Involvement of a Cytochrome P4502D Subfamily in Human Liver Microsomal Bunitrolol 4-Hydroxylation

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The oxidative metabolism of bunitrolol, an adrenergic β-receptor antagonist, was examined in human liver microsomes fortified with an NADPH-generating system. The microsomal fractions (n=11) showed bunitrolol 4-hydroxylase activities, which correlated well with CYP2D6 contents (correlation coefficient, r=0.854), debrisoquine 4-hydroxylase (r=0.953) and imipramine 2-hydroxylase (r=0.976) activities. On the other hand, the bunitrolol 4-hydroxylase activity showed relatively poor correlations with CYP3A4 content (r=0.552) and testosterone 6β-hydroxylase activity (r=0.668). The bunitrolol 4-hydroxylase activity was significantly inhibited by quinidine, a selective inhibitor for CYP2D6. Polyclonal antibodies raised against rat liver microsomal cytochrome P450BTL, which is thought to belong to the CYP2D subfamily, effectively inhibited bunitrolol 4-hydroxylation. In contrast, polyclonal antibodies raised against human liver microsomal CYP3A4 did not show any inhibitory effect on the activity. These results suggest that CYP2D6 is involved in the bunitrolol 4-hydroxylase activity in human liver microsomes.

Keywords human liver microsome; bunitrolol 4-hydroxylation; CYP2D6; quinidine; imipramine 2-hydroxylation

Much attention has been focused on genetic polymorphism in drug oxidative reactions, and extensive studies have been made on two particular types of polymorphism, debrisoquine/sparteine and mephenytoin types.1,2) The debrisoquine/sparteine-type poor metabolizers are deficient in oxidative metabolism of more than 30 drugs.3,4) Oxidation of these drugs is catalyzed by CYP2D6 in human liver microsomes and its absence causes the poor metabolism of the above drugs.1) Genetic variation in the oxidative metabolism of debrisoquine has also been investigated in rats,3, 5–7) and CYP2D1 as a debrisoquine 4-hydroxylase in the rat together with 13 other isozymes from various animal species including CYP2D6 in man has been classified in the CYP2D subfamily on the basis of the homology of their amino acid sequences.8)

Bunitrolol is one of the β-adrenergic receptor blocking agents used in the treatment of hypertension or arrhythmia. Its oxidative metabolism consists primarily of p-hydroxylation of the phenyl ring forming 4-hydroxybunitrolol (Fig. 1), and desalkylation of the side-chain followed by oxidative deamination.9) The bunitrolol 4-hydroxylation is the major oxidation pathway in rats and mice.9) It is also a main pathway in humans,10) whereas the side-chain oxidation pathway is dominant in dogs (unpublished data from Nippon C.H. Boehringer-Sohn Co.).

We investigated the oxidative metabolism of bunitrolol in rat liver microsomes, and found that bunitrolol 4-hydroxylase activity was deficient in the female Dark Agouti rat,11) an animal model for debrisoquine poor metabolizers.12) This indicates that the bunitrolol 4-hydroxylase activity is mediated by the CYP2D subfamily in the rat. There has been no report, however, indicating participation of the CYP2D subfamily in bunitrolol metabolism in humans.

In the present study we examined oxidative metabolism of bunitrolol using microsomal fractions obtained from autopsied human liver samples, and compared bunitrolol 4-hydroxylase activity with contents of CYP2D6 and CYP3A4, and activities of debrisoquine 4-hydroxylase, imipramine 2-hydroxylase, and testosterone 6β-hydroxylase. Results obtained suggest the involvement of CYP2D6 in the oxidative metabolism of bunitrolol in human liver microsomes.

