CYP2D6-Mediated Mexiletine-Protein Adduct Formation

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An antiarrhythmic drug mexiletine, which is metabolized by CYP2D6 and, to a lesser extent, by CYP1A2, is known to sometimes induce allergic reactions. Since mexiletine itself is chemically inert, metabolic activation of the drug, required for the formation of protein adduct, is thought to be a first step in the induction of the allergic reactions. In the present study, we screened several cytochrome P450 (CYP) enzymes for their ability to form protein adducts with mexiletine. Of the 10 CYPs examined (CYP1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5), CYP2D6 was most efficient in adduct formation with microsomal proteins. On autoradiographs of SDS-PAGE-separated proteins, a 52 kDa band was detected in the CYP2D6-expressing microsomes. These results suggest that the mexiletine-protein adduct is formed mainly by CYP2D6-dependent metabolic activation.

Key words —— protein adduct, mexiletine, CYP2D6, metabolic activation

INTRODUCTION

Many therapeutic drugs are known to cause allergic reactions such as skin eruption, fever, hepatic toxicity and, more severely, Stevens-Johnson syndrome.[1] These drugs, mostly low molecular weight compounds, are thought to become immunogenic upon binding to proteins. Most drugs are chemically inert, and their metabolic activation to a reactive intermediate capable of forming protein adducts has been proposed to be a necessary first step in the induction of an allergic reaction, especially for sensitization.[2] The role of human cytochrome P450 (CYP) enzymes in the metabolic activation of these drugs is being increasingly recognized. For example, protein adduct formation of the antiarrythmic and local anesthetic drug lidocaine is mediated by CYP2D,[3,4] and that of the anticonvulsant drug phenytoin is catalyzed by CYP2C9 and 2C19.[5]

Mexiletine is a class Ib antiarrythmic drug whose pharmacological effect is attributed to its inhibitory activity toward the cardiac sodium channel. In humans, mexiletine undergoes extensive metabolism in the liver to several pharmacologically inactive metabolites.[6] A CYP enzyme CYP2D6 has predominant roles in oxidation of aromatic rings and aromatic methyl groups.[7] On the other hands, N-hydroxylation was recently shown to be mediated mainly by CYP1A2.[8]

It has been reported that mexiletine causes skin eruptions.[9–11] Since mexiletine is apparently inert chemically, we hypothesized that mexiletine might undergo metabolic activation by CYP enzyme and become capable of forming protein adducts, which would function as a neoantigen inducing allergic reactions. Here, we demonstrated that mexiletine-protein adduct formation was mediated mainly by CYP2D6.

MATERIALS AND METHODS

Materials —— Mexiletine was purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). [ring methyl(n)-^{3}H]-mexiletine (111 GBq/mmol) was obtained from Amersham Biosciences (Buckinghamshire, U.K.). $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were from Oriental Yeast Co. (Tokyo, Japan). Pooled human liver microsomes (HLM) and insect cell microsomes (SUPERSOMES™) expressing individual human recombinant CYP forms (CYP1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5) plus cytochrome P450 reductase or the P450 reductase alone were all obtained from BD Gentest (Woburn, MA, U.S.A.). Other reagents were of the highest quality commercially available.

Covalent Binding Assay —— The reaction mixture contained HLM (2 mg) or microsomes expressing one of the human CYP forms (150 pmol), 100 μM [^{3}H]-mexiletine (74 kBq), and 5 mM $MgCl_2$ in 0.25 ml of 100 mM potassium phosphate buffer

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(pH 7.4). After preincubation for 6 min at 37°C, the reaction was started by the addition of the NADPH-generating system (1 mM $NADP^+$, 10 mM glucose-6-phosphate, and 2 units/ml glucose-6-phosphate dehydrogenase). Each mixture was incubated for 30 min at 37°C unless otherwise indicated, and the reaction was terminated by adding 0.25 ml of 20% trichloroacetic acid. Each mixture was loaded onto a glass-fiber filter disk (Millipore, Billerica, MA, U.S.A.). The filters were washed 8 times with ethanol (until no further radioactivity was released) and dried, and the radioactivity was counted after addition of 10 ml scintillation fluid.

Autoradiographic Analysis —— Microsomes expressing CYP1A2, CYP2D6 or P450 reductase alone (control) (150 pmol) were incubated for 90 min at 37°C with 100 μM [^{3}H]-mexiletine (740 kBq), 5 mM $MgCl_2$ and the NADPH-generating system in 0.25 ml of 100 mM potassium phosphate buffer (pH 7.4). After the reaction was terminated with 0.25 ml of 20% trichloroacetic acid, the precipitated proteins were washed 8 times with ethanol and solubilized in Laemmli sample buffer (150 μl). An aliquot (20 μl) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 8% gels. The dried gels were exposed for approximately 40 days to a BAS imaging plate and analyzed with a BAS-1500 imaging system (Fuji film, Tokyo, Japan).

RESULTS AND DISCUSSION

Since mexiletine is metabolized by CYP2D6 and CYP1A2, we first determined the ability of these CYP forms to induce covalent binding of [^{3}H]-mexiletine with the microsomal proteins. Incubation of the drug with CYP2D6-expressing microsomes in the presence of an NADPH-generating system resulted in the covalent binding of [^{3}H]-mexiletine to the proteins. The amount of the covalent binding increased almost linearly during a 30 min incubation (Fig. 1). CYP1A2-expressing microsomes also generated weak binding of [^{3}H]-mexiletine to the proteins, approximately 1/3 that of the CYP2D6-expressing microsomes.

