Pergamon

Biochemical Pharmacology, Vol. 48, No. 10, pp. 1891–1898, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)00362-9

ROLE OF THE CYP2D SUBFAMILY IN METABOLISM-DEPENDENT COVALENT BINDING OF PROPRANOLOL TO LIVER MICROSOMAL PROTEIN IN RATS

YASUHIRO MASUBUCHI, SHIZUO NARIMATSU,* SHIN HOSOKAWA and TOKUJI SUZUKI

Laboratory of Biopharmaceutics, Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263, Japan

(*Received* 17 *March* 1994; *accepted* 8 *August* 1994)

Abstract—*In vitro* covalent binding of a chemically reactive metabolite of propranolol to microsomal macromolecules, which is presumed to cause inhibition of its own metabolism in rats, was diminished in liver microsomes from rats pretreated with propranolol. Covalent binding was suppressed by the addition of an antibody against P450BTL, which is a cytochrome P450 (P450) isozyme belonging to the CYP2D subfamily. SDS–PAGE of microsomal proteins after incubation with [^{3}H]propranolol and NADPH indicated that the binding was non-selective but prominent at the molecular mass of approx. 50 kDa, corresponding to those of the P450 protein. The radioactivity peak was markedly but not completely diminished by the addition of reduced glutathione. In a reconstituted system containing P450BTL, NADPH-cytochrome P450 reductase (fp_2) and dilauroylphosphatidylcholine, propranolol 4-, 5- and 7-hydroxylase activities decreased time dependently following preincubation with propranolol in the presence of NADPH, indicating time-dependent inactivation of P450BTL. The covalent binding of a reactive metabolite of [^{3}H]propranolol to the proteins was also observed in this system. SDS–PAGE showed that among the three proteins in the reconstituted system, fp_2 and P450BTL consisting of two polypeptides with molecular masses of 49 and 32 kDa, the binding was specific for a polypeptide corresponding to the P450 isozyme with a molecular mass of 49 kDa. In addition, the ratio of the amount of covalently bound radiolabelled materials to that of P450BTL which was estimated from each impaired propranolol hydroxylase activity under the same reconstitutional conditions was calculated to be approx. 1.0. These findings indicate that propranolol is a mechanism-based inactivator of a cytochrome P450 isozyme(s) belonging to the CYP2D subfamily.

Key words: metabolic activation; cytochrome P450; mechanism-based inactivation; reduced glutathione; debrisoquine; propranolol 4-hydroxylation

Propranolol is a β-adrenergic blocking agent used in the treatment of hypertension, angina pectoris, cardiac arrhythmias and other diseases. The metabolism of propranolol has been quantitatively studied in experimental animals and humans [1, 2]. Propranolol is metabolized to a number of products, some of which are pharmacologically active [3, 4]. Major primary metabolites of propranolol are ring-hydroxylated products, 4-, 5- and 7-hydroxypropranolol (4-OH-P, 5-OH-P and 7-OH-P†, respectively), and a side-chain-oxidised product, NDP in rat liver microsomes [5, 6]. *In vivo* observation in human subjects showed that propranolol 4-hydroxylation co-segregated with debrisoquine-type polymorphic drug oxidation [7–9]. Studies using human liver microsomes indicated that CYP2D6, a human debrisoquine 4-hydroxylase, was involved in propranolol 4-hydroxylation [10, 11]. Recently, we found that a rat hepatic P450 isozyme(s) belonging to the CYP2D subfamily participated not only in propranolol 4-hydroxylation but also in 5- and 7-hydroxylations [12, 13].

Previous reports showed that propranolol was a potent inhibitor of P450-mediated drug metabolism in rats and humans [14, 15]. Furthermore, repetitive oral administration of propranolol to rats caused a marked decrease in liver microsomal propranolol 4-, 5- and 7-hydroxylations, which are catalysed by the CYP2D subfamily, without loss of spectrally detectable P450 [16, 17]. We also found that debrisoquine 4-hydroxylase and imipramine 2-hydroxylase activities [17], both of which are catalysed by the CYP2D subfamily [18, 19], were decreased by propranolol pretreatment in rats. From these findings, it was considered that a P450 isozyme(s) in the CYP2D subfamily was decreased and/or inactivated by the pretreatment.

