ABSTRACT:
Dihydralazine is known to induce immunoallergic hepatitis, and the anti-liver microsome (anti-LM) autoantibodies found in the serum of the patients have been reported to react with cytochrome P450 1A2 (CYP1A2). It is thus suggested that a reactive metabolite of dihydralazine covalently binds to the P450 protein and triggers an immunological response as a neoantigen. We investigated the selectivity of inactivation of P450 enzymes during the metabolism of dihydralazine to evaluate the target protein of its reactive metabolite. Liver microsomes from male Wistar rats were preincubated with dihydralazine in the presence of NADPH, followed by assays of several monooxygenase activities. Preincubation of microsomes of β-naphthoflavone-treated rats with dihydralazine resulted in time-dependent loss of phenacetin O-deethylase activity (an indicator of CYP1A2 activity), showing inactivation of CYP1A2 during the dihydralazine metabolism. The preincubation with dihydralazine was less effective on ethoxyresorufin O-deethylase activity in microsomes of β-naphthoflavone-treated rats (CYP1A1) and pentoxyresorufin O-depentylase activity in microsomes of phenobarbital-treated rats (CYP2B). On the other hand, preincubation of microsomes of untreated rats with dihydralazine caused time-dependent loss of testosterone 2α-, 16α- (CYP2C11), and 6β-(CYP3A) hydroxylase activities. These results demonstrated that dihydralazine was metabolically activated by CYP1A2, and the chemically reactive metabolite bound to the enzyme itself and inactivated it, as was suggested by the appearance of anti-LM antibodies in dihydralazine-hepatitis, whereas CYP2C and -3A enzymes were also suggested to be the enzymes that activate dihydralazine and lead to the target of the reactive intermediates.

Liver is often a target of drug-induced toxicity, which is generally attributed to the following two causes. One is a direct toxicity in which reactive metabolites are formed and damage critical cell targets (Nelson and Pearson, 1990; Hinson and Roberts, 1992; Boelsterli, 1993). Another is an indirect toxicity in which reactive metabolites covalently bind to proteins, which then behave as neoantigens, and trigger an abnormal immunological response leading to the disease (Boelsterli et al., 1993; Pohl et al., 1988; Pirmohamed et al., 1996). An example of the latter is hepatitis induced by halothane (Pohl et al., 1989; Pohl, 1990) or tienilic acid (Homberg et al., 1984; Beaune et al., 1987). Antimicrobial autoantibodies commonly appear in the sera of the patients with the drug-induced hepatitis. The formation of reactive metabolites has been proposed as an initial step of the disease (Pohl, 1990; Beaune et al., 1987; Leceœur et al., 1994). The step may be followed by covalent binding of the metabolites to the protein(s) generating the reactive metabolite(s) and/or other proteins, whereas it has not been always elucidated what the targets of the reactive metabolite are.

Dihydralazine, an antihypertensive drug, is known to induce immunoallergic hepatitis (Pariente et al., 1983; Nataf et al., 1986). The autoantibodies reacting with liver microsomes (anti-LM) found in the serum of the patients have been reported to be directed against cytochrome P450 1A2 (CYP1A2) (Bourdi et al., 1990). It is thus suggested that a reactive metabolite of dihydralazine covalently binds to the P450 protein and triggers an immunological response as a neoantigen. In practice, it was demonstrated that dihydralazine was activated into a chemically reactive metabolite that covalently binds to liver microsomal protein by CYP1A2 (Bourdi et al., 1994). However, it remains unknown whether the appearance of anti-LM resulted from covalent binding only to CYP1A2 because its selectivity as the target protein of dihydralazine-reactive metabolite has not been evaluated. In the present study, we investigated the selectivity of inactivation of P450 enzymes during the metabolism of dihydralazine to evaluate the P450 protein(s) as the specific target protein of the reactive metabolite.

**Materials and Methods**

**Chemicals.** Dihydralazine dihydrochloride and β-naphthoflavone were purchased from Aldrich; hydralazine hydrochloride, sodium phenobarbital, and resorufin were from Tokyo Chemical Industry (Tokyo, Japan); phenacetin, 4-acetamidophenol, ethoxyresorufin, pentoxyresorufin, testosterone, and 2α- and 16α-hydroxytestosterones were from Sigma; 6β-hydroxytestosterone was from Steraloids Inc. (Wilton, NH); glucose 6-phosphate (G-6-P), glucose 6-phosphate dehydrogenase (G-6-PDH), and NADPH were from Oriental Yeast Co., Ltd. (Tokyo, Japan); and reduced glutathione (GSH) was from Wako Pure Chemical (Osaka, Japan). All other chemicals and solvents used were of analytical grade.

