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SHORT COMMUNICATIONS

Activation of propranolol and irreversible binding to rat liver microsomes: strain differences and effects of inhibitors

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Repetitive oral administration of propranolol to rats caused a marked decrease in propranolol hydroxylase activities of hepatic microsomes [1, 2]. Covalent binding of a reactive metabolic intermediate of propranolol to microsomal macromolecules was proposed in the mechanism of impairment of these activities following administration of propranolol [1, 3]. The reaction forming the reactive intermediate was suggested to be catalysed by cytochrome P450 (P450*) [1], but the species of P450 which catalyses the activation of propranolol is not clear. Our previous study [2] showed that the impairment of monooxygenase activity following treatment with propranolol was selective for propranolol 4-hydroxylase and debrisoquine 4-hydroxylase. From this finding, it is suggested that a P450 isozyme(s) that metabolizes debrisoquine is involved in the activation of propranolol. To confirm this suggestion, we investigated the effects of strain and sex differences and of inhibitors of debrisoquine 4-hydroxylase on irreversible binding of the reactive propranolol metabolite to microsomal macromolecules in rat liver. Wistar and DA strain [4, 5] rats were used as extensive and poor metabolizer animal models for debrisoquine 4-hydroxylase, respectively.

Materials and Methods

Chemicals. Propranolol hydrochloride was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). 4-[^{3}H]Propranolol hydrochloride (sp. act. 21 Ci/mmol) was purchased from Amersham International (Amersham, U.K.). The radiochemical purity of the labelled drug was stated to be at least 97.0% as determined by HPLC on a MCP C18 protein column using a gradient of 0.01 M trifluoroacetic acid to 0.01 M trifluoroacetic acid–acetonitrile (25:75). Debrisoquine hemisulfate was supplied by F. Hoffmann-La Roche Ltd (Basel, Switzerland). Sparteine sulfate, quinidine sulfate and phenacetin were purchased from Sigma. G-6-P, G-6-PDH and NADPH were purchased from the Oriental Yeast Co., Ltd (Tokyo, Japan). All other chemicals and solvents were of analytical grade.

Preparation of hepatic microsomes. Male and female Wistar rats were obtained from Takasugi Experimental Animal (Kasukabe, Japan). Male and female DA rats were obtained from Shizuoka Laboratory Co. (Shizuoka, Japan). All were 8 weeks old when used. The rats were killed by decapitation, and hepatic microsomal fractions were prepared according to the method of Omura and Sato [6]. Protein concentrations were determined by the method of Lowry *et al.* [7].

Incubation of labelled propranolol with hepatic microsomes. A 1-mL incubation mixture contained 10 mM G-6-P, 2 U G-6-PDH, 0.5 mM NADPH, 8 mM $MgCl_2$, 1 mg microsomal protein obtained from the rats described above and 2 μM (0.2 μCi) 4-[^{3}H]propranolol in 1 mL of 0.15 M potassium phosphate buffer (pH 7.4). In metabolic inhibition studies, debrisoquine, sparteine, quinidine or phenacetin was added to the mixture, and inhibitor concentrations ranged from 50 to 400 μM. After 5-min preincubation under air at 37°, the reaction was started by the addition of propranolol and NADPH. Incubation was performed for 5 min, and was stopped by the addition of 1 mL of 10% TCA.

Determination of irreversible binding of radioactivity to hepatic microsomes. Irreversible binding of radioactivity to hepatic microsomes after incubation of 4-[^{3}H]propranolol was measured as described by Nakagawa *et al.* [8]. After termination of the reaction, 4 mL of 10% TCA were added to the reaction mixture. The resultant precipitate was collected by centrifugation (2000 *g*, 10 min) and was resuspended in 4 mL of 7.5% TCA. After centrifugation, the washed pellet was extracted with 4 mL of 80% methanol (twice), 80% hot methanol (twice), methanol–ether (1:1, v/v, twice) and 80% methanol (twice). After the last extraction, no further radioactivity could be removed from the pellet. The thoroughly extracted precipitate was dissolved in 0.5 mL of 1 N NaOH, and was mixed with 10 mL of a scintillation medium. The medium consisted of one volume of Triton X-100 and two volumes of toluene solution including 4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis[2-(4-methyl-5-diphenyloxazolyl)]benzene per 1000 mL of toluene. The radioactivity in these samples was measured by a liquid scintillation counter (Beckman LS-1800).