MATERIALS AND METHODS

Materials Bunitrolol, 4-hydroxybunitrolol and N-desertiaributylbunitrolol [1-amin-2-hydroxy-3-(o-cyanophenoxyprene)propane] as hydrochlorides were donated by Nippon C.H. Boehringer-Sohn Co. (Osaka, Japan); 2-hydroxyimipramine hydrochloride was by Geigy Co. (Basel, Switzerland); debrisoquine and 4-hydroxydebrisoquine as hemisultates were by Hoffmann-La Roche Ltd. (Basel, Switzerland); imipramine hydrochloride, quinidine hydrochloride and testosterone were purchased from Sigma Chemical Co. (St. Louis, MO); NADPH, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were from Oriental Yeast Co. (Tokyo, Japan). 6β-Hydroxylated testosterone was the generous gift of Dr. D. N. Kirk, Queen Mary College, University of London. Other chemicals and reagents used were of analytical and biochemical grade. Microsomal fractions were prepared from autopsy liver samples of 11 male Japanese who

Fig. 1. Formation of 4-Hydroxybunitrolol from Bunitrolol as a Major Oxidative Metabolic Route

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ranged in age from 2 to 72 years (mean age of 45.4 years) according to the method described elsewhere,¹³ and were stored at −80 °C until use. Total cytochrome P450 contents in the samples varied from 0.21 to 0.51 nmol/mg of microsomal protein among the 11 livers.

**Assay of Enzymatic Activities** Bunitrolol 4-hydroxylase activity was assayed essentially by the high-performance liquid chromatography (HPLC) previously described¹¹ with a slight modification. An incubation mixture contained 0.5 mg/ml microsomal protein, 10 mM glucose 6-phosphate, 2 IU/ml glucose 6-phosphate dehydrogenase, 0.5 mM NADPH, 8 mM MgCl₂, 10 μM bunitrolol, and 150 mM potassium phosphate buffer (pH 7.4) to make a final volume of 1.0 ml. Incubation at 37 °C was started by adding NADPH, and terminated by adding 0.5 ml of 1 M carbonate buffer (pH 9.6) 10 min later. α-Naphthylethylenediamine (50 ng) as internal standard was added to the medium, and metabolites were extracted into ethyl acetate. After evaporation of the solvent, residue was dissolved in 100 μl of a mobile phase, and an aliquot (10 μl) was subjected to HPLC. The HPLC apparatus consisted of a Hitachi 655 liquid chromatograph equipped with a 650-10S fluorescence detector and an Inertsil octadeyl silica (ODS) column (4.6 mm i.d. × 25 cm, GL Science Ltd., Tokyo, Japan). Other conditions were: mobile phase, methanol–acetonitrile–H₂O–acetic acid (22:18:60:1, by volume); flow rate, 1 ml/min; excitation/emission wavelength, 310/380 nm.

Debrisoquine 4-hydroxylase,¹⁴ testosterone 6β-hydroxylase¹⁵ and imipramine 2-hydroxylase¹⁶ activities were measured by the published HPLC methods using a substrate concentration of 1, 0.2 and 1 μM, respectively. In the inhibition experiments using quinidine, the inhibitor (2 μM) was added to the reaction medium, followed by preincubation at 37 °C for 5 min. The incubation was sequentially started by adding NADPH and the following manipulation was run as described above. Immunoinhibition studies on bunitrolol 4-hydroxylase activity in human liver microsomes were conducted using antisera against cytochrome P450BTL purified from native rat liver microsomes,¹⁷ and that against CYP3A4 purified from human liver microsomes.¹⁶

**Others** CYP2D6 contents were measured by Western blot analysis¹⁸,¹⁹ using polyclonal anti-rat CYP2D1 (cytochrome P450 UT-H) IgG fraction, which was cross-reacted with human orthologous CYP2D6. A major protein band with a molecular mass of ca. 51 kDa together with faint band with a much lower molecular mass were visualized on a transblot membrane under the conditions used. The staining intensity of the major band measured with 5 pmol of rat liver microsomal cytochrome P450 was defined as 1 unit of immunoreactive protein. CYP3A4 contents were measured by the method using anti-CYP3A4 antibodies as described elsewhere.¹⁶ Protein concentrations were determined by the standard methods. Statistical significance was calculated by Student’s t-test.

**RESULTS**

CYP2D6 contents in liver microsomes containing total cytochrome P450 ranging from 0.21 to 0.51 nmol/mg of microsomal protein were measured as 0.8 to 9.7 units/mg of microsomal protein using polyclonal antibodies to rat CYP2D1. CYP3A4 contents in the autopsied liver samples ranged from 0.65 to 98.8 pmol/mg of microsomal protein. 4-Hydroxybunitrolol was the only prominent metabolite peak detected on the chromatogram under the conditions used in the HPLC analysis (Fig. 2). Bunitrolol 4-hydroxylase activities together with other monoxygenase activities and cytochrome P450 contents for the 11 liver microsomal samples are listed in Table I. The bunitrolol 4-hydroxylase activities varied from 1.74 to 81.4 pmol/min/mg of microsomal protein.

