MECHANISM-BASED INACTIVATION OF CYP2C11 BY DICLOFENAC

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ABSTRACT:

It has been known that diclofenac is biotransformed into chemically reactive metabolites, which bind covalently to liver microsomal proteins, including cytochrome P450 enzyme(s). We have investigated the ability and selectivity of diclofenac to inactivate P450 enzymes. Preincubation of microsomes of untreated rats with diclofenac in the presence of NADPH resulted in time-dependent loss of testosterone 2α- and 16α-hydroxylating activities. No effect of the preincubation was observed on ethoxyresorufin O-deethylase, pentoxyresorufin O-depentylase, or testosterone 6β-hydroxylating activity. The time-dependent decreases in testosterone 2α- and 16α-hydroxylation activities followed the pseudo-first order kinetics and were saturable with increasing diclofenac concentrations. Reduced glutathione was not capable of protecting against the decrease in the enzyme activities. These data establish that a mechanism-based inactivation of CYP2C11 occurs during the oxidative metabolism of diclofenac. The diclofenac concentrations required to achieve the half-maximal rate of inactivation (Km) were 3 to 4 μM, which were close to Km for the low-Km components for diclofenac 4′- and 5-hydroxylation activities (7.29 and 4.43 μM, respectively). Anti-CYP2C11 IgG inhibited diclofenac 4′- and 5-hydroxylation activities, indicating that CYP2C11 is a major isozyme responsible for these aromatic oxidations. The preincubation of microsomes with 4′- or 5-hydroxydiclofenac did not cause a decrease in testosterone 2α- or 16α-hydroxylation activity, suggesting that neither of the primary metabolites is a precursor of the metabolite that inactivates CYP2C11. Therefore, a highly reactive intermediate(s) inactivating CYP2C11, probably arene-oxide, appears to be generated during the process of diclofenac 4′- and/or 5-hydroxylation. Diclofenac metabolism in human liver microsomes did not cause inactivation of CYP2C9, a major isozyme involved in diclofenac 4′-hydroxylation. Because the human microsomes have high diclofenac 4′-hydroxylation but not 5-hydroxylation activity, importance of the latter pathway in the inactivation is suggested.

Diclofenac is one of the nonsteroidal anti-inflammatory drugs widely used clinically. In relation to the diclofenac-induced hepatotoxicity, extensive studies have focused on biotransformation of diclofenac into chemically reactive metabolites capable of binding covalently to liver macromolecules (Boelsterli et al., 1995). Some protein targets of the reactive metabolites have been identified in the liver of animals administrated the drug (Pumford et al., 1993; Hargus et al., 1995; Wade et al., 1997; Seitz et al., 1998). In vitro studies with hepatocytes also showed the formation of the reactive metabolites (Kretz-Rommel and Boelsterli, 1994b; Gil et al., 1995). UDP-glucuronosyltransferase and cytochrome P450 CYP1 isozyme responsible for these aromatic oxidations. The preincubation of microsomes with 4′- or 5-hydroxydiclofenac did not cause inactivation of CYP2C9, a major isozyme involved in diclofenac 4′-hydroxylation. Because the human microsomes have high diclofenac 4′-hydroxylation but not 5-hydroxylation activity, important of the latter pathway in the inactivation is suggested. If a product formed by CYP-dependent metabolism is highly reactive, it should bind to the site of formation in the enzyme, resulting in mechanism-based inactivation of the CYP enzyme. The diclofenac metabolism to generate the benzoquinone metabolites mentioned and Benet, 1992; Boelsterli et al., 1995). The reactivity and potential toxicity of acyl glucuronide products are widely recognized (Kretz-Rommel and Boelsterli, 1993, 1994a; Hargus et al., 1994). It is known that diclofenac is oxidized mainly into two phenolic metabolites, 4′-hydroxydiclofenac and 5-hydroxydiclofenac (Sterlin et al., 1979) (Fig. 1). Although quinone imine metabolite of 5-hydroxydiclofenac has been proposed as a reactive metabolite of diclofenac (Brune and Lindner, 1992), definitive proof for the chemical structure of reactive metabolite by CYP enzymes had not been available. Recent studies provided information about the chemical nature of the reactive metabolite and CYP enzymes involved in its formation. Tang et al. (1999a) found benzoquinone imines as their reduced glutathione (GSH) conjugates, which were formed from 4′- and 5-hydroxydiclofenac in rats and human hepatocytes. They also isolated the GSH conjugates of benzoquinone metabolites in incubations of human liver microsomes with diclofenac in the presence of NADPH and GSH (Tang et al., 1999b). Shen et al. (1999) reported that covalent binding of diclofenac to human liver microsomes was CYP3A4-dependent, and benzoquinone imine, a decomposition product of 5-hydroxydiclofenac, bound covalently to human liver microsomes. Bort et al. (1999) reported that N,5-dihydroxydiclofenac was also found as a further metabolite of 5-hydroxydiclofenac, which was proposed to contribute to the hepatotoxicity of diclofenac.

If a product formed by CYP-dependent metabolism is highly reactive, it should bind to the site of formation in the enzyme, resulting in mechanism-based inactivation of the CYP enzyme. The diclofenac metabolism to generate the benzoquinone metabolites mentioned...
Protein concentrations were assayed by the method of Lowry et al. (1951). Fractions were prepared according to the method of Omura and Sato (1964). Untreated rats were killed 24 h after the final doses, and liver microsomes from Sigma (St. Louis, MO); 6 were from Takasugi Experimental Animals (Saitama, Japan). The remaining enzyme activity against the preincubation time. The reciprocal of the initial slopes of the linear regression lines of the semilogarithmic plots of the enzymatic activities was started by the addition of a test substrate, testosterone, ethoxyresorufin, pentoxyresorufin, or tolbutamide. Testosterone 2α-, 16α-, and 6β-hydroxylation activities of the preincubated microsomes were determined according to the high-performance liquid chromatography (HPLC) method previously described (Miners et al., 1988) at the tolbutamide concentration of 2 mM. Liver microsomes from untreated rats were used for assays of testosterone oxidation activities; those from phenobarbital-treated rats were for PROD activity assays; those from phenytin-treated rats were for EROD activity assays; and human liver microsomes were for tolbutamide hydroxylation activity. All of the assays were performed under linear conditions of metabolite formation with regard to incubation time and protein concentration. Immuno inhibition of Diclofenac Metabolism by an Antibody against CYP2C11. A polyclonal antibody against CYP2C11 raised in a goat was obtained from Daiichi Pure Chemicals (Tokyo, Japan). In immuno inhibition studies, microsomes were preincubated with various amounts of the antibody or preimmune serum at 25°C for 30 min, followed by adding other components of the incubation mixture and assay of diclofenac 4'- and 5-hydroxylation activities. Data Analysis. Enzyme kinetic parameters (\( V_{\text{max}} \), \( K_{\text{m}} \)) were analyzed according to a nonlinear least-squares regression analysis based on a simple method (Yamaoka et al., 1981). Best fittings of the data were performed by weighting them with the reciprocal of the square of the activity. Pseudo-first order kinetic constants for the enzyme inactivation (\( k \)) were calculated from the initial slopes of the linear regression lines of the semilogarithmic plots of the remaining enzyme activity against the preincubation time. The reciprocal of \( k \) thus obtained was plotted against the reciprocal of the diclofenac concentration and then a concentration required for a half-maximum inactivation (\( K_i \)) for the inactivation and a maximum inactivation rate constant (\( k_{\text{max}} \)) were determined from the intercepts on the abscissa and the ordinate, respectively. Results were represented as means ± S.E. Statistical significance was calculated by the Student’s t-test. For experiments involving more than two experimental groups, the groups were compared by analysis of variance, followed by Newman-
Hydroxylation Activities.

Diclofenac inhibited testosterone 2α- and 16α-hydroxylation activities, which were used as indicators of CYP2C11 activity, with an IC₅₀ value of approximately 60 μM at the testosterone concentration of 50 μM (data not shown). Preincubation of liver microsomes from untreated male rats in the presence of NADPH, followed by assay of testosterone 2α- and 16α-hydroxylation activities. Results are means ± S.E. (n = 3).

Keuls multiple comparison test to determine significant differences between the group means.

Results

Effect of Diclofenac Metabolism on Testosterone 2α- and 16α-Hydroxylation Activities.

Diclofenac inhibited testosterone 2α- and 16α-hydroxylation activities, which were used as indicators of CYP2C11 activity, with an IC₅₀ value of approximately 60 μM at the testosterone concentration of 50 μM (data not shown). Preincubation of liver microsomes from untreated male rats in the presence of NADPH with diclofenac intensified the inhibitory effect of diclofenac (Fig. 2). The time-dependent decreases in the activities indicate inactivation of CYP2C11 during metabolism of diclofenac. The decreases in testosterone 2α- and 16α-hydroxylation activities were exponential against the preincubation time and depended on the diclofenac concentration. The first order kinetic constants for the enzyme inactivation (k) were calculated from the initial slopes of the linear regression lines of the semilogarithmic plots of the remaining enzyme activity against the preincubation time as shown in Fig. 2. The reciprocal of k thus obtained was plotted against the reciprocal of the diclofenac concentration. Results are means ± S.E. (n = 3).

Effect of GSH on Diclofenac-Induced Decreases in Testosterone 2α- and 16α-Hydroxylation Activities.

Liver microsomes were preincubated with diclofenac and NADPH in the presence or absence of GSH to determine its protective effect against the inhibition of testosterone 2α- and 16α-hydroxylation activity by the diclofenac metabolism. The decrease in the activity by the preincubation of microsomes was observed both without and with GSH at the concentration of 5 mM (Fig. 4).

Effect of Diclofenac Metabolism on Other Monoxygenase Activities.

The preincubation of microsomes with diclofenac did not affect EROD activity in liver microsomes of β-naphthoflavone-treated rats (an indicator of CYP1A), PROD activity in microsomes of phenobarbital-treated rats (CYP2B), or testosterone 6β-hydroxylation activity in microsomes of untreated rats (CYP3A) (Fig. 5). These results indicated selectivity for CYP2C11 in the inactivation during the diclofenac metabolism.

Kinetics for Diclofenac 4’- and 5-Hydroxylation Activities.

Kinetic analysis for diclofenac 4’- and 5-hydroxylation activities obtained in the substrate concentration range of 1 to 320 μM indicated that both of the reactions catalyzed by more than two enzyme systems. Kₘ values for the low-Kₘ components for diclofenac 4’-hydroxylation and 5-hydroxylation activities (7.29 and 4.43 μM, respectively; Table 2) were close to the Kᵢₗ for the inactivation (3–4 μM; Table 1). Partition ratio of sum of diclofenac 4’- and 5-hydroxylation (diclofenac elimination is almost equivalent to the sum) versus the rate of inactivation was calculated to be 8.50.

Effects of Primary Metabolites of Diclofenac on Testosterone 2α- and 16α-Hydroxylation Activities.

Addition of primary metabolites of diclofenac, 4’-hydroxydiclofenac, and 5-hydroxydiclofenac, at the concentration that diclofenac inhibited testosterone 2α- and 16α-hydroxylation activities, did not affect either of the activities (Fig. 6). Preincubation of microsomes with the metabolite in the presence of NADPH did not cause a decrease in testosterone 2α- or 16α-hydroxylation activity (Fig. 6). Similar results were obtained by the preincubation of microsomes with the metabolite but in the absence of NADPH (data not shown).

Diclofenac Metabolism and Inactivation of CYP2C11 Expressed in Microsomes of Insect Cells with Baculovirus System.

Effects of diclofenac metabolism on CYP2C11 were also tested using microsomes from insect cells expressing CYP2C11 instead of rat liver.
Microsomal reaction mixture was preincubated with NADPH in the absence or presence of diclofenac (10 μM) and GSH (5 mM) for 10 min, followed by assay of testosterone 2α- and 16α-hydroxylation activities. Results are means ± S.E. (n = 3). The groups were compared by analysis of variance, followed by Newman-Keuls multiple comparison test. **, significantly different from the “control” activity obtained without diclofenac (p < 0.01).

Microsomes from β-naphthoflavone-treated, phenobarbital-treated, and untreated male rats were preincubated without (○) or with (●) diclofenac (10 μM) in the presence of NADPH, followed by assay of EROD, PROD, and testosterone 6β-hydroxylation activities, respectively. Results are means ± S.E. (n = 3).

TABLE 2
Kinetic parameters for diclofenac 4'- and 5-hydroxylation in liver microsomes of male rats

Parameters were calculated from the metabolic activities obtained at the substrate concentration range of 1 to 320 μM. Results are means ± S.E. (n = 3).

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<tr>
<td></td>
<td>Km1</td>
<td>Vmax1</td>
<td>Km2</td>
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<tr>
<td>4'-Hydroxylation</td>
<td>7.29 ± 0.29</td>
<td>0.338 ± 0.026</td>
<td>80.3 ± 24.2</td>
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<tr>
<td>5-Hydroxylation</td>
<td>4.43 ± 1.02</td>
<td>0.389 ± 0.069</td>
<td>78.6 ± 13.4</td>
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Microsomes. Testosterone 16α-hydroxylation activity rapidly decreased during the preincubation of expressed CYP2C11 with diclofenac in the presence of NADPH (Fig. 7). The first order kinetic constants for the enzyme inactivation thus obtained from the initial slope were 0.645 min⁻¹. It was observed that CYP2C11 had an ability to generate 4'- and 5-hydroxydiclofenac (1.8 pmol/min/pmol of CYP for 4'-hydroxylation, 2.2 pmol/min/pmol of CYP for 5-hydroxylation, at the diclofenac concentration of 10 μM).

Effect of Antibody against CYP2C11 on Diclofenac 4'- and 5-Hydroxylation Activities. Effect of addition of a polyclonal anti-
Discussion

A previous immunochemical study has demonstrated that CYP2C11 is one of the microsomal target proteins of covalent binding of the diclofenac reactive metabolite (Shen et al., 1997). It was demonstrated that diclofenac inactivated CYP2C11 in a mechanism-based manner according to the following observations (Figs. 2–4): 1) NADPH dependence for the inhibition, 2) pseudo-first order kinetics for the time-dependent inactivation, 3) saturation of inactivation with increasing diclofenac concentrations, and 4) lack of protection against the inhibition by GSH. Selectivity toward one particular CYP enzyme, CYP2C11 in this case, is characteristic of suicide inactivation. More rapid inactivation of CYP2C11, which was observed by using expressed CYP2C11 instead of liver microsomes (Fig. 7), supported the conclusion.

Microsomes from untreated male rats were preincubated with anti-CYP2C11 antiserum (○) or preimmune serum (□), followed by assay of diclofenac 4’- and 5-hydroxylation activities at diclofenac concentrations of 10 μM. Relative activities are represented as percentage of the activity obtained without the antibody, and are means of duplicate determinations.

The diclofenac concentrations required to achieve the half-maximal rate of inactivation (K1/2) were 3 to 4 μM. Kinetic analysis for diclofenac 4’- and 5-hydroxylation activities indicated that both of the reactions were catalyzed by more than one enzyme system (Table 2). The K1/2 value for the inactivation were close to Km values for the low-Km components for diclofenac 4’- and 5-hydroxylation activities (7.29 and 4.43 μM, respectively), suggesting that the pathway(s) is relevant to the inactivation of CYP2C11.

There can be two ways to generate the reactive metabolite(s). One possibility is that the process of diclofenac 4’- and/or 5-hydroxylation is directly involved in generation of a reactive intermediate(s). Arene-oxide is one of the proposed metabolic intermediates generated during the aromatic hydroxylations, which are highly reactive and are involved in enzyme inactivation. A second possibility is that further metabolites of 4’- and 5-hydroxydiclofenac, which include benzoquinones and hydroxylamine and have been already proposed in relation to the diclofenac hepatotoxicity (Bruno and Lindner, 1992; Bort et al., 1999; Shen et al., 1999; Tang et al., 1999a,b). However, the preincubation of microsomes with 4’- or 5-hydroxydiclofenac instead of diclofenac did not cause decrease in testosterone 2α- or 16α-hydroxylation activity (Fig. 6), indicating that the proposed further metabolite is not responsible for the inactivation of CYP2C11. We found that both aromatic hydroxylations of diclofenac were mediated by CYP2C11 (Fig. 7). Thus, it is concluded that diclofenac inactivates CYP2C11 during the 4’- and/or 5-hydroxylation processes, probably via the arene-oxide formation.

Human liver microsomes have higher diclofenac 4’-hydroxylation activity compared with rats, whereas 5-hydroxylation activity was very low. There was no evidence for mechanism-based inactivation of CYP2C9, a major CYP isozyme responsible for diclofenac 4’-hydroxylation, during the diclofenac metabolism in human liver microsomes (Fig. 9). It is reasonable to postulate that the reactive metabolite relevant to inactivation of CYP enzymes is not generated during diclofenac 4’-hydroxylation. Thus, diclofenac 5-hydroxylation rather
than 4'-hydroxylation seems to be closely related to formation of arene-oxide, a possible candidate to inactivate CYP2C11. However, the possibility could not be excluded that diclofenac 4'-hydroxylation was mediated by CYP2C11 and CYP2C9 via different intermediates.

In summary, diclofenac is demonstrated to be a selective and mechanism-based inactivator of CYP2C11. Formation of a chemically reactive metabolite of diclofenac that inactivates CYP2C11 was a low-$K_{inact}$ reaction. The major pathways leading to aromatic hydroxylations, especially 5-hydroxylation, appear to be directly involved in the formation of the intermediate that binds to CYP2C11, resulting in loss of catalytic activity.

References