Covalent binding of a metabolic intermediate of propranolol to liver microsomal protein was proposed as a mechanism of the impairment of monooxygenase activities following repetitive administration of propranolol [16, 20]. The covalent binding required NADPH and oxygen, and was inhibited by SKF-525A, a classical inhibitor of P450 [16]. These data suggest that propranolol is converted by P450-dependent monooxygenation to a chemically reactive metabolic intermediate which binds covalently to rat

* Corresponding author. Tel. (81)43-290-2936; FAX (81)43-255-1574.

† Abbreviations: X-OH-P, X-hydroxypropranolol; NDP, *N*-desisopropylpropranolol; P450, cytochrome P450; fp_2, NADPH-cytochrome P450 reductase; DLPC, dilauroylphosphatidylcholine; G-6-P, glucose 6-phosphate; G-6-PDH, glucose 6-phosphate dehydrogenase; GSH, reduced glutathione.

liver microsomal protein [16]. Furthermore, we found that the formation of the reactive species was markedly lower in Dark Agouti rats than in Wistar rats, the former having a lesser ability to metabolise debrisoquine than the latter [20]. Inhibition of metabolism-dependent covalent binding by typical substrates and inhibitors of the CYP2D subfamily was also observed [20]. On the basis of these observations, we presumed that a reactive intermediate was formed through propranolol metabolism by a P450 isozyme(s) belonging to the CYP2D subfamily, resulting in the inactivation of the P450 species by covalent binding of the intermediate to the enzyme itself.

To investigate the hypothetical mechanism of the propranolol-induced impairment of monooxygenase activities, the following studies were conducted: (1) effects of *in vivo* propranolol pretreatment and *in vitro* addition of the antibody against P450BTL, a P450 isozyme belonging to the CYP2D subfamily [21], on the covalent binding of a reactive metabolite of propranolol to liver microsomal protein were examined to clarify the involvement of the CYP2D isozyme in the formation of a reactive intermediate; (2) microsomal proteins incubated with [^{3}H]-propranolol were separated by SDS–PAGE, and localization of the radioactivities were measured; (3) effects of preincubation of P450BTL with propranolol on the P450BTL-catalysed propranolol hydroxylase activities were examined in a reconstituted system containing P450BTL, fp_2, DLPC and NADPH; and (4) covalent binding of a [^{3}H]propranolol metabolite to P450BTL in the reconstituted system was also measured, and the relationship between the enzyme activities and covalent binding of ^{3}H-labelled materials to the enzyme was investigated.

MATERIALS AND METHODS

Chemicals. Propranolol hydrochloride was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). [^{3}H]Propranolol hydrochloride labelled at the 4-position (specific activity 21 Ci/mmol) was obtained from Amersham International (Amersham, U.K.). The radiochemical purity of [^{3}H]propranolol was stated to be at least 97.0% as determined by HPLC on an MCP C18 protein column using a gradient of 0.01 M trifluoroacetic acid to 0.01 M trifluoroacetic acid/acetonitrile (25:5 v/v). 4-OH-P and NDP were supplied by the ICI Pharmaceuticals Co. (Macclesfield, U.K.). 5-OH-P and 7-OH-P were synthesized according to the method of Oatis *et al.* [3]. Bunitrolol hydrochloride and 4-hydroxybunitrolol hydrochloride were supplied by the Nippon C.H. Boehringer Sohn Co., Ltd (Osaka, Japan). G-6-P, G-6-PDH and NADPH were purchased from the Oriental Yeast Co., Ltd (Tokyo, Japan) and GSH from the Wako Chemical Co. (Osaka, Japan). All other chemicals and solvents used were of analytical grade.

Pretreatment of animals and preparation of liver microsomes. Male Wistar rats (2 months old) were obtained from Takasugi Experimental Animals (Kasukabe, Japan). The rats were pretreated with propranolol (100 mg/kg/day p.o. for 5 days) and were killed by decapitation along with non-treated rats 30 hr after final administration. Liver microsomal fractions were prepared according to the method of Omura and Sato [22].

