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Participation of microsomal electron transport systems in nicotine metabolism by livers of guinea pigs

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It has been suggested that nicotine is metabolized predominantly in liver microsomes by cytochrome P-450 and FAD-containing monooxygenase [1–4]. These two enzymes have important roles in drug, insecticide and other xenobiotic metabolism [5, 6]. Since it has been shown that many kinds of substrates oxidized by FAD-containing monooxygenase are also metabolized by cytochrome P-450, the definition of the relative contribution of these two enzymes to the metabolism of common substrates is necessary [5, 6]. Recently, we showed that phenobarbital (PB)-inducible cytochrome P-450 (PB-P-450) in rat and guinea pig livers catalyzes nicotine oxidation in reconstituted systems and microsomes [7, 8]. In addition, constitutive forms of cytochrome P-450 have been assumed to participate in microsomal nicotine oxidation since PB-P-450 is scarcely detectable in liver microsomes of untreated animals [8]. Many kinds of cytochrome P-450 have been purified from microsomes of animal livers, whereas only one type of NADPH–cytochrome P-450 reductase, the other component of microsomal electron transport systems, has been found to be present in liver microsomes. By using antibody against NADPH–cytochrome P-450 reductase, therefore, we have investigated the contribution of microsomal electron transport systems to nicotine oxidation in livers of untreated and PB-treated guinea pigs.

Materials and methods

Male Hartley guinea pigs (30–42 days of age) were injected intraperitoneally each day for 5 days with PB (60 mg/kg). Microsomal fractions were prepared as previously described [8]. NADPH–Cytochrome P-450 reductase was purified by the method of Yasukochi and Masters [9] with some modifications. The purified enzymes showed a single protein band when submitted to polyacrylamide gel electrophoresis by the method of Laemmli [10]. Cytochrome P-450 and b_5 were not detected in the purified reductase fractions.

Antibody against NADPH–cytochrome P-450 reductase was produced in rabbits as previously described for PB-P-450 [8] and purified by fractionation with ammonium sulfate and chromatography with DE-52. The purified immunoglobulin fractions were designated as anti-NADPH–cytochrome P-450 reductase. The Ouchterlony double diffusion test showed that anti-NADPH–cytochrome P-450 reductase cross-reacted with the purified reductase and with the microsomal components, and formed a single precipitation line. Control immunoglobulin G was prepared by the same method using blood obtained from non-immunized rabbits. When inhibition of liver microsomal nicotine oxidase activity 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; then $MgCl_2$, NADPH and phosphate buffer were added, followed by an additional 5-min incubation at 37°. After this preincubation, the reaction was started at 37° by the addition of nicotine.

Nicotine oxidase activity was determined spectrophotometrically at 259 nm as previously described [8]. NADPH–Cytochrome P-450 reductase activity was assayed by its ability to catalyze cytochrome *c* reduction in 100 mM potassium phosphate buffer (pH 7.4) at 25° [11]. Di-

methylaniline N-oxidation was assayed at 37° by the method of Zeigler and Pettit [12]. One unit of enzyme activity is arbitrarily defined as that amount of enzyme catalyzing the formation of 1 nmole of a product or the decrease of 1 nmole of a substrate per 1 min. Protein was determined by the method of Lowry *et al.* [13].

Results and discussion

Figure 1 shows anti-NADPH–cytochrome P-450 reductase inhibition of NADPH-dependent cytochrome *c* reduction and nicotine oxidation in liver microsomes of untreated or PB-treated guinea pigs. The antibody inhibited the NADPH-dependent cytochrome *c* reduction by about 95% at a concentration of 16.3 mg of the antibody per mg of microsomal protein. In addition, the antibody also inhibited the microsomal aminopyrine demethylation by more than 90%, but did not inhibit microsomal dimethylaniline N-oxidation (data not shown). 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 [6]. These results show that the antibody was a useful tool to approximate the contribution of microsomal electron transport systems to nicotine oxidation in liver microsomes. The antibody maximally inhibited the nicotine oxidase activity in liver microsomes of untreated and PB-treated guinea pigs by about 45 and 70% respectively. Control immunoglobulin G inhibited less than 5% of the nicotine oxidase activities. α-Naphthoflavone, an effector of cytochrome P-450 reductase, also maximally inhibited the nicotine oxidase activity in liver microsomes of untreated guinea pigs by about 55%, but did not inhibit dimethylaniline N-oxidation (Fig. 2). Dimethylaniline N-oxidation was not inducible by PB.

