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IMPAIRMENT OF DEBRISOQUINE 4-HYDROXYLASE AND RELATED MONOOXYGENASE ACTIVITIES IN THE RAT FOLLOWING TREATMENT WITH PROPRANOLOL

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Abstract—The effect of repetitive oral administration of propranolol on hepatic microsomal drug metabolizing enzyme activities in the rat was investigated. Propranolol ring (4-, 5- and 7-)hydroxylase activities were markedly decreased, but, interestingly, *N*-desisopropylase activity was increased after propranolol administration. A marked decrease in enzyme activity after propranolol pretreatment was also observed with debrisoquine 4-hydroxylation. In addition, a similar decrease was observed with imipramine 2-hydroxylation which co-segregates with debrisoquine/sparteine type polymorphic drug oxidation, but not with imipramine N-demethylation. These results suggest the selective impairment of debrisoquine 4-hydroxylase by propranolol pretreatment.

Repetitive oral administration of propranolol to rats caused a marked decrease of hepatic microsomal propranolol 4-hydroxylase activity [1]. Covalent binding of a metabolic intermediate of propranolol with the molecular form of cytochrome P450 was suggested as its cause. But metabolic activities for ethylmorphine *N*-demethylase and aryl hydrocarbon hydroxylase were not affected in these rats. The total cytochrome P450 content in microsomal preparations derived from propranolol-pretreated rats was also reported to be unaffected [1]. It was proposed, therefore, that selective covalent binding of the reactive metabolic intermediate to cytochrome P450 that ring hydroxylates propranolol would account for the marked inhibition of propranolol metabolism [1]. Shaw *et al.* [2] also observed that a product(s) of propranolol oxidation bound irreversibly but non-selectively to human liver microsomal protein, and that the extent of binding was decreased to a considerable extent by the addition of debrisoquine, antipyrine and phenacetin.

Genetic deficiency in oxidative metabolism is known for several drugs such as debrisoquine and mephenytoin in humans [3–6]. Propranolol 4-hydroxylation was found to co-segregate with debrisoquine/sparteine type polymorphic oxidation in humans from *in vivo* observation [7–9]. In human liver microsomes, antibodies prepared to a form of cytochrome P450 responsible for debrisoquine 4-hydroxylation in rats were found to inhibit propranolol 4-hydroxylation [10]. Furthermore, quinidine, a potent specific inhibitor of debrisoquine 4-hydroxylation was reported to markedly reduce propranolol 4-hydroxylation [11]. From these results, the species of cytochrome P450 impaired by propranolol pretreatment can be presumed to be cytochrome P450 IID1 [12–14], the human analogue of which catalyses both debrisoquine 4-hydroxylation and propranolol 4-hydroxylation. However, Shaw *et al.* [2] observed the lack of any inhibitory effect of debrisoquine on the rate of propranolol 4-hydroxylation in human liver microsomes. Instead, they observed very potent inhibitory action of phenacetin. They concluded that the enzyme system responsible for the irreversible binding and 4-hydroxylation of propranolol appears to be related more closely to the cytochrome P450 system which metabolizes phenacetin than to that metabolizing debrisoquine. Their conclusion is at variance with the clinical observations [7–9, 11] and with the immunological study [10] described above.

In the present work, we studied the effect of propranolol pretreatment on propranolol, debrisoquine and imipramine metabolism in rat liver microsomes to present the evidence that propranolol pretreament selectively inhibits the cytochrome P450 isozyme(s) that catalyse(s) the polymorphic oxidation of debrisoquine and other drugs.