Purification of P450BTL and preparation of anti-P-450BTL IgG. P450BTL was purified from liver microsomes of male Sprague–Dawley rats (2 months old, the Shizuoka Laboratory Co., Shizuoka, Japan) as described elsewhere [21]. SDS–PAGE of the purified P450BTL showed two protein bands corresponding to molecular masses of 49 and 32 kDa. The N-terminal amino acid sequence of the 49 kDa protein (GLLIGXDLMAVVXFXAIXLL) [21] was very similar to that of CYP2D2 [23]. P450BTL exhibited high debrisoquine 4-hydroxylase activity (2.22 and 5.63 nmol/min/nmol P450 at substrate concentrations of 50 μM and 2 mM, respectively) [21]. Thus, P450BTL was indicated to belong to the CYP2D subfamily.

The specific content of P450BTL was 5.02 nmol/mg protein. This relatively low value may be due to involvement of the 32 kDa protein. The protein with the low molecular mass is thought to be the same species which was contained in other P450 preparations belonging to the CYP2D subfamily [18, 24, 25]. The N-terminal amino acid sequence of the 32 kDa protein (MWLYLLALVGLXN) did not show high homology to any protein reported so far. The nature of the protein remains to be characterized, but was shown to be tightly bound to the P450 protein and to be essential to the expression of the maximum metabolic activities of the CYP2D subfamily [24].

The antibody against P450BTL was raised in female Japanese white rabbits (3 months old, Takasugi Experimental Animals) and the IgG fraction was prepared as described elsewhere [21]. The anti-P450BTL IgG suppressed microsomal debrisoquine 4-hydroxylase activity almost completely [21]. In immunoblot analysis, only two protein bands corresponding to 49 and 32 kDa proteins were clearly visualized on a membrane. Immunoblot analysis using purified rat liver P450 isoforms revealed that the antibodies did not cross-react with the CYP1A, 2B, 2C, 2E or 3A subfamily. The specificity of the antibody was also confirmed by regioselective immunoinhibition of CYP2D-dependent reactions using propranolol (ring-hydroxylations at 4, 5 and 7-positions) [13], lidocaine (3-hydroxylation) [26] and imipramine (2-hydroxylation, unpublished data) as substrates.

Incubation of liver microsomes with propranolol. A 1-mL incubation mixture containing 10 mM G-6-P, 2 U G-6-PDH, 0.5 mM NADPH, 8 mM $MgCl_2$, 1 mg microsomal protein, 2 μM (0.2 μCi) [^{3}H]-propranolol and 154 mM potassium phosphate buffer (pH 7.4) was used. After 5-min preincubation under air at 37°, reaction was started by adding the NADPH and was stopped 5 min later by adding 1 mL of 10% trichloroacetic acid. In immunoinhibition studies, various amounts of anti-P450BTL IgG or preimmune IgG were preincubated with liver microsomes at 25° for 30 min, followed by incubation with other components of the mixture. To determine localization of covalently bound radioactivity, 0.1 mL of incubation mixture containing 2 μM (1 μCi) [^{3}H]-propranolol and the ingredients 10-fold those

described above were incubated for 30 min with or without NADPH and a portion of the sample was subjected to SDS–PAGE. In some experiments, 2 mM GSH was added in the presence of NADPH. Microsomes were also incubated with unlabelled propranolol and NADPH in the presence or absence of GSH, followed by assay of bunitrolol 4-hydroxylase activity according to the reported method [27].

Determination of covalent binding of radioactivity to liver microsomal protein. Covalent binding of labelled material to microsomal protein after incubation of liver microsomes with [^{3}H]propranolol was measured as described by Nakagawa *et al.* [28]. After incubation was terminated by adding 1 mL of 10% trichloroacetic acid, 4 mL of 10% trichloroacetic acid was added to the reaction mixture. The resultant precipitate was collected by centrifugation (2000 *g*, 10 min) and resuspended in 4 mL of 7.5% trichloroacetic acid. 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 thus obtained was dissolved in 1 N NaOH, and was mixed with 10 mL of scintillation medium for the determination of radioactivity of a material(s) which bound irreversibly to microsomal protein. The radioactivity in these samples was measured by a liquid scintillation counter (Beckman LS-1800). The scintillation medium used consisted of one volume of Triton X-100 and two volumes of toluene phosphor including 4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene per 1000 mL of toluene.