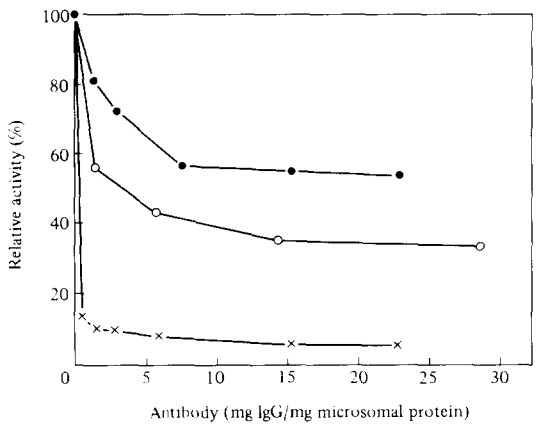

Fig. 1. Effects of anti-NADPH–cytochrome P-450 reductase on NADPH-dependent cytochrome *c* reduction and nicotine oxidase activities in liver microsomes of untreated or PB-treated guinea pigs. Anti-NADPH–cytochrome P-450 reductase was preincubated with liver microsomes of untreated or PB-treated guinea pigs for 10 min at room temperature. The control activities of NADPH-dependent cytochrome *c* reduction in untreated guinea pigs and nicotine oxidase in untreated and PB-treated animals were determined in the absence of the antibody and were 3.36, 1.58 and 1.60 units respectively. The activities of NADPH-dependent cytochrome *c* reduction (×) in untreated guinea pigs and nicotine oxidase in untreated (●) and PB-treated (○) animals are shown in the figure as percent of control.

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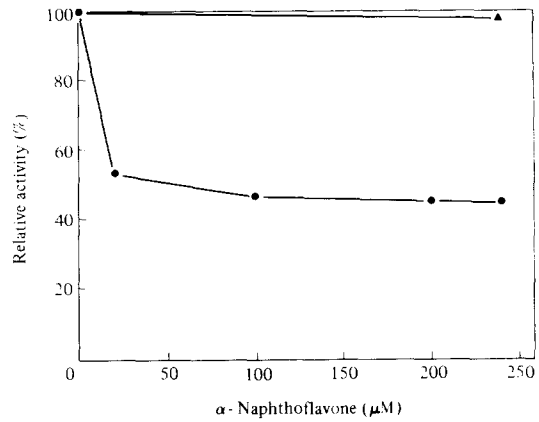

Fig. 2. Effects of α-naphthoflavone on nicotine oxidase activity and dimethylaniline N-oxidation in liver microsomes of untreated guinea pigs. α-Naphthoflavone (in 20 μl methyl alcohol) was added to give a final concentration of 20, 100, 200 and 240 μM. The control activities of nicotine oxidase and dimethylaniline N-oxidation in the absence of α-naphthoflavone were 1.58 and 2.8 units respectively. The activities of nicotine oxidase (○) and dimethylaniline N-oxidation (▲) in the absence of α-naphthoflavone are shown in the figure as percentages.

We previously reported that antibody against PB-P-450 maximally inhibited liver microsomal nicotine oxidase activities in PB-treated and untreated guinea pigs by about 30% and less than 5% respectively [8]. The degree of the anti-NADPH–cytochrome P-450 reductase inhibition observed in the present study was higher than that of the anti-PB-P-450 inhibition, showing that constitutive forms of cytochrome P-450 also participate in liver microsomal nicotine oxidation in both PB-treated and untreated guinea pigs. The increase of anti-NADPH–cytochrome P-450 reductase inhibition with PB treatment seems to correlate with the increase of PB-P-450 content. Administration of many kinds of drugs to animals was found to induce cytochrome P-450, whereas FAD-containing monooxygenase is not induced by most drugs or other xenobiotic agents [6]. Therefore, the ratio of cytochrome P-450-dependent nicotine oxidation to microsomal nicotine metabolism is changeable with drug treatment.

In summary, microsomal electron transport systems have an important role in nicotine metabolism in both untreated and PB-treated guinea pigs. PB treatment increased the ratio of participation of microsomal electron transport systems to all microsomal nicotine metabolism.

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Characterization of the microsomal cytochrome P-450 species inhibited in rat liver in the course of fascioliasis

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Experimentally induced fascioliasis has been described to decrease significantly hepatic mixed function oxidase in rats 3–8 weeks after infestation [1, 2]. More recently, the incidence of this parasitism was investigated in rats infected for 5 weeks and untreated or pretreated with either phenobarbital, 3-methylcholanthrene, β-naphthoflavone or arochlor 1254 [3]. This consisted in decreasing liver microsomal P-450,* aminopyrine *N*-demethylase, aniline hydroxylase and mutagenic activation of cyclophosphamide which were specifically induced by both PB and arochlor [4].