MATERIALS AND METHODS

Chemicals. Propranolol hydrochloride and imipramine hydrochloride was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). 4-Hydroxypropranolol and *N*-desisopropylpropranolol were provided by ICI Pharmaceuticals Co. (Macclesfield, U.K.). 5- and 7-Hydroxypropranolol were synthesized according to the method of Oatis *et al.* [15]. 2-Hydroxyimipramine and *N*-desmethylimipramine hydrochloride were provided by Geigy (Basel, Switzerland) and nortriptyline was supplied by the Dainippon Pharmaceutical Co., Ltd (Osaka, Japan). 4-Hydroxybunitrolol was supplied by Nippon C.H. Boehringer Sohn Co., Ltd (Osaka, Japan). Debrisoquine hemisulfate and 4-hydroxydebrisoquine hemisulfate were supplied by Hofmann-La

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Roche Co. (Basel, Switzerland). Other chemicals and cofactors used were of analytical or biochemical grade.

Pretreatment of animals and preparation of hepatic microsomes. Male Wistar rats weighing 250–300 g were treated with propranolol (100 mg/kg/day p.o. for 5 days) according to the treatment regimen employed by Schneck and Pritchard [1]. These rats were killed by decapitation along with non-treated rats 30 hr after the final administration, instead of 18 hr employed by Schneck and Pritchard to avoid residual propranolol in hepatic microsomes prepared from pretreated rats. Hepatic microsomal fractions were prepared according to the method of Omura and Sato [16]. Protein concentrations were determined by the method of Lowry *et al.* [17].

Assay methods of enzymatic activities. Propranolol 4-, 5-, 7-hydroxylase and *N*-desisopropylase activities were assayed by the high-performance liquid chromatographic (HPLC) method [18] with modifications which enabled the simultaneous determination of propranolol and its four major metabolites described below. The rates of oxidation of propranolol were measured in a 1-mL incubation mixture containing 5 μM or 1 mM of propranolol, 10 mM glucose-6-phosphate (G-6-P), 2 units glucose-6-phosphate dehydrogenase (G-6-PDH), 0.5 mM NADPH, 8 mM $MgCl_2$ and 0.5 mg microsomal protein in 1 mL of 0.15 M potassium phosphate buffer (pH 7.4). After 5 min preincubation under air at 37°, reaction was started by the addition of propranolol and incubation was performed for 30 sec. The reaction was stopped by the addition of 1 mL of 1 N NaOH containing 25 mg of sodium bisulfite as an antioxidant to the assay mixture. 4-Hydroxybunitrolol was then added as internal standard. After extraction with ethyl acetate, the organic phase was evaporated to dryness and the residue was dissolved in HPLC mobile phase (CH_3CN :CH_3OH : H_2O : CH_3COOH = 22 : 22: 56:1). The sample was applied to a reversed-phase column (Inertsil ODS, Gasukuro Kogyo Ltd, Tokyo, Japan). The fluorescent intensity of propranolol and its metabolites was continuously monitored with an excitation wavelength at 310 nm and an emission wavelength at 380 nm.

Imipramine 2-hydroxylase and *N*-demethylase activities were also assayed by an HPLC method [19] after 30 sec incubation of 5 μM of imipramine with the microsomal assay mixture. The extracted samples were applied to a reversed-phase column (Inertsil ODS) using the pH 7.4 mobile phase (CH_3CN : CH_3OH : H_2O : di-*n*-butylamine = 48:50:53:2). The UV absorption intensity was monitored at 254 nm.

Debrisoquine 4-hydroxylase assay will be described elsewhere, but briefly, 50 μM of debrisoquine was incubated for 5 min with a 0.5-mL assay mixture containing 10 mM G-6-P, 1 unit G-6-PDH, 0.5 mM NADPH, 10 mM $MgCl_2$ and 0.5 mg microsomal protein in 0.15 M potassium phosphate buffer (pH 7.4) at 37°. Reaction was stopped with 0.5 mL of 12 N NaOH. After extraction of debrisoquine and its metabolites with ethyl acetate, glycinexylidide as internal standard and 0.01 N H_2SO_4 were added. After vortex mixing and centrifugation, the upper layer was removed. It was neutralized by 0.01 N NaOH, evaporated to dryness and was dissolved in HPLC mobile phase (CH_3CN:0.1 M potassium phosphate buffer pH 3.0 = 15:85). The sample was applied to a reversed-phase HPLC column (Inertsil ODS). The UV absorption intensity was monitored at 210 nm. The limit of detection of the assay was 0.025 nmol/mL.