Incubation of P450BTL with propranolol in a reconstituted system. A 1-mL incubation mixture contained 50 pmol P450BTL, 0.05 U fp_2, 0.5 mM NADPH, 5 μg DLPC, 0.1 mg sodium cholate, 20 μM propranolol and 154 mM potassium phosphate buffer (pH 7.4). After 5-min preincubation under air at 37°, reaction was started by adding the NADPH. The incubations were performed for 0, 15, 30, 45 and 60 min. To remove unbound propranolol and metabolites in the reconstituted system, each sample was dialysed for 48 hr at 4° against 2 × 3-L portions of 50 mM Tris–acetate buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA and 1 mM dithiothreitol according to the regimen employed by Halpert and Neal [29]. After dialysis, a small portion corresponding to 25 pmol P450 of each sample was taken for the assay of propranolol hydroxylase activities in a reconstituted system containing the same components described above except for the addition of 0.25 U fp_2. Incubation was started by adding NADPH and stopped 5 min later by adding 1 mL of 1 N NaOH including sodium bisulphite (25 mg/mL) as an antioxidant to avoid degradation of 4-OH-P.

To compare the covalent binding of radiolabelled material with the loss of enzyme activity, a 0.5 mL incubation mixture containing 250 pmol P450BTL, 0.125 U fp_2, 20 μM (2 μCi) [^{3}H]propranolol and the same components of the reconstituted system described above were incubated for 45 min at 37°, followed by dialysis as described above. A portion corresponding to 50 pmol P450BTL was taken for the assay of propranolol hydroxylase activities in the reconstituted system containing 0.5 U fp_2 and the other components. Remaining samples of P450BTL were used for the measurement of radioactivity covalently bound to the proteins in the mixture, and for the SDS–PAGE analysis described below.

Measurement of propranolol and its metabolites. Propranolol, 4-OH-P, 5-OH-P, 7-OH-P and NDP in the incubation mixture were assayed by the HPLC method described previously [17]. After the termination of the reaction with 1 N NaOH, 4-hydroxybunitrolol was added as internal standard. Propranolol and its metabolites were extracted with ethyl acetate. The organic phase was evaporated to dryness and the residue dissolved in HPLC mobile phase ($CH_3CN:CH_3OH:H_2O:CH_3COOH$ = 22:22:56:1 by vol.). The sample was applied to a reversed-phase column (Inertsil ODS, GL Sciences, Tokyo, Japan). The fluorescent intensity of propranolol and its metabolites was continuously monitored with excitation/emission wavelength at 310/380 nm.

SDS–PAGE analysis. The samples (50–100 μL) were solubilized and subjected to electrophoresis using a 10% acrylamide slab gel (2 mm thickness) by the method of Laemmli [30]. The gel was stained with Coomassie Brilliant Blue, and was cut into 0.25-cm strips. The gel fragments were solubilized with hydrogen peroxide, followed by liquid scintillation counting.

Other methods. Protein concentrations and P450 contents were determined by the methods of Lowry *et al.* [31], and of Omura and Sato [22], respectively. Fp_2 was purified from liver microsomes of phenobarbital-pretreated (80 mg/kg/day i.p. for 4 days) male Sprague–Dawley rats according to the method of Yasukochi and Masters [32]. Statistical significance was calculated by the Student's *t*-test.

RESULTS

Effect of in vivo *propranolol pretreatment on the metabolism-dependent* in vitro *covalent binding of propranolol*

Incubation of rat liver microsomes with [^{3}H]-propranolol and NADPH resulted in the covalent binding of a propranolol metabolite to microsomal protein as reported previously [16, 20]. Propranolol pretreatment of male Wistar rats (100 mg/kg/day p.o. for 5 days) did not affect spectrally detectable microsomal P450 content (control, 0.655 ± 0.023 and propranolol-pretreatment, 0.799 ± 0.061 nmol/mg protein, N = 4) as reported previously [16, 17]. On the other hand, the pretreatment decreased the metabolism-dependent covalent binding of [^{3}H]-propranolol by 78% (control, 130 ± 13 and propranolol pretreatment, 29 ± 3 pmol bound/5 min/mg protein, N = 4, $P < 0.01$). This finding indicates that *in vivo* propranolol pretreatment causes a decrease in the ability of rat liver microsomes to activate propranolol and produce a reactive metabolic intermediate able to bind covalently to microsomal protein.

