combination of microsomes and partially purified aldehyde oxidase showed an appearance of three distinct spots corresponding to nicotine, cotinine and nicotine-1′-oxide. Though we failed to detect hydroxynicotine and the corresponding aldehyde, the formation of cotinine by the addition of aldehyde oxidase is in accord with the view that these two compounds may be intermediates of the pathway from nicotine to cotinine [9]. Aldehyde oxidase has been reported to be the enzyme responsible for the formation of cotinine [8, 9]. Prough and Ziegler [27] showed that cytochrome P-450 and FAD-containing monooxygenase catalyze the oxidation of *tert*-amines to the demethylated compounds and amine oxides respectively. In this case, it was assumed that cytochrome P-450 catalyzes an oxidative attack on a carbon alpha to the nitrogen, while FAD-containing monooxygenase catalyzes N-oxidation of *tert*-amines. On the other hand, *s*-oxide of thiobenzamide is formed by both cytochrome P-450 and FAD-containing monooxygenase [28]. In nicotine oxidation, SKF-525A has been reported to inhibit cotinine formation by rat post-mitochondrial supernatant [10] but to have little effect on the formation by hamster post-mitochondrial supernatant [11]. Cytochrome P-450 is a mixture of different related proteins, whereas only one type of NADPH–cytochrome P-450 reductase has been found to be present in liver microsomes. The present study, therefore, used not only inhibitors of cytochrome P-450 but also anti-NADH–cytochrome P-450 reductase as inhibitors of

microsomal electron transport systems and gave clear evidence of the participation of microsomal electron transport systems in the formation of cotinine. Cytochrome-P-450-participating nicotine metabolism may have species differences in response to SKF-525A. Recently, McCoy *et al.* [11] showed that cysteamine, a substrate for FAD-containing monooxygenase, partially inhibits the conversion of nicotine to nicotine-1′-oxide. Together with these reports, the present inhibition studies with the antibody and methimazole strongly suggest the involvement of FAD-containing monooxygenase in the formation of nicotine-1′-oxide.

There have been many reports of tissue and species differences in nicotine metabolism. For example, in mice, liver homogenates were found to metabolize nicotine to cotinine and nicotine-1′-oxide, whereas lung and kidney homogenates produce only nicotine-1′-oxide [8]. In the case of rabbits, nicotine is converted primarily to cotinine [3]. The amount of nicotine-1′-oxide excreted by smokers in 24 hr under normal conditions was shown to be about half that of the cotinine excreted [31]. Though analysis of nicotine metabolites was carried out under such different conditions that direct comparisons were impossible, the tissue and species differences in nicotine metabolism seem to connect with those in contents and properties of cytochrome P-450 and FAD-containing monooxygenase. Tissue and species differences were found in the relative contribution of cytochrome P-450 and FAD-containing monooxygenase to the metabolism of thiobenzamide [28]. Large species and tissue differences of FAD-containing monooxygenase have been found in the enzyme activities and the substrate specificity [32, 33], and the enzyme occurs in different molecular forms, which may be differentially expressed in liver and lung [34, 35]. Cytochrome P-450 is composed of many distinct molecular forms and which types of cytochrome-450 participate in nicotine metabolism has not been confirmed with purified enzymes except for PB-inducible cytochrome P-450 [12, 13]. To clarify the tissue and species differences in nicotine metabolism, further studies with the purified enzymes and specific inhibitors against both enzymes are necessary.

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