RESULTS AND DISCUSSION

As shown in Fig. 1, propranolol 4-hydroxylase activity at a propranolol concentration of 5 μM was markedly decreased in rat liver microsomes by propranolol pretreatment. On the other hand, propranolol *N*-desisopropylase activity was markedly increased by propranolol pretreatment. These data agree with the data of Schneck and Pritchard. [1]. Furthermore, propranolol 5- and 7-hydroxylase activities were also significantly decreased. These data indicate that propranolol pretreatment selectively inhibits naphthalene ring oxidation but not N-desisopropylation of the side-chain. Similar position selective impairment and enhancement of propranolol metabolism were obtained at a propranolol concentration of 1 mM (Fig. 1). *N*-Desisopropylase activity was increased by propranolol pretreatment even at a propranolol concentration of 1 mM which is well above its K_m value of 137 μM [1], suggesting that this increase is due to the induction of propranolol *N*-desisopropylase.

Debrisoquine 4-hydroxylase activity in the microsomes used in the experiments of Fig. 1 was also investigated to confirm whether it is indeed decreased by propranolol pretreatment. Michaelis–Menten analysis of microsomal debrisoquine 4-hydroxylase activity in the rat has been reported to be biphasic [20]. Debrisoquine 4-hydroxylase by liver microsomes from female DA rats was found to be substantially lower than by microsomes from other strain rats, and a major difference between DA and other strain rats was observed in V_{max} of a high-affinity and low-capacity component whose K_m was about 10 μM [20]. Therefore, we employed 50 μM as substrate concentration, because at this concentration the contribution of low-affinity and high-capacity component (K_m = 1 mM [20]) is negligible and debrisoquine 4-hydroxylase activity determined corresponds to be more than 80% of the V_{max} of the high-affinity component. As shown in Fig. 2, mean debrisoquine 4-hydroxylase activity was decreased to 40% of the mean value of the non-treated control by propranolol pretreatment. This result is similar to the case of propranolol ring hydroxylation.

Figure 3 shows imipramine 2-hydroxylase and *N*-demethylase activities in liver microsomal fractions obtained from propranolol-pretreated and non-treated rats when incubated at low imipramine concentration (5 μM). Imipramine 2-hydroxylase activity was markedly decreased by propranolol pretreatment, whereas the decrease of imipramine N-demethylation was not statistically significant ($P < 0.05$). The effect of propranolol pretreatment on imipramine metabolism was pathway selective similarly to the case of propranolol.

A recent human study indicated that propranolol

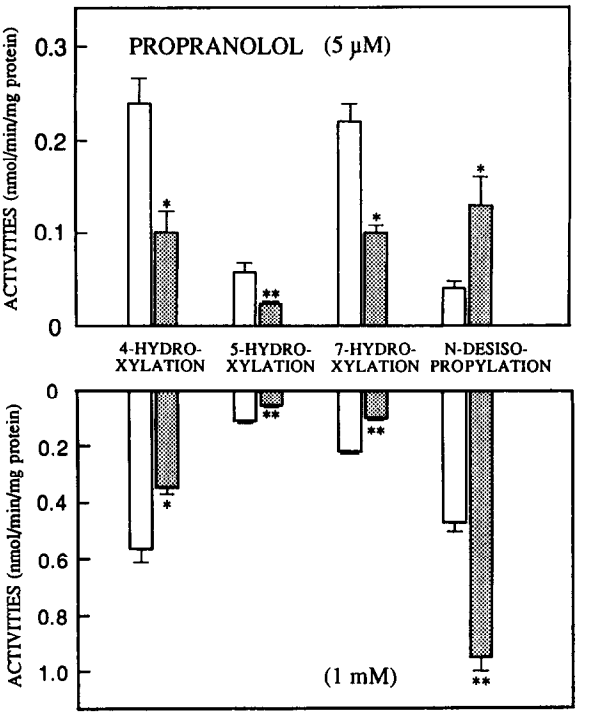

Fig. 1. Effect of propranolol pretreatment on propranolol 4-, 5-, 7-hydroxylase and *N*-desisopropylase activities in rat liver microsomes. Microsomes were obtained from non-treated (□) and propranolol pretreated (100 mg/kg/day p.o. 5 days, ▨), rats. Activity was assayed at a propranolol concentration of 5 μM or 1 mM. Data represent the means ± SE from three determinations. * $P < 0.05$, ** $P < 0.01$, compared with non-treated rats by Student's *t*-test.

