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, 1993; Pohl et al., 1988; Pirmohamed et al., 1996). An example of the latter is hepatitis induced by halothane (Pohl, 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 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.

Materials and Methods

Dihydralazine dihydrochloride and β-naphthoflavone were purchased from Aldrich; hydroxyresorufin, sodium phenobarbital, and resorufin were from Tokyo Chemical Industry (Tokyo, Japan); phenacetin, 4-acetamidophenol, ethoxyresorufin, pentoxyresorufin, testosterone, and 2- and 16-hydroxytestosterone 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). The materials 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
corn oil) or sodium phenobarbital (80 mg/kg in physiological saline) was given to the rats intraperitoneally for 4 days. The rats were killed along with untreated rats by decapitation 24 hr after the final doses, and liver microsomal fractions were prepared according to the method of Omura and Sato (1964). Protein concentrations were assayed by the method of Lowry et al. (1951).

**Protocols for Preincubation of Liver Microsomes with Hydralazine Derivatives.** Liver microsomes of male Wistar rats were preincubated with dihydralazine in the presence of NADPH to determine effects of its metabolites on microsomal monoxygenase activities. A 1-ml incubation mixture contained 0.5 mg of liver microsomal protein, 10 mM G-6-P, 2 units G-6-PDH, 5 mM MgCl₂, 0.1 mM EDTA, and various concentrations of dihydralazine in 0.15 M Tris-HCl buffer (pH 7.4). After temperature equilibration (37°C, 5 min), preincubation of microsomes with dihydralazine was started by adding NADPH (final 0.5 mM) and performed for various time periods. The subsequent incubation of the microsomes for the assay of enzymatic activities was started by the addition of each test substrate, phenacetin, ethoxyresorufin, pentoxysorufin, or testosterone. Liver microsomes from β-naphthoflavone-treated rats were assayed for phenacetin O-deethylase (POD) activity and ethoxyresorufin O-deethylase (EROD) activity assays; those from phenobarbital-treated rats were to POD activity and pentoxysorufin O-depentylation activity (PROD) assays; those from untreated rats were to POD and testosterone oxidation assays. In some experiments, hydralazine was employed instead of dihydralazine. In the control experiments, the same reaction mixture in the absence of dihydralazine as above was preincubated for the corresponding time periods; or, the mixture including the corresponding concentrations of dihydralazine was not preincubated, and the incubation for the enzyme assay was started by adding NADPH.

**Assay of Enzymatic Activities.** POD (Masubuchi et al., 1994) and testosterone 2α-, 16α-, and 6β-hydroxylase (Masubuchi et al., 1995) activities were determined according to the high pressure liquid chromatographic method previously described. EROD and PROD activities were assayed by the fluorometric method to determine the resorufin formation (Burke et al., 1985). All of the assays were performed under linear conditions of metabolite formation with regard to incubation time and protein concentration.

**Data Analysis.** Pseudo-first order kinetic constants for the enzyme inactivation (k_inact) were calculated from the initial slopes of the linear regression lines of the semilogarithmic plots of the remaining enzyme activity against the preincubation time. Results were represented as means ± SE. Statistical significance was calculated by the Student’s t test.

**Results**

**Time-Dependent Decrease in POD Activity of Liver Microsomes.** POD activity was determined with microsomes from β-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 IC₅₀ values for the inhibition than that obtained without the preincubation (with preincubation, 28.8 ± 3.1 μM, mean ± SE of three determinations; without preincubation, >200 μM). 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_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 was 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 V₅₀ 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 β-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 β-naphthoflavone-treated rats. The k_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 2α-, 16α-, and 6β-hydroxylase activities were determined with microsomes from untreated rats. Dihydralazine (50 μM) inhibited all of the activities measured here in a
Microsomes were not preincubated (white bar) or preincubated for 10 min with dihydralazine (50 μM) in the presence of NADPH (stippled bar), followed by assay of POD activity. Results are represented as per cent of the activity obtained without dihydralazine and are means ± SE (N = 3). **, significantly different from “without preincubation” (p < 0.01).

Fig. 3. Inhibition of POD activity of various microsomes by dihydralazine.

Addition of hydralazine, a metabolite of dihydralazine, induced by the preincubation of microsomes with dihydralazine (fig. 8).

Fig. 4. Time-dependent decrease in EROD and PROD activities during preincubation of microsomes with dihydralazine.

Microsomes were preincubated without (○) or with (●) dihydralazine (50 μM) in the presence of NADPH, followed by assay of alkoxyresorufin O-dealkylase activities. First-order inactivation constants for EROD and PROD activities were 0.0172 ± 0.0004 and 0.0200 ± 0.0068 min⁻¹, respectively. Results are represented as per cent of the activity obtained without the preincubation and are means ± SE (N = 3).

Fig. 5. Inhibition of testosterone oxidation by dihydralazine.

Testosterone 2α- (●), 16α- (○), and 6β- (●) hydroxylase activities were determined in the absence or presence of various amounts of dihydralazine. Results are represented as per cent of the activity obtained without dihydralazine and are means ± SE (N = 3).

Fig. 6. Time-dependent decrease in testosterone oxidation activities during preincubation of microsomes with dihydralazine.

Microsomes were preincubated without (○) or with (●) dihydralazine (50 μM) in the presence of NADPH, followed by assay of testosterone oxidation activities. First-order inactivation constants for testosterone 2α-, 16α-, and 6β-hydroxylase activities were 0.0664 ± 0.0030, 0.0696 ± 0.0059, and 0.0347 ± 0.0055 min⁻¹, respectively. Results are represented as per cent of the activity obtained without the preincubation and are means ± SE (N = 3).

Fig. 7. Effect of GSH on decreases in testosterone oxidation activities during preincubation of microsomes with dihydralazine.

Microsomes were preincubated for 10 min with dihydralazine (50 μM) and NADPH in the absence (white bar) or presence (stippled bar) of GSH (5 mM), followed by assay of testosterone 2α-, 16α-, and 6β-hydroxylase activities. Results are represented as per cent of the activity obtained without dihydralazine and are means ± SE (N = 3).

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 that of dihydralazine. On the other hand, preincubation of the microsomes with hydralazine 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...
for the disease because none of the other antisera tested behaved in the same manner as anti-LM, even those from patients treated with dihydralazine and without hepatic disease. The sera recognized a single polypeptide in human liver microsomes as judged by immunoblotting, and the antigen was identified as CYP1A2 (Bourdi et al., 1990). The antibodies were found to react specifically with CYP1A2 but not with CYP1A1 expressed in yeast and bacteria (Bourdi et al., 1992). Incubation of microsomes from rat and human livers resulted in covalent binding of dihydralazine metabolites to the microsomes. Formation of these metabolites was shown to be mediated by the CYP1A isoenzyme(s) (Bourdi et al., 1990). On the other hand, no significant inhibitory effect was obtained on the activity in microsomes of phenobarbital-treated rats, which was shown to be mediated by CYP2B1/2 (Kahn et al., 1987), indicating no evidence for the inactivation of the enzymes. In addition, 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 \( k_{\text{nact}} \) 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 generate 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 sera from patients with tienilic acid-induced hepatitis called anti-LKM2 (liver-kidney microsomes) (Beaune et al., 1987), and 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, behave 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, 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., 1992), 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