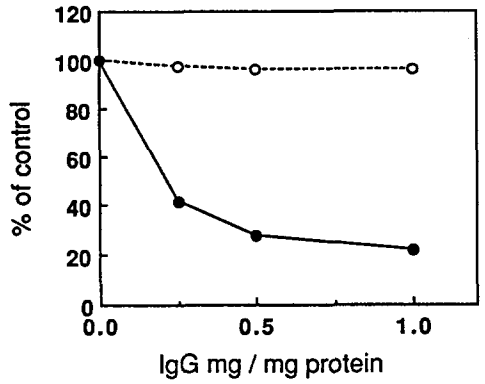

Fig. 1. Effects of antibody against P450BTL on the covalent binding of a [^{3}H]propranolol metabolite to rat liver microsomal proteins. Microsomes (1 mg) were preincubated with different amounts (0–1 mg/mg microsomal protein) of anti-P450BTL IgG (●) or preimmune IgG (○) for 30 min at 25°, followed by incubation of [^{3}H]propranolol (2 μM, 0.2 μCi) in the presence of NADPH for 5 min. Results are expressed as per cent of radioactivity in the absence of the antibody.

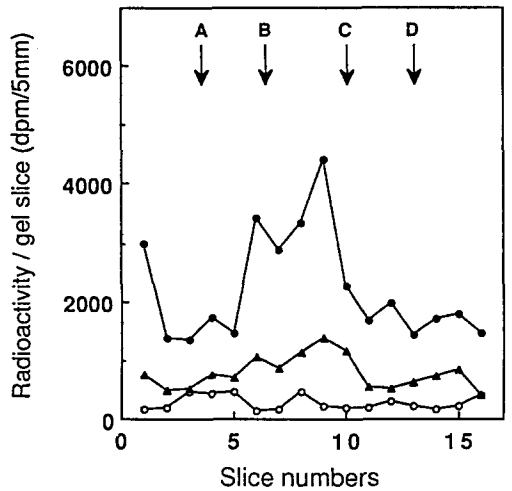

Fig. 2. SDS–PAGE of ^{3}H-labelled microsomal protein incubated with [^{3}H]propranolol. Microsomes (1 mg) were incubated with [^{3}H]propranolol (2 μM, 1 μCi) for 30 min in the presence (●) and absence (○) of NADPH, and were also incubated in the presence of NADPH and GSH (▲). The samples were subjected to SDS–PAGE as described in Materials and Methods. Lettered arrows indicate the following molecular weight markers: A, phosphorylase b (94 kDa); B, bovine serum albumin (67 kDa); C, ovalbumin (43 kDa); D, carbonic anhydrase (30 kDa).

Effect of anti-P450BTL antibody on the metabolism-dependent in vitro *covalent binding of propranolol*

Figure 1 shows the effect of antibody against P450BTL, a P450 isozyme belonging to the CYP2D subfamily [21], on the metabolism-dependent covalent binding of [^{3}H]propranolol to microsomal protein. The anti-P450BTL IgG suppressed the covalent binding concentration dependently, while preimmune IgG exhibited no effect. The anti-P450BTL IgG at a protein ratio (IgG to microsomes) of 1.0 inhibited the covalent binding by 78%.

Electrophoretic separation of covalently bound radioactivity in microsomal protein

After incubation of rat liver microsomes with [^{3}H]propranolol and NADPH, the microsomes were subjected to SDS–PAGE. There was a relatively broad peak of covalently bound radioactivity in the molecular mass range of 43–71 kDa which included the molecular masses of P450, but binding also occurred in other regions (Fig. 2). The peak of radioactivity was essentially absent in incubations without NADPH.

Preliminary experiments showed that GSH (0.1–2 mM) inhibited the covalent binding of a [^{3}H]-propranolol metabolite to microsomal proteins and the maximum inhibitory effect was observed at a GSH concentration of 2 mM (data not shown). The addition of GSH (2 mM) in the incubation system resulted in a decrease in radioactivity all over the range of protein migration on the gel, but a small peak of radioactivity remained in the molecular mass range of 43–56 kDa which corresponds to that of P450 (Fig. 2). GSH at the same concentration did not protect the microsomal CYP2D activity, bunitrolol 4-hydroxylase, against impairment following preincubation with propranolol in the presence of NADPH (data not shown).