The activity of bunitrolol oxidation was then compared

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**Table I. Drug Oxidation Activities and Cytochrome P450 Contents in Human Liver Microsomes**

<table>
<thead>
<tr>
<th>Metabolic pathway or P450 content</th>
<th>Activity (pmol/min/mg)</th>
<th>Correlation coefficient (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bunitrolol 4-hydroxylation</td>
<td>27.5 ± 9.1</td>
<td>0.953</td>
</tr>
<tr>
<td>Debrisoquine 4-hydroxylation</td>
<td>15.8 ± 4.0</td>
<td>0.976</td>
</tr>
<tr>
<td>Imipramine 2-hydroxylation</td>
<td>114.2 ± 35.6</td>
<td>0.668</td>
</tr>
<tr>
<td>Testosterone 6β-hydroxylation</td>
<td>1.13 ± 0.38</td>
<td></td>
</tr>
<tr>
<td>CYP2D6 content</td>
<td>4.69 ± 0.93</td>
<td>0.854</td>
</tr>
<tr>
<td>CYP3A4 content</td>
<td>28.78 ± 8.88</td>
<td>0.552</td>
</tr>
</tbody>
</table>

a) Values are mean ± S.E. (n=11). b) Correlation coefficient with bunitrolol 4-hydroxylase activity. c) nmol/min/mg of protein. d) Arbitrary unit/mg of protein. e) pmol/mg of protein.
with the CYP2D6 content immunochemically determined (Fig. 3A). A good correlation was observed between the bunitrolol 4-hydroxylase activity and the CYP2D6 content ($Y = 0.090X + 2.207$, $r = 0.854$, $p < 0.001$). In contrast, the bunitrolol 4-hydroxylation showed a relatively poor correlation ($Y = 0.541X + 13.907$, $r = 0.552$) with the CYP3A4 content (Fig. 3B). Figure 3C shows a comparison of the bunitrolol 4-hydroxylase activity with desbrisoquine 4-hydroxylase activity, which was in the range of 5.37 to 41.2 pmol/min/mg of microsomal protein. In this case also, a high correlation coefficient was obtained ($Y = 0.421X + 4.190$, $r = 0.953$, $p < 0.001$).

Imipramine 2-hydroxylase and testosterone 6β-hydroxylase activities for the 11 liver microsomal samples are also listed in Table I. The imipramine 2-hydroxylase activity ranging from 5.5 to 484.8 pmol/min/mg of microsomal protein correlated well with the desbrisoquine 4-hydroxylase activity ($Y = 13.33X - 34.48$, $r = 0.943$, $p < 0.001$). The bunitrolol 4-hydroxylase activity also was observed to correlate well with the imipramine 2-hydroxylase activity ($Y = 3.845X + 8.698$, $r = 0.976$, $p < 0.001$) (Fig. 3D). Testosterone 6β-hydroxylase activity ranging from 0.03 to 4.00 nmol/min/mg of microsomal protein correlated strongly with the CYP3A4 content ($Y = 21.715X + 4.2394$, $r = 0.926$, $p < 0.001$). In contrast, the testosterone hydroxylase activity showed a poor correlation with bunitrolol 4-hydroxylase activity as shown in Fig. 3E. The correlation coefficient ($Y = 0.027X + 0.369$, $r = 0.668$) between these activities was much lower than the values of imipramine 2-hydroxylase activity with bunitrolol 4-hydroxylase activity ($r = 0.976$) or desbrisoquine 4-hydroxylase ($r = 0.943$) activity.