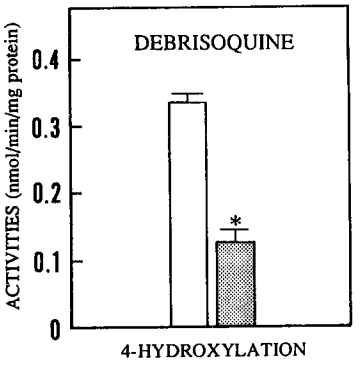

Fig. 2. Effect of propranolol pretreatment on debrisoquine 4-hydroxylase activities in rat liver microsomes. Shading patterns of columns correspond to those in Fig. 1. Activity was assayed at a debrisoquine concentration of 50 μM. Data represent the means ± SE from three determinations. * $P < 0.05$, compared with non-treated rats by Student's *t*-test.

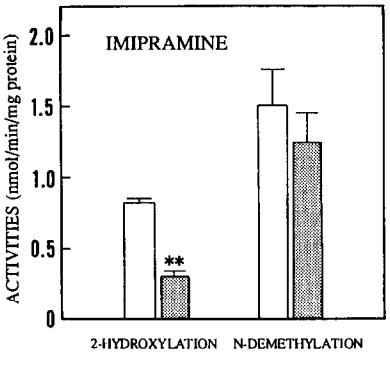

Fig. 3. Effect of propranolol pretreatment on imipramine 2-hydroxylase and *N*-demethylase activities in rat liver microsomes. Shading patterns of columns correspond to those in Fig. 1. Activity was assayed at an imipramine concentration of 5 μM. Data represent the means ± SE from three determinations. ** $P < 0.01$, compared with non-treated rats by Student's *t*-test.

4-hydroxylation and N-desisopropylation co-segregated separately with polymorphic debrisoquine 4-hydroxylation and mephenytoin 4-hydroxylation, respectively [9]. Imipramine 2-hydroxylation was shown to co-segregate with debrisoquine/sparteine type polymorphic oxidation in humans [21–23]. We found that a male specific P450 isozyme was predominantly responsible for imipramine N-demethylation in male rats [24]. In the present study, we found that not only propranolol 4-hydroxylase

activity, but debrisoquine 4-hydroxylase and imipramine 2-hydroxylase activities were markedly decreased. In addition, the inhibitory effect of propranolol pretreatment on its own metabolism and imipramine metabolism showed pathway selectivity. These data suggest that the species of cytochrome P450 impaired by propranolol pretreatment is cytochrome P450 IID1, the human analogue of which catalyses the polymorphic oxidation of debrisoquine and other drugs. It is also suggested that the possibility of the impairment of propranolol 5- and 7-hydroxylation is related to debrisoquine 4-hydroxylase.

Shaw *et al.* [2] observed that debrisoquine at 10-fold the concentration of propranolol did not inhibit propranolol 4-hydroxylase activity. They concluded from these data that the cytochrome P450 isozyme involved in the irreversible binding of propranolol to human liver microsomes appears to be other than debrisoquine 4-hydroxylase, despite the fact that propranolol 4-hydroxylation is closely linked to debrisoquine 4-hydroxylation in humans [7–10]. This lack of inhibition is probably due to an insufficient concentration of the inhibitor caused by a large difference between the K_m values of these two reactions. Indeed the K_m value (0.2 μM) of propranolol 4-hydroxylation was observed to be much lower [25] than that of debrisoquine 4-hydroxylation [20].