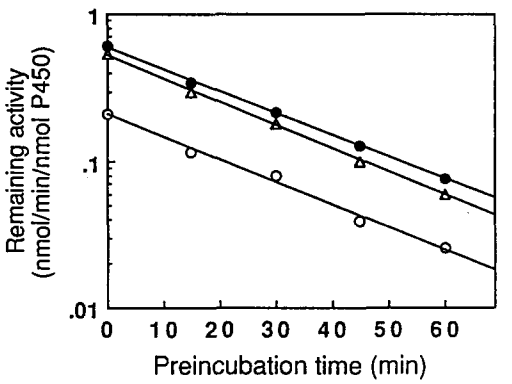

Fig. 3. Time-dependent loss of propranolol 4-, 5- and 7-hydroxylase activities of purified P450BTL in a reconstituted system by preincubation with propranolol. P450BTL (50 pmol) and fp_2 (0.05 U) were preincubated with propranolol (20 μM) in a reconstituted system for appropriate times. An aliquot of the mixture of each sample was then taken and dialysed against 50 mM Tris–acetate buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA and 1 mM dithiothreitol. Propranolol 4- (●), 5- (○) and 7-(△) hydroxylase activities of the fraction corresponding to 25 pmol of P450BTL at a substrate concentration of 20 μM were measured in a reconstituted system containing fp_2 (0.25 U). Results are typical for two experiments.

Time-dependent loss of propranolol 4-, 5- *and* 7-*hydroxylase activities of P450BTL by preincubation with propranolol*

We previously showed that P450BTL, a P450

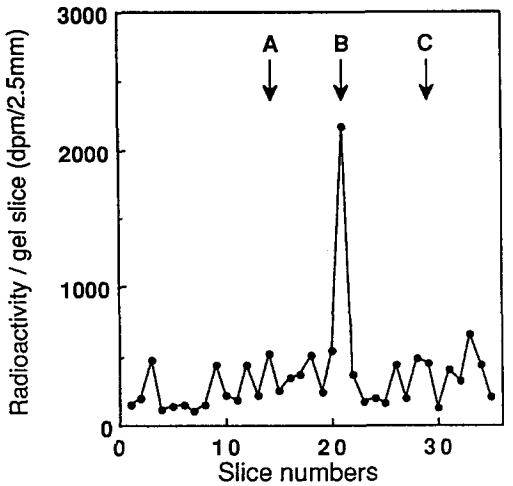

Fig. 4. SDS–PAGE of ^{3}H-labelled protein in a reconstituted system incubated with [^{3}H]propranolol. P450BTL (250 pmol) and fp_2 (0.125 U) were incubated with [^{3}H]-propranolol (20 μM, 2 μCi) for 45 min in the presence of NADPH, followed by dialysis against 50 mM Tris–acetate buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA and 1 mM dithiothreitol. The sample was subjected to SDS–PAGE as described in Materials and Methods. Lettered arrows indicate: A, fp_2; B, P450BTL with a molecular mass of 49 kDa; C, 32 kDa protein.

isozyme belonging to the CYP2D subfamily, had a high activity to oxidise propranolol at naphthalene 4-, 5- and 7-positions [12]. Preincubation of P450BTL with propranolol in a reconstituted system containing fp_2, DLPC and NADPH caused a time-dependent loss of propranolol 4-, 5- and 7-hydroxylase activities of P450BTL in the reconstituted system (Fig. 3). In contrast, controls similarly preincubated but without propranolol did not show a notable decrease in the activities. Approximately 90% of each enzyme activity was lost after 60 min preincubation of the enzyme with propranolol. In addition, first-order kinetics were observed for the loss of the hydroxylase activities (mean rate constant, 0.035 min^{-1}).

Covalent binding of a [^{3}H]propranolol metabolite to P450BTL in a reconstituted system

Incubation of P450BTL and fp_2 with [^{3}H]-propranolol and NADPH in a reconstituted system led to covalent binding of a ^{3}H-labelled material to microsomal proteins. Figure 4 shows the SDS–PAGE of ^{3}H-labelled proteins in the reconstituted system incubated with [^{3}H]propranolol. The proteins existing in this system were fp_2 (a molecular mass of 72 kDa) and P450BTL, which consists of the two polypeptides with molecular masses of 49 and 32 kDa. However, only one prominent peak of covalently bound radioactivity was observed at a molecular mass of 49 kDa corresponding with that of P450. The radioactivities associated with fp_2 and the protein with the lower molecular mass (32 kDa) were at negligible levels.