Figure 4 depicts the inhibitory effect of quinidine, a selective inhibitor of CYP2D6, on bunitrolol 4-hydroxylase activity. The inhibitor (2 μM) significantly decreased

![Fig. 3. Correlation of Bunitrolol 4-Hydroxylase Activity with Cytochrome P450 Isozyme Contents and Other Drug Oxidation Activities in Human Liver Microsomes](image)

- A to E show the correlation of bunitrolol 4-hydroxylase activity (pmol/min/mg of protein) with CYP2D6 content (A, unit/mg of protein), CYP3A4 content (B, pmol/mg of protein), desbrisoquine 4-hydroxylase activity (C, 4-OH DB, pmol/min/mg of protein), imipramine 2-hydroxylase activity (D, 2-OH IMI, pmol/min/mg of protein), and testosterone 6β-hydroxylase activity (E, 6β-OH T, nmol/min/mg of protein).

![Fig. 4. Inhibitory Effect of Quinidine on Bunitrolol 4-Hydroxylase Activity in Human Liver Microsomes](image)

- Quinidine (a final concentration of 2 μM) was added to the incubation medium followed by preincubation for 5 min at 37 °C. Incubation was then started by adding NADPH as described in Materials and Methods. Open and hatched columns (mean ± S.E., each n = 3) are control (C) and quinidine added (Q) groups, respectively. A mean value of the control group was 69.8 ± 9.5 pmol/min/mg of protein, which was taken as 100%.

- Significant difference from the control value ($p < 0.01$).

The bunitrolol 4-hydroxylase activity by 72% ($p < 0.01$). Effect of the polyclonal antibodies raised against rat cytochrome P450BTL and human CYP3A4 on bunitrolol 4-hydroxylation is shown in Fig. 5. The antibodies against cytochrome P450BTL suppressed the bunitrolol 4-hydroxylase activity in a concentration-dependent manner, and the remaining activity at the highest serum/microsomes protein ratio of 5 was 7.2% that of the control incubation including microsomal fraction preincubated with preimmune serum. On the other hand, the antibodies against CYP3A4 showed no effect on the bunitrolol 4-hydroxylation even at the highest concentration.
DISCUSSION

In our previous studies we examined the oxidative metabolism of bunitrolol in rat liver microsomes, and found that bunitrolol 4-hydroxylase activities in Dark Agouti rats of both sexes were significantly lower than those in Wistar rats of corresponding sex.\(^{11}\)

We then purified a cytochrome P450 isozyme (cytochrome P450BTL) using bunitrolol 4-hydroxylase activity as index for the enzymatic activity, and thought the isozyme to belong to the CYP2D subfamily on the basis of its N-terminal amino acid sequence.\(^{17}\) In reconstituted systems including NADPH-cytochrome P450 reductase and each of 12 isoforms of cytochrome P450 purified from rat liver microsomes, cytochrome P450BTL was the only isozyme exhibiting debrisoquine 4-hydroxylase activity at a substrate concentration of 50 \(\mu\)M, but all the isoforms showed 4-hydroxylase activities toward debrisoquine at 2 nM.\(^{17}\)

On the other hand, only two isoforms, cytochrome P450BTL and MC-5 (CYP1A1) were found to have bunitrolol 4-hydroxylase activities at bunitrolol concentrations of 10 \(\mu\)M and 1 nM.\(^{17}\) From these findings, bunitrolol is a more specific substrate for cytochrome P450BTL than debrisoquine in the rat, and a particularly interesting substrate for enzymatic studies on the CYP2D isoforms. Immunochromatography studies using polyclonal antibodies raised against cytochrome P450BTL indicated that bunitrolol 4-hydroxylase activity was predominantly catalyzed by cytochrome P450BTL and/or its immunologically related isozyme(s), namely the CYP2D subfamily, in native rat liver microsomes.\(^{17}\) This prompted us to examine the possibility that CYP2D6, an isozyme belonging to the CYP2D subfamily in human liver microsomes, mediated the bunitrolol 4-hydroxylase activity.

The human liver microsomal samples examined in the present study had bunitrolol 4-hydroxylase activities ranging from 1.74 to 81.4 pmol/min/mg of protein. An \(N\)-desalkylated metabolite [1-amino-2-hydroxy-3-(o-cyanoxypropane), if produced, would be eluted (a retention time of 2.62 min) just before the peak of 4-hydroxybunitrolol; however, 4-hydroxybunitrolol was the only metabolite peak detected in the HPLC analysis under the conditions used. In preliminary experiments we had confirmed that a deaminated metabolite subsequently formed from the desalkylated metabolite, probably by monoamine oxidase(s), was not produced in microsomes fortified with an NADPH-generating system, and that bunitrolol 4-hydroxylation was responsible for almost all elimination (above 95%) of the substrate in rat and human liver microsomes during the incubation. It is therefore indicated that 4-hydroxybunitrolol is a major and exclusive metabolite formed from bunitrolol under the conditions used.