Oxidation of sparteine and debrisoquine was practically abolished in quinidine-treated patients [26, 27]. This was presumed to be due to an inhibition of sparteine and debrisoquine hydroxylation by quinidine. It is possible that a similar drug–drug interaction may occur after propranolol administration due to residual propranolol in liver microsomes. In the present study, however, residual propranolol carried over from the pretreated doses was not detected in liver microsomes (detection limit of 3 pmol/mL) from propranolol-pretreated rats. So the inhibition of metabolism is likely to be due to irreversible binding of a propranolol metabolite(s) to a cytochrome P450 isozyme(s) [1] or to the destruction of a cytochrome P450 isozyme(s) by a propranolol metabolite(s).

In summary, propranolol and imipramine ring hydroxylase and debrisoquine 4-hydroxylase activities in rat liver microsomes were selectively inhibited by propranolol pretreatment. Irreversible binding of isotope labeled propranolol similar to that observed in rats [1] was reported also in human liver microsomes [2]. Furthermore, when propranolol was given chronically, an enhancement of oral availability and propranolol accumulation were observed in humans [28]. These reports seem to suggest the possibility that cytochrome P450 isozymes responsible for debrisoquine 4-hydroxylation may be inhibited after chronic use of propranolol in humans also. One should cautiously pay attention to the drug history of a patient in discriminating debrisoquine extensive and poor metabolizer phenotypes.

REFERENCES

1. Schneck DH and Pritchard JF, The inhibitory effect of propranolol pretreatment on its own metabolism in the rat. *J Pharmacol Exp Ther* **218**: 575–581, 1981.
2. Shaw L, Lennard MS, Tucker GT, Bax NDS and Woods HF, Irreversible binding and metabolism of propranolol by human liver microsomes—relationship to polymorphic oxidation. *Biochem Pharmacol* **36**: 2283–2288, 1987.
3. Mahgoub A, Idle JR, Dring LG, Lancaster R and Smith RL, Polymorphic hydroxylation of debrisoquine in man. *Lancet* **2**: 584–586, 1977.
4. Idle JR and Smith RL, Polymorphism of oxidation at carbon centers of drugs and their clinical significance. *Drug Metab Rev* **9**: 301–317, 1979.
5. Eichelbaum M, Defective oxidation of drugs: pharmacokinetics and therapeutic implications. *Clin Pharmacokin* **7**: 1–22, 1982.
6. Kupfer A and Preisig R, Pharmacogenetics of mehenytoin: a new drug hydroxylation polymorphism in man. *Eur J Clin Pharmacol* **26**: 753–759, 1984.
7. Lennard MS, Jackson PR, Freestone S, Tucker GV, Ramsay LE and Woods HF, The relationship between debrisoquine oxidation phenotype and pharmacokinetics and pharmacodynamics of propranolol. *Br J Clin Pharmacol* **17**: 679–685, 1984.
8. Raghuram TC, Koshakji RP, Wilkinson GR and Wood AJJ, Polymorphic ability to metabolize propranolol alters 4-hydroxypropranolol levels but not beta blockade. *Clin Pharmacol Ther* **36**: 51–56, 1984.
9. Ward SA, Walle T, Walle K, Wilkinson GR and Branch RA, Propranolol's metabolism is determined by both mephenytoin and debrisoquin hydroxylase activities. *Clin Pharmacol Ther* **45**: 72–79, 1989.
10. Distlerath LM and Guengerich FP, Characterization of a human liver cytochrome P-450 involved in the oxidation of debrisoquine and other drugs by using antibodies raised to the analogous rat enzymes. *Proc Natl Acad Sci USA* **81**: 7348–7352, 1984.
11. Zhou HH, Anthony LB, Roden DM and Wood AJJ, Quinidine reduced clearance of (+)-propranolol more than (−)-propranolol through marked reduction in 4-hydroxylation. *Clin Pharmacol Ther* **47**: 686–693, 1990.
12. Gonzalez FJ, Matsunaga T, Nagata K, Meyer UA, Nevert DW, Pastewka J, Kozak CA, Gillette J, Gelboin HV and Hardwick JP, Debrisoquine 4-hydroxylase: characterization of a new P450 gene subfamily, regulation, chromosomal mapping, and molecular analysis of the DA rat polymorphism. *DNA* **6**: 149–161, 1987.
13. Nebert DW, Nelson DR, Adesnik M, Coon MJ, Estabrook RW, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF, Kemper B, Levin W, Phillips IR, Sato R and Warterman MR, The P450 superfamily: updated listing of all genes and recommended nomenclature for the chromosal loci. *DNA* **8**: 1–13, 1989.
14. Matsunaga E, Zanger UM, Hardwick JP, Gelboin HV, Meyer UA and Gonzalez FJ, The CYP2D gene subfamily: analysis of molecular basis of the debrisoquine 4-hydroxylase deficiency in DA rats. *Biochemistry* **28**: 7349–7355, 1989.
15. Oatis JE, Russel MP, Knapp DR and Walle T, Ring-hydroxylated propranolol: synthesis and beta-receptor antagonism and vasodilator activities of the seven isomers. *J Med Chem* **24**: 309–314, 1981.
16. Omura T and Sato R, The carbon monooxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J Biol Chem* **239**: 2370–2378, 1964.
17. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
18. Fujita S, Usui T, Suzuki M, Hirasawa M, Mori Y, Naganuma H and Suzuki T, Induction of hepatic