The relationship between covalent binding of ^{3}H-labelled materials and loss of propranolol hydroxylase activities of P450BTL under the same reconstitutional conditions is summarized in Table 1. No covalently bound radioactivity was observed after preincubation of P450BTL with [^{3}H]propranolol in the absence of NADPH. After 45 min preincubation of the purified proteins with [^{3}H]propranolol and NADPH in the reconstituted system, the ratio of a covalently bound [^{3}H]propranolol metabolite to P450 was calculated to be 0.43. At the same time, propranolol 4-, 5- and 7-hydroxylase activities of P450BTL were decreased by about 40% of each activity of the control incubated in the absence of NADPH. Consequently, the ratio of covalently bound radiolabelled material to P450BTL inactivated, which was estimated from each impaired catalytic activity, was calculated to be approx. 1.0.

DISCUSSION

We previously reported that repetitive administration of propranolol caused impairment of propranolol 4-, 5- and 7-hydroxylase activities, suggesting impairment of the CYP2D isozyme(s) [17]. In the present study, the covalent binding of a reactive metabolite of [^{3}H]propranolol to microsomal protein, which was proposed by Schneck and Pritchard [16] as a mechanism of propranolol-induced impairment of drug metabolizing enzyme activities, was reduced in liver microsomes from rats pretreated with propranolol. This result showed that the ability of rat liver microsomes to activate propranolol was impaired by the *in vivo* pretreatment of the drug itself, indicating that a P450 isozyme(s) activating propranolol and one impaired by propranolol is the same species. It is thus suggested that the CYP2D isozyme(s) is involved in the activation of propranolol.

Recently, we purified a P450 isozyme from rats by tracing bunitrolol 4-hydroxylase activity [21], and this isozyme was considered to belong to the CYP2D subfamily as described in Materials and Methods [21]. The antibody against P450BTL suppressed the metabolic activation of propranolol forming a reactive metabolite able to bind covalently to microsomal proteins (Fig. 1). This observation shows that P450BTL and/or its immunorelated P450 isozyme(s) belonging to the CYP2D subfamily is responsible for the production of a reactive intermediate. It is compatible with our previous results showing inhibition of the metabolic activation by substrates and inhibitors of CYP2D1 [20], and also a markedly low ability to produce a reactive intermediate of propranolol in Dark Agouti rats [20], in which CYP2D1 is deficient [23].

Separation of microsomal proteins by SDS–PAGE following incubation with [^{3}H]propranolol revealed that covalent binding occurred with various proteins only in the presence of NADPH, and the radioactivities of proteins within the molecular mass range of 43–71 kDa were prominent (Fig. 2). These results suggest that the binding of a reactive metabolite of [^{3}H]propranolol to microsomal protein is not selective toward P450 proteins. This is in agreement with the previous report by Shaw *et al.* [33] using human liver microsomes.

GSH markedly suppressed covalent binding (Fig. 2) as reported by Schneck and Pritchard [16].

Table 1. Covalent binding of ^{3}H-labelled materials and loss of propranolol hydroxylase activities in a reconstituted system incubated with [^{3}H]propranolol

^{3}H-labelled covalent binding (nmol/nmol P450)		Propranolol hydroxylase activity (nmol/min/nmol P450)		
		4-OH†	5-OH	7-OH
NADPH (−)[a]	ND*	0.420	0.258	0.560
NADPH (+)[b]	0.426	0.245 (41.7)‡	0.156 (39.5)	0.328 (41.4)

A reconstituted system containing 250 pmol P450BTL and 0.125 U fp_2 was preincubated with [^{3}H]propranolol (20 μM, 2 μCi) for 45 min in the presence (a) and absence (b) of NADPH and dialysed against 50 mM Tris–acetate buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA and 1 mM dithiothreitol. Aliquots corresponding to 50 pmol P450 of each sample were then taken for the assay of propranolol hydroxylase activities at a substrate concentration of 20 μM in a reconstituted system containing 0.5 U fp_2. Results are typical for two experiments.

