CHLORZOXAZONE 6-HYDROXYLASE AND P-NITROPHENOL HYDROXYLASE AS THE MOST SUITABLE ACTIVITIES FOR ASSAYING CYTOCHROME P450 2E1 IN CYNOMOLGUS MONKEY LIVER

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ABSTRACT:

Western blot analyses of liver microsomes from 13 male and 12 female monkeys demonstrated that in each sample a variable amount of a cytochrome P450 (P450) protein, likely monkey P450 2E1, cross-reacted with anti-rat P450 2E1 antibodies. Therefore, the involvement of monkey 2E1 in the oxidation of typical substrates for 2E1 from other species, such as dimethylnitrosamine (DMN), p-nitrophenol (pNP), chlorzoxazone (CLZ), and aniline, was investigated. Kinetic studies using microsomes from five male and five female monkeys showed that CLZ and pNP hydroxylations were monophasic, with apparent $K_M$ values of 77 and 14 $\mu$M, respectively, whereas aniline hydroxylation and DMN demethylation were multiphasic, suggesting that P450s other than 2E1 were involved in catalyzing the latter two reactions. When correlation analyses were performed using several monooxygenase activities determined in male and female monkey liver specimens, it was found that immunodetectable 2E1 contents were highly correlated ($r = 0.75$) with CLZ and pNP hydroxylations, weakly correlated ($r = 0.6$) with aniline hydroxylation, and not correlated with DMN demethylation or other monooxygenase activities; CLZ hydroxylation was strongly correlated with pNP hydroxylation, weakly correlated with aniline hydroxylation, and not correlated with DMN demethylation. Inhibition experiments showed that CLZ and pNP hydroxylations were immunoinhibited by 60–80% by anti-rat P450 2E1 and were inhibited by the prototypical 2E1 inhibitor 4-methylpyrazole with $IC_{50}$ values of 1.5 and 13 $\mu$M, respectively. In conclusion, the findings provide evidence that P450 2E1 is constitutively and equally expressed in male and female monkey liver and it exerts a major role only in hydroxylation of CLZ and pNP.

Metabolism studies, both in vivo and in vitro, have mainly used mice or rats as the experimental animal model. However, it is difficult to extrapolate to humans from data obtained with rodents, essentially because of differences among species in many physiological functions. Accordingly, nonhuman primates, which are genetically closer to humans than are rodents, could be better species for comparative studies. The pharmaceutical industry often includes monkeys in preclinical and toxicological studies.

Among enzymes involved in xenobiotic metabolism, the most important system is represented by the P450 superfamily (Parkinson, 1996), which has been studied mostly in rodents and humans. In monkeys (Nelson et al., 1996). To date, P450 isoforms closely related to the P450 1A (Komori et al., 1992a), 2A (Ohmori et al., 1993a), 2B (Ohmori et al., 1993b), 2C (Ohi et al., 1989; Ohmori et al., 1994), 2D (Wu et al., 1993), and 3A (Ohmori et al., 1993a; Dalet-Beluche et al., 1992) subfamilies have been purified from the livers of some monkey species and their biochemical properties have been compared with those of the corresponding human and rodent enzymes. On the other hand, although the sequence of the P450 2E1 gene from cynomolgus monkeys has been reported and found to be similar (90%) to the human orthologue (Komori et al., 1992b), the function of the 2E1 protein has not been described. Because P450 2E1 has a considerable influence on the metabolism of low-molecular weight xenobiotics and the metabolic activation of several hepatotoxins, including benzene, halothane, and carbon tetrachloride (Guengerich et al., 1991), interest in the development of model substrates and inhibitors of this enzyme in monkeys appears warranted.

The rate of demethylation of DMN, determined with substrate concentrations of 1–4 mM, is generally accepted as a selective activity to assay the contribution of 2E1 in rats, rabbits, and humans (Tu and Yang, 1983; Thomas et al., 1987; Yang et al., 1990; Raucy et al., 1987). Some authors (Stevens et al., 1993; Sharer et al., 1995) also used this activity, with DMN at concentrations of 1 mM, to probe 2E1 in monkey species. However, a selective DMNδ role