The existence of several different isozymes of cytochrome P-450 differing in substrate specificity, aminoacid sequence, electrophoretic mobility and immunological properties is now well-established [5]. In the present investigation, we have compared the isozymes of P-450 more probably destroyed or repressed in the course of experimental fascioliasis in untreated and PB or MC induced rats, on the basis of substrate specificity, sensitivity to different inhibitors and immunological properties. In order to recognize a possible dual effect of *Fasciola hepatica* infection on microsomal cytochrome P-450 species in the course of parasitic disease, all studies were carried out in both 3 and 6 week-infected rats, since at these stages, parasitism was characterized by respectively the beginning and the end of the histophagous migration of juvenile larvae through the liver parenchyma [6].

Materials and methods

Phenobarbital sodium, 3-methylcholanthrene, metyrapone, α-naphthoflavone and electrophoresis reagents were bought from Serva (Heidelberg, F.R.G.). Peroxidase conjugated immunoglobulins were purchased by Dako (Copenhagen, Denmark). All other chemicals were the highest quality available. Distilled deionized water was used in all studies.

In the study of induction in *Fasciola hepatica* infected animals, male Sprague–Dawley rats (140–160 g) were randomly distributed into control or infested groups of 9 rats and housed in cages of 3 or 4 animals. Each infected rat received by gastric tubing 20 metacercariae of *Fasciola hepatica* suspended in a 1% Tween aqueous solution. Parallel studies were carried out on uninfected control animals receiving the same solution without metacercariae. By weeks 3 and 6 after the infection, PB (80 mg/kg) dissolved in saline was administered (i.p.) daily for 3 days; MC dissolved in corn oil was injected by the same route at a single dose of 80 mg/kg. PB- and MC-treated rats were killed 24 and 48 hr respectively after the last injection.

After killing, hepatic microsomes were prepared by differential ultracentrifugation and stored as previously described [2]. The microsomal protein concentration was determined according to Lowry *et al.* [7] with bovine albumin as the standard. Cytochrome P-450 was measured in microsomes as described by Omura and Sato [8]. Aminopyrine *N*-demethylase and benzphetamine *N*-demethylase, activities were measured by the Nash reaction and ethoxycoumarin *O*-deethylase was determined by direct fluorimetry [9]. This reaction was also measured in presence of α-naphthoflavone [0.01 mM].

UT-A, the main constitutive cytochrome P-450 form, PB-B the main phenobarbital inducible form and BNF-B the main β-naphthoflavone inducible form were purified as previously described [10, 11]; in those papers, A_2NI corresponded to UT-A, B2PB to PB-B and B2MC to BNF-B. They were shown to be identical to those initially described [5] by western blotting [12]. Antibodies were raised in female New Zealand rabbits. Anti-UT-A was purified by immunoaffinity chromatography, it recognized in "western blots" male rat liver microsomes as a single band. Monoclonal anti-PB-B [13] recognized microsomes from PB-treated rats as a single band in "western blots". Anti-BNF-B were those described [11] and recognized liver microsomes as two bands, one corresponding to BNF-B and another one corresponding to ISF-G, the major form induced by isosafrole [5]. It was not possible to measure this form because of the lack of pure antigen.

The immunoquantification was performed as previously described [12] and the statistical analysis was carried out using analysis of variance followed by the Dunnett test [14].

Results and discussion

At autopsy, lesions characteristic of fascioliasis [2] were present in livers of all infected rats. As reported in Table 1, the sum of immunochemically determined specific contents of the three P-450s was greater than the conventional spectral measurement, which is in agreement with Dannan *et al.* [15].

Total cytochrome P-450 was decreased in untreated infected animals because of the decrease in UT-A form which is predominant in such untreated rats [5]. The slight decrease in PB-treated infected animals in comparison with corresponding controls would correspond to the decrease in $P\text{-}450_{UT\text{-}A}$ which was lowered by treatment with PB. Since this form is much more decreased, whereas $P\text{-}450_{BNF\text{-}B}$ becomes the essential form in MC-treated rats [15], fascioliasis provoked no change in these animals. The invariability of both $P\text{-}450_{PB\text{-}B}$ and $P\text{-}450_{BNF\text{-}B}$ inducibility in the course of fascioliasis leads us to reject the hypothesis [3] that incidence of fascioliasis could be due to a particular localization of tissue damages in relation to the specific localization of cytochrome P-450 isozymes within the liver lobule. The high sensitivity of aminopyrine *N*-demethylase

* Abbreviation used: P-450, cytochrome P-450; MC, 3-methylcholanthrene; PB, phenobarbital; BNF, β-naphthoflavone; ISF, isosafrole; UT-A: P-450 isozyme from untreated rats (form A).