The bunitrolol oxidation activity well correlated with the CYP2D6 content and debrisoquine 4-hydroxylase activity. The debrisoquine 4-hydroxylase activity has been demonstrated to be predominantly mediated by the CYP2D6 in human liver microsomes.\(^{13}\) This previous finding and the present results suggest that bunitrolol 4-hydroxylase activity also is catalyzed largely by CYP2D6. It has been reported that in humans imipramine 2-hydroxylase activity was mainly mediated by CYP2D6.\(^{16,23,24}\) We thus compared the bunitrolol 4-hydroxylase activity with the imipramine 2-hydroxylase activity and found a very high correlation (\(r>0.97\)) between the two.

In contrast, a relatively low correlation coefficient (\(r<0.56\)) was obtained between the bunitrolol 4-hydroxylase activity and the CYP3A4 content in the human autopsied liver samples. Testosterone 6β-hydroxylase activity is known to be primarily catalyzed by CYP3A4 in the human liver.\(^{25}\) This activity showed a relatively poor correlation (\(r<0.67\)) with the bunitrolol 4-hydroxylase activity in the present study. Consequently, CYP3A4 seems likely to have a small part, if any, in bunitrolol 4-hydroxylase in the human liver microsomes.

To further confirm the possible participation of CYP2D6 in the microsomal bunitrolol oxidation, the effect on the bunitrolol 4-hydroxylation of quinidine, a selective inhibitor of CYP2D6, was examined. The inhibitor (final concentration of 2 \(\mu\)M) significantly decreased the bunitrolol 4-hydroxylase activity in human liver microsomes.

Moreover, the effects of polyclonal antibodies raised against cytochrome P450BTL, which was purified from native rat liver microsomes by pursuing bunitrolol 4-hydroxylase activity,\(^{17}\) were examined on human liver microsomal bunitrolol 4-hydroxylation. Because cytochrome P450BTL has an \(N\)-terminal amino acid sequence highly homologous to that of CYP2D2, this isozyme is thought to belong to the CYP2D subfamily.\(^{17}\) Therefore, it is feasible that polyclonal antibodies raised against cytochrome P450BTL suppress bunitrolol 4-hydroxylation mediated by CYP2D6. As expected, the antibodies against cytochrome P450BT almost completely suppressed bunitrolol 4-hydroxylation in the human liver microsomes, while preincubation of the antibodies raised against CYP3A4 with the microsomes showed no effect on the bunitrolol 4-hydroxylation.
Koymans et al.\textsuperscript{26} proposed that substrates of CYP2D6 held some common features, \textit{i.e.}, they had at least one nitrogen atom in their chemical structures, and their oxidation sites by the CYP2D6 were located 5 to 7 Å apart from the nitrogen atom, which was thought to interact with a carboxyl group located near an active site of the CYP2D6 protein. In this context, binitrolothol seems to fit these criteria. Taking into account the use of polyclonal antibodies raised against human or rat cytochrome P450 isozyme, it is reasonable to think that cytochrome P450 isozyme(s) belonging to the CYP2D (probably CYP2D6) but not the CYP3A (probably CYP3A3-5) subfamily is involved in binitrolothol 4-hydroxylation in human liver microsomes under the conditions employed in the present study.

To examine the correlations between binitrolothol 4-hydroxylase activity and CYP2D6 content or the oxidation activities for other substrates known to be catalyzed by CYP2D6, we used the human autopsy liver samples in the present study. We have no reliable information whether any of the deceased Japanese from whom the autopsy liver samples were obtained were CYP2D6-deficient or not. Pharmacokinetic data demonstrated that 4-hydroxylation followed by glucuronidation is a major metabolic pathway of binitrolothol in humans.\textsuperscript{10} Further studies are necessary to confirm that what we found is the direct cause of the polymorphism in oxidative metabolism of binitrolothol in humans.

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