* Not detected.

† X-OH indicates X-hydroxylation.

‡ % loss of activity to NADPH (−).

However, binding in the molecular mass range of 43 to 56 kDa could not be diminished completely (Fig. 2) even in the presence of GSH at a concentration producing the maximum protective effect against covalent binding. Furthermore, GSH did not protect against impairment of monooxygenase activity of the CYP2D subfamily, examined using bunitrolol 4-hydroxylation. Nucleophiles such as GSH react only with the reactive species that diffuses away from the active site of the enzyme [34, 35]. The reactive metabolite of [^{3}H]propranolol diffusing away from the active site of P450, which are able to bind to other microsomal proteins, is trapped as a GSH-adduct, resulting in a marked decrease in the binding of radioactivity. It is very likely that some of the reactive species do not diffuse away and do bind to the active site of the P450 isozyme which metabolically activates [^{3}H]propranolol. This can be primarily responsible for the remaining bound radioactivity in the presence of GSH. This is compatible with the observation that GSH did not protect microsomal CYP2D activity against impairment following preincubation with propranolol and NADPH.

We proceeded with further experiments using a reconstituted incubation system including P450BTL, fp_2 and DLPC in an attempt to confirm whether propranolol is a mechanism-based inactivator of the P450 isozyme. The NADPH-dependent irreversible inhibition of propranolol hydroxylase activities was observed in this system, and time-dependent inhibition showed a first-order kinetics (Fig. 3). This finding indicates that P450BTL is inactivated through the metabolism of propranolol.

The reconstituted system used in the present study included three proteins, fp_2 and two proteins of P450BTL with molecular masses of 49 and 32 kDa. The separation of the proteins in the reconstituted system by SDS–PAGE showed that the reactive metabolite from [^{3}H]propranolol bound to P450BTL with a molecular mass of 49 kDa but not to the protein with a molecular mass of 32 kDa or fp_2 (Fig. 4). Thus, the reactive metabolite of propranolol bound only to the protein corresponding to P450 under the conditions used, suggesting that covalent binding of a reactive metabolite of propranolol to P450BTL itself, probably to the active site of the enzyme, is a major cause of the impairment of propranolol hydroxylase activities. Moreover, the molecular ratio of covalently bound radiolabelled material to P450BTL inactivated, which was estimated from each propranolol hydroxylase activity, was calculated to be approx. 1.0 (Table 1). These findings indicate that propranolol is a mechanism-based inactivator of P450BTL, a P450 isozyme belonging to the CYP2D subfamily. In addition, the reactive metabolite of propranolol was considered to bind to the apoprotein of P450BTL rather than the heme moiety, because the washing procedure employed in the present study was able to denature P450 and cause the release of the heme moiety from the apoprotein.

Our previous kinetic study indicated that the CYP2D isozyme(s) was involved in the high-affinity phases for propranolol ring-hydroxylations [13]. In addition, we recently showed that the sum of V_{max}/K_m for the primary metabolic reactions of propranolol was markedly decreased in propranolol-treated rats [36]. Consequently, inactivation of the CYP2D isozyme(s) by a reactive metabolite of propranolol might result in reduction of *in vivo* elimination of propranolol in rats [16] and humans [37].

Since propranolol 4-hydroxylation was suggested to proceed mostly via a 3,4-arene oxide intermediate [33], this epoxide was proposed as one of the reactive intermediates [38]. We recently found that incubation of microsomes with 4-OH-P in the presence of NADPH resulted in potent inhibition of microsomal propranolol 5- and 7-hydroxylase activities, suggesting that a further metabolite of 4-OH-P is one of the possible reactive species that binds to and impairs a P450 isozyme(s) catalysing propranolol ring-hydroxylations [39]. Further studies are required to determine the structure of the reactive species.

In summary, propranolol is metabolized by a P450 isozyme(s) belonging to the CYP2D subfamily to a chemically reactive metabolite which can bind to the P450 isozyme(s) itself. The reactive metabolite binds to and inactivates the P450 isozyme(s) which activates

propranolol, indicating that propranolol is a mechanism-based inactivator of a P450 isozyme in the CYP2D subfamily in rats.

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