Next we screened the ability of various CYP forms [P450 reductase alone (control), CYP1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5] to induce covalent binding of [^{3}H]-mexiletine with microsomal proteins (Fig. 2). After 30 min incubation, significant binding was observed in the

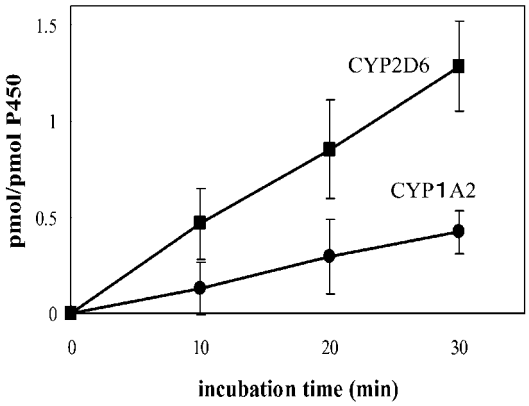

Fig. 1. Time–Dependent Covalent Binding of [^{3}H]-Mexiletine by CYP2D6 and CYP1A2

Microsomes expressing CYP2D6 or CYP1A2 were incubated for 10, 20 or 30 min at 37°C with [^{3}H]-mexiletine in the presence or absence of the NADPH-generating system. The mixture was loaded onto glass-fiber filter disks, and the filters were washed, dried and counted for radioactivity with scintillation fluid. Data (mean ± S.D.) from 5 independent experiments are shown after subtracting the values of samples without the NADPH-generating system from those with the NADPH-generating system.

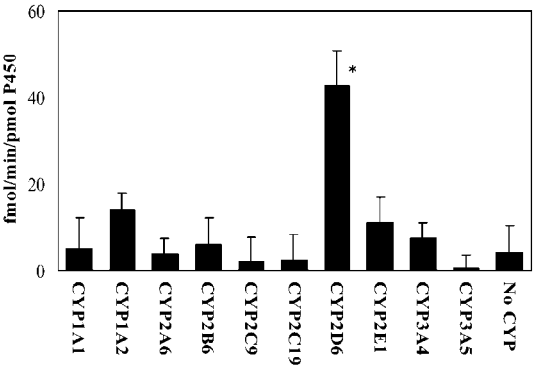

Fig. 2. Covalent Binding of [^{3}H]-Mexiletine to Microsomal Proteins Containing Various Recombinant Human CYP Forms

Microsomes expressing each CYP form were incubated for 30 min at 37°C with [^{3}H]-mexiletine in the presence or absence of the NADPH-regenerating system. The mixture was loaded onto glass-fiber filter disks, and the filters were washed, dried and counted for the radioactivity with scintillation fluid. Data (mean ± S.D.) from 3–5 independent experiments are shown after subtracting the values from samples without the NADPH-generating system from those with NADPH-generating system. *Statistically different from the control (No CYP) ($p < 0.001$).

CYP2D6-expressing microsomes ($p < 0.001$ by one-way analysis of variance (ANOVA) followed by Scheffe test). Weak binding was also apparent in the CYP1A2, CYP2E1 and CYP3A4-expressing microsomes, though the binding was not statistically different from that in the control microsomes (No CYP). The covalent binding activity of the other microsomes was negligible. In addition, pooled

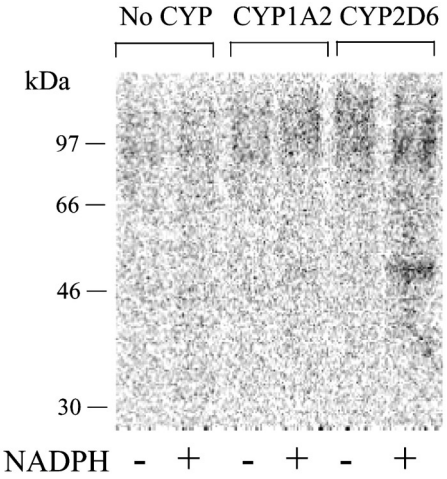

Fig. 3. Autoradiographic Analysis of the Protein Covalently Bound with [^{3}H]-Mexiletine

Microsomes expressing CYP1A2, CYP2D6 or P450 reductase alone (No CYP) were incubated for 90 min at 37°C with [^{3}H]-mexiletine in the presence (+) or absence (–) of the NADPH-generating system. Acid-precipitated proteins were washed, solubilized, and subjected to SDS-PAGE, followed by analysis with a BAS-1500 imaging system. One representative result from 3 independent experiments is shown.

HLM demonstrated activity in the covalent binding of [^{3}H]-mexiletine (1.08 ± 0.35 pmol/min/mg protein), with linearity, during a 30 min incubation (data not shown).

We then attempted to detect [^{3}H]-mexiletine-bound proteins by autoradiography. A 52 kDa band was detected with the CYP2D6-expressing microsomes in an NADPH-dependent manner (Fig. 3). On the other hand, no specific band was detected with the CYP1A2-expressing microsomes or the control microsomes (No CYP). We could not detect the [^{3}H]-mexiletine-bound protein when pooled HLM was used as an enzyme source, probably due to limited protein amounts applicable to the gels (data not shown).

CYP2D6 metabolizes mexiletine mainly into *p*-hydroxy- and 2-hydroxy-forms. The mechanism of the covalent mexiletine binding remains unknown, but it is assumed that a reactive intermediate preceding the above metabolites might be involved in the protein adduct formation.

It has been suggested that a uricosuric diuretic, tienilic acid, covalently bound to CYP2C9 through its transformation into a reactive metabolite, and antisera from patients with tienilic acid-hepatitis recognize CYP2C9.[12] Dihydralazine is suggested to form adducts with CYP1A2, which is also recognized by autoantibodies from patients with dihydralazine-induced hepatitis.[13] Though we could not yet identify the protein covalently bound with mexiletine, similarity in the molecular weight (52 kDa) suggested that the mexiletine-bound protein might be human CYP2D6 itself.[14]

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