**Preparation of Liver Microsomes.** Male Wistar rats (2 months old) were obtained from Takasugi Experimental Animals (Saitama, Japan). The animals were housed in an air-conditioned room (25°C) under a 12-hr light-dark cycle for 1 week prior to use. Food (commercially available pellet, Oriental Yeast Co., Ltd.) and water were given ad libitum. β-Naphthoflavone (80 mg/kg in
Time-Dependent Decrease in POD Activity of Liver Microsomes. POD activity was determined with microsomes from \( \beta \)-naphthoflavone-treated rats as an indicator of the activity for CYP1A2. Dihydralazine inhibited POD activity in a concentration-dependent manner (fig. 1A). Preincubation of the microsomes with dihydralazine in the presence of NADPH intensified the inhibitory effect of the compound, resulting in one-fifth less IC\(_{50}\) values for the inhibition than that obtained without the preincubation (with preincubation, 28.8 ± 3.1 \( \mu \)M, mean ± SE of three determinations; without preincubation, >200 \( \mu \)M). The inhibitory effect was also found to be time-dependent, i.e. the enzymatic activity decreased exponentially vs. the preincubation time of the microsomes with dihydralazine in the presence of NADPH (fig. 1B), indicating inactivation of CYP1A2 during oxidative metabolism of dihydralazine. The pseudo-first-order kinetic constant for the inactivation (\( k_{\text{inact}} \)) thus obtained was 0.0729 ± 0.0124 min\(^{-1}\), whereas that of control was 0.0100 ± 0.0030 min\(^{-1}\).

The kinetic analysis revealed inhibition of POD activity by dihydralazine in a typical competitive manner (fig. 2A). On the other hand, the type of the inhibition changed to noncompetitive type by the preincubation of microsomes with dihydralazine and NADPH (fig. 2B), resulting in a marked decrease in the \( V_{\text{max}} \) value. In addition, the marked inhibition in the latter condition was obtained within a lower dihydralazine concentration range than that obtained without the preincubation.

POD activity was also determined with microsomes from untreated and phenobarbital-treated rats. Intensification of the inhibition of POD activity by the preincubation of microsomes with dihydralazine and NADPH was also observed in microsomes of untreated rats but not in those of phenobarbital-treated rats (fig. 3).

Time-Dependent Decrease in EROD and PROD Activities of Liver Microsomes. Time-dependent effects of the preincubation of microsomes with dihydralazine and NADPH were also studied on EROD activity in microsomes of \( \beta \)-naphthoflavone-treated rats and PROD activity in phenobarbital-treated rats, which are indicators for the activities of CYP1A1 and CYP2B1/2, respectively. The preincubation of microsomes with dihydralazine caused time-dependent decreases in these activities (fig. 4) but was less effective than that on POD activity in the microsomes of \( \beta \)-naphthoflavone-treated rats. The \( k_{\text{inact}} \) values for EROD and PROD activities thus obtained were 0.0172 ± 0.0004 and 0.0200 ± 0.0068 min\(^{-1}\), respectively.

Time-Dependent Decrease in Testosterone Oxidation Activities of Liver Microsomes. Testosterone 2\( \alpha \), 16\( \alpha \), and 6\( \beta \)-hydroxylase activities were determined with microsomes from untreated rats. Dihydralazine (50 \( \mu \)M) inhibited all of the activities measured here in a
concentration-dependent manner, and the inhibitory effect was more potent on 6β-hydroxylase activity than on 2α- and 16α-hydroxylase activities (fig. 5). The enzymatic activities decreased exponentially vs. the preincubation time of the microsomes with dihydralazine in the presence of NADPH, and the decreases were more pronounced in 2α- and 16α-hydroxylase activities than in 6β-hydroxylase activity (fig. 6). The $k_{\text{inact}}$ for testosterone 2α-, 16α-, and 6β-hydroxylase activities thus obtained was 0.0664 ± 0.0030, 0.0696 ± 0.0059, and 0.0347 ± 0.0055 min⁻¹, respectively.

**Effect of GSH on Dihydralazine-Induced Decreases in Testosterone Oxidation Activities.** Liver microsomes of untreated rats were preincubated with dihydralazine and NADPH in the presence or absence of GSH to determine its protective effect against the inhibition of testosterone oxidation activities by dihydralazine metabolites. Addition of GSH (5 mM) exhibited no significant effect on the decreases in testosterone 2α-, 16α-, and 6β-hydroxylase activities induced by the preincubation of microsomes with dihydralazine (fig. 7).

**Effect of Hydralazine on POD and Testosterone Oxidation Activities.** Addition of hydralazine, a metabolite of dihydralazine, significantly decreased POD activity. However, no additional effect of the preincubation of microsomes in the presence of NADPH was obtained with hydralazine (fig. 8).

Inhibitory effect of addition of hydralazine on testosterone 2α-, 16α-, and 6β-hydroxylase activities was more pronounced than that of dihydralazine. On the other hand, preincubation of the microsomes with dihydralazine in the presence of NADPH resulted in the intensification of the inhibitory effect, but the preincubation with hydralazine did not (fig. 9).

**Discussion**

Sera from patients with dihydralazine-induced hepatitis were shown to contain anti-liver microsomal antibodies (anti-LM) (Pariente et al., 1983; Nataf et al., 1986). The anti-LM antibodies were specific
The antibodies against CYP1A2.