microsomal propranolol *N*-desisopropylase activity by 3-methylcholanthrene and sudan III. *Biochem Biophys Res Commun* **105**: 1233–1239, 1982.
19. Chiba M, Nishihara E, Fujita S and Suzuki T, Position selective sex difference in imipramine metabolism in rat liver microsomes. *Biochem Pharmacol* **34**: 898–900, 1984.
20. Kahn GC, Rubenfield M, Davis DS, Murray S and Boobis AR, Sex and strain difference in hepatic debrisoquine 4-hydroxylase activity in the rat. *Drug Metab Dispos* **13**: 510–516, 1985.
21. Brosen K, Otton V and Gram LF, Imipramine demethylation and hydroxylation: impact of the sparteine oxidation phenotype. *Clin Pharmacol Ther* **40**: 543–549, 1986.
22. Brosen K, Recent developments of hepatic drug oxidation—implications for clinical pharmacokinetics. *Clin Pharmacokin* **18**: 220–239, 1990.
23. Brosen K and Gram LF, Quinidine potently inhibits the 2-hydroxylation of imipramine and desipramine but not the demethylation of imipramine. *Eur J Clin Pharmacol* **37**: 155–160, 1989.
24. Fujita S, Morimoto R, Chiba M, Kitani K and Suzuki T, Evaluation of the involvement of a male specific cytochrome P-450 isozyme in senesence-associated decline of hepatic drug metabolism in male rat. *Biochem Pharmacol* **38**: 3925–3931, 1989.
25. Fujita S, Ishida R, Kagimoto N, Suzuki K, Masubuchi Y, Chiba M, Funae Y and Suzuki T, Mechanism of alterations of non-linearity in hepatic first-pass metabolism of propranolol: alterations in the relative abundance of cytochrome P-450 isozymes in the liver microsomes in relation to the organ-level metabolic activities. *J Pharmacobio-Dyn* **13**: s-98, 1990.
26. Brinn R, Brosen K, Gram LF, Haghfelt T and Otton SV, Sparteine oxidation is practically abolished in quinidine-treated patients. *Br J Clin Pharmacol* **22**: 194–197, 1986.
27. Brosen K, Gram LF, Haghfelt T and Bertilsson L, Extensive metabolizers of debrisoquine become poor metabolizers during quinidine treatment. *Pharmacol Toxicol* **60**: 312–314, 1987.
28. Evans GH and Shand DG, Disposition of propranolol. V. Drug accumulation and steady-state concentrations during chronic oral administration in man. *Clin Pharmacol Ther* **13**: 181–195, 1973.