Results and Discussion

Irreversible binding of radioactivity to microsomal macromolecules after incubation of liver microsomes with

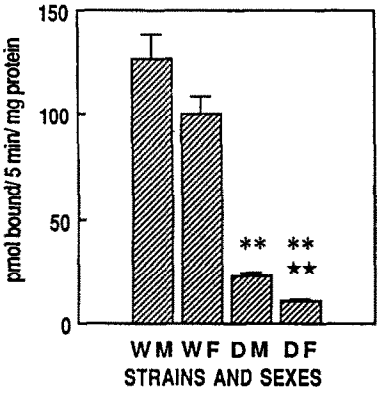

Fig. 1. Strain and sex differences in irreversibly bound radioactivity to microsomal macromolecules after incubation of 4-[^{3}H]propranolol with rat liver microsomes. WM, WF, DM and DF indicate the enzyme sources which were obtained from Wistar male, Wistar female, DA male and DA female rats, respectively. Incubation conditions were described in Materials and Methods. Values represent mean ± SE from nine rats. ** Significantly different from mean values of Wistar rats for the respective sexes ($P < 0.01$) and ★★ significantly different from mean values of DA male rats ($P < 0.01$) by Student's *t*-test.

* Abbreviations: P450, cytochrome P450; DA, Dark Agouti; G-6-P, glucose 6-phosphate; G-6-PDH, glucose 6-phosphate dehydrogenase; TCA, trichloroacetic acid.

4-[^{3}H]propranolol was observed in both Wistar and DA rats. No bound radioactivity was observed in the absence of NADPH necessary for propranolol metabolism (data not shown). The extent of binding for the corresponding sexes was markedly smaller in DA rats than in Wistar rats ($P < 0.01$, Fig. 1). In Wistar rats, the average bound radioactivity seemed to be higher in male than in female rats, but this was not significant ($P > 0.05$). On the other hand, the average bound radioactivity in male DA rats was 2-fold higher than that in females ($P < 0.01$). These findings show that the ability to convert propranolol to a reactive metabolite(s) is much lower in DA rats than in Wistar rats. Propranolol was, therefore, suggested to be mainly activated by a P450 isozyme(s) deficient in DA rats that belongs to the P450IID subfamily [9, 10].

Effects of the addition of debrisoquine, sparteine, quinidine and phenacetin on NADPH-dependent irreversible binding of radioactivity to hepatic microsomes obtained from male Wistar rats were examined. As shown in Fig. 2, irreversible binding was inhibited markedly by debrisoquine, sparteine and quinidine, but not by phenacetin. These data indicate that debrisoquine, sparteine and quinidine bind to the active site of the enzyme(s) responsible for the activation of propranolol, but phenacetin does not. Debrisoquine and sparteine are archetypal substrates of P450IID isozymes [9–11]. Quinidine is a specific inhibitor of P450IID isozymes [12], and phenacetin is a substrate of P450IA2 [13]. It is, therefore, suggested that a P450 isozyme(s) classified into the P450IID subfamily catalyses this activation reaction, being consistent with the strain difference in the activation of propranolol described above.

Shaw *et al.* [14] observed that incubation of human liver microsomes with isotope-labelled propranolol gave rise to NADPH-dependent irreversible binding of radioactivity to the microsomal protein. As compared with our results, the extent of binding was much smaller in humans than in rats. The binding was considerably decreased by the addition of debrisoquine and antipyrine, and was abolished completely by the addition of phenacetin [14]. The inhibitory effect of phenacetin on the binding has not been observed in our study, though inhibitor/substrate concentration ratios (25:1–200:1) are much larger in our study at a propranolol concentration of 2 μM than in their study (10:1) at a propranolol concentration of 100 μM. Therefore, this lack of inhibition is probably not due to an insufficient concentration of the inhibitor. Considering these experimental conditions, it is not clear whether the cause of the presence of inhibition is due to a species difference or not.

Our previous work [2] indicated that repetitive oral administration of propranolol selectively inhibits propranolol 4-hydroxylation and debrisoquine 4-hydroxylation. The P450 isozyme catalysing these reactions is classified into the P450IID subfamily [9, 15]. From these findings and the results obtained here, it is thought that binding of some reactive metabolite of propranolol to a P450 isozyme(s) belonging to the IID gene subfamily is one of the causes of impaired oxidation activities in rat liver microsomes. In that case, a P450 isozyme(s) of the IID subfamily is probably involved in a metabolic pathway of propranolol forming the reactive metabolite, since this isozyme(s) is situated in a position easily accessible to the reactive metabolite.

In summary, strain difference and inhibition studies showed that an enzyme(s) converting propranolol to a reactive metabolite capable of irreversible binding to microsomal macromolecules appeared to be a P450 isozyme(s) which catalyses debrisoquine 4-hydroxylation in rats. It seems likely that cytochrome P450 isozymes responsible for debrisoquine 4-hydroxylation activate propranolol and may be impaired after chronic use of propranolol also in human subjects. The findings obtained in the present study provide a clue for the elucidation of the mechanism of propranolol-induced impairment of the drug metabolizing enzyme system. Further studies using purified debrisoquine 4-hydroxylase are required to identify a P450 isozyme(s) responsible for the metabolic activation of propranolol. We are now performing experiments along this line.

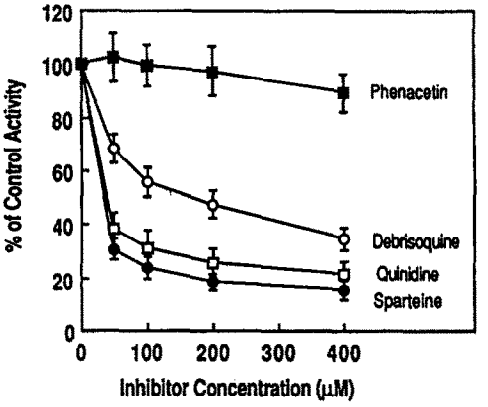

Fig. 2. Effects of various inhibitors on irreversibly bound radioactivity to microsomal macromolecules after incubation of 4-[^{3}H]propranolol with rat liver microsomes. Microsomes used were obtained from Wistar male rats. Incubation conditions were described in Materials and Methods. Results are expressed as per cent of the activity in the case of no inhibitor (control), and the control bound radioactivity was 123.5 ± 20.8 pmol/5 min/mg protein. Values represent mean ± SE from four rats.

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Species and congener specific induction of hepatic cytochrome P4504A by polychlorinated biphenyls

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Cytochrome P450 is a large gene family of haemoproteins responsible for the metabolism of a variety of substrates ranging from highly toxic environmental pollutants to biologically important endogenous substances, such as fatty acids in both man and experimental animals [1]. The cytochrome P4504AI isoenzyme is responsible for fatty acid 12-hydroxylation [2], and is a constitutive isoenzyme, which can be induced by treatment with hypolipidaemic agents [3], phthalate ester plasticizers [4] and by the pathophysiological state of diabetes [5]. Induction of cytochrome P4504AI by the ubiquitous environmental pollutants, polychlorinated biphenyls (PCBs*), was first reported in rats by Borlakoglu *et al.* [6] after treatment with Aroclor 1254. However, Aroclor 1254 is a technical grade mixture of PCBs, containing many different PCB congeners and isomers and previous work has demonstrated that different sub-families and particular cytochrome P450 isoenzymes are inducible by these isomers [7, 8]. We now report on the PCB-congener specific induction of the cytochrome P4504A sub-family as assessed by its associated lauric acid hydroxylase activity in the rat and the guinea pig, the latter species being chosen because of our recent arachidonic acid hydroxylase induction studies by a PCB in this species [9].

Materials and Methods

2,4,2′,4′-Tetrachlorobiphenyl (TCB) (99% pure) and 3,4,5,3′,4′-pentachlorobiphenyl (PENCB) (99% pure) were purchased from the Greyhound Chromatography & Allied Chemical Co. (Birkenhead, Merseyside, U.K.). Although it is unlikely because of the high purity of these chemicals, we cannot exclude the possibility that contaminants may be present which may have biological activity. All other chemicals were obtained from commercial sources and were of the highest purity available.

Male Wistar albino rats (100–130 g initial body weight, University of Surrey Breeders) were administered a single i.p. injection of either TCB (88 mg/kg in corn oil) or PENCB (5 mg/kg in corn oil), the control rats receiving an equivalent amount of the corn oil vehicle. Male Dunkin Hartley guinea pigs (130–190 g initial body weight, University of Surrey Breeders) were given a single i.p. injection of either a high dose (88 mg/kg) or low dose (29 mg/kg) of TCB in corn oil. PENCB was given to guinea pigs by a single i.p. injection at the dose levels of 0.025, 0.05, 0.05, 0.1, 0.5 and 1.0 mg/kg. These dose levels in the rat and guinea pig were chosen because they caused maximal induction of cytochrome P450 in a previous study [9]. The animals were maintained in a 12 hr light/12 hr dark cycle with feed provided *ad lib.* and killed 5 days after dosing by cervical dislocation. The livers were immediately removed and perfused with 0.9% (w/v) ice-cold saline, blotted dry, weighed and scissor-minced in 0.25 M ice-cold sucrose and a 25% (w/v) homogenate prepared. Liver microsomal fractions were isolated by ultracentrifugation as described previously [9], and microsomal protein [10], total cytochrome P450 [11] and lauric acid 11- and 12-hydroxylase activities [12] determined as indicated.

Results and Discussion

TCB and PENCB are representatives of the phenobarbital-type and 3-methylcholanthrene-type of PCB inducers, respectively [7, 8], and both significantly increased the total hepatic cytochrome P450 content, but differentially influenced laurate 11- and 12-hydroxylase activities in the rat (Table 1). At a dose of 88 mg/kg, TCB significantly

* Abbreviations: PCB, polychlorinated biphenyls; TCB, 2,4,2′,4′-tetrachlorobiphenyl; PENCB, 3,4,5,3′,4′-pentachlorobiphenyl.