Neoantigen that triggers an immune response characterized by autoantibodies.

Addition of GSH did not affect either POD activity or testosterone oxidation activities. These data indicate that CYP2C11 was inactivated to a similar extent of CYP1A2.

The preincubation with dihydralazine was less effective on EROD in microsomes of β-naphthoflavone-treated rats and PROD in microsomes of phenobarbital-treated rats, a marker of the activity of CYP1A1 and CYP2B1, respectively (Burke et al., 1985). These apparently indicate selective inactivation of CYP1A2. However, dihydralazine inhibited the testosterone 2α- and 16α-hydroxylase activities, both of which were known to be catalyzed by CYP2C11 (Sonderfan et al., 1987; Imaoka et al., 1988), and also testosterone 6β-hydroxylase activity, a marker of the P450 enzymes in the CYP3A subfamily (Sonderfan et al., 1987; Imaoka et al., 1988) in liver microsomes of untreated rats, indicating that dihydralazine is a substrate and/or an inhibitor of the CYP2C11 and CYP3A enzymes. In addition, preincubation of the microsomes with dihydralazine in the presence of NADPH caused time-dependent loss of the testosterone oxidation activities, demonstrating that CYP2C and CYP3A enzymes were inactivated during oxidative metabolism of dihydralazine. Moreover, the k\textsubscript{nact} value obtained from remaining activity vs. time profile clearly shows that CYP2C11 was inactivated to a similar extent of CYP1A2.

It has been known that GSH can react only with the reactive metabolites which can diffuse from the active site of the enzyme that generates the metabolites. In the present study, addition of GSH did not prevent dihydralazine-induced and NADPH-dependent inactivation of the CYP2C11 or CYP3A enzyme. Thus, the inhibition of the CYP2C11 and CYP3A enzymes was demonstrated not to be a result of an unstable inhibitory metabolite formed by CYP1A2 but to the covalent binding of reactive metabolites in a mechanism-based manner. It was thus suggested that the CYP2C and CYP3A enzymes as well as CYP1A2 metabolized dihydralazine into a chemically reactive metabolite and led to the target of the reactive metabolite.

The autoantibodies reacting with the CYP2C enzyme have been observed in serum from patients with tienilic acid-induced hepatitis called anti-LKM2 (liver-kidney microsomes) (Beaune et al., 1987; Imaoka et al., 1988), and also those reacting with the CYP3A enzyme have been observed with anticonvulsants (Riley et al., 1993). The present results suggest that the reactive metabolite of dihydralazine binds to the CYP2C and -3A enzymes as well as CYP1A2 and inactivates them. However, in humans, the autoantibody that recognizes the CYP2C or CYP3A enzyme has not been found in the sera from patients with dihydralazine-induced hepatitis. The reason is not presently understood, but if a CYP2C enzyme(s) really plays a role in development of dihydralazine-induced autoimmune hepatitis, the autoantibodies directed to other P450 enzymes than CYP1A2, such as CYP2C9, could be found in sera from patients with dihydralazine-induced hepatitis in future clinical trials. However, there may be a possibility that the covalently bound product, i.e. the CYP1A2-reactive metabolite complex, behaves as an antigen, but the product with other P450 enzymes does not. In any event, the selectivity of covalent binding and/or antigenicity of P450 enzymes may play a role in drug-induced autoimmune hepatitis. However, it remains to be clarified whether the autoantibodies are causative of the hepatitis or are only a marker of the disease.

The preincubation of the microsomes with hydralazine, a metabolite of dihydralazine, in the presence of NADPH did not affect either POD or testosterone oxidation activities. These data indicate that
Dihydralazine but not hydralazine is a precursor of the reactive intermediate(s) that binds to the P450 enzymes and inactivates them and suggest that the reactive species is formed during transformation of dihydralazine into hydralazine by CYP1A2, CYP2C, and CYP3A enzymes (fig. 10). Bourdi et al. (1994) speculated that a radical intermediate from dihydralazine to hydralazine could be a candidate to adduct with CYP1A2. The present results described here are conceivable with this speculation.

The present results may provide other clinical importance than the drug-induced hepatitis, i.e., the inactivation of the P450 enzymes might result in elevation of the blood concentration of the drugs metabolized by CYP1A2, CYP2C, or CYP3A enzyme, which are co-and/or postadministered with dihydralazine, suggesting an importance in view of such drug-drug interactions. On the other hand, as dihydralazine is reported to induce CYP1A2 (Bourdi et al., 1994), the induction also should be taken into account for the drug-drug interactions.

In conclusion, the present study demonstrated that dihydralazine was metabolically activated not only by CYP1A2 but also by CYP2C and CYP3A enzymes, and the chemically reactive metabolite inactivated the enzymes themselves, probably by the covalent binding to the enzymes. Further studies with radiolabeled dihydralazine are required to confirm the formation of the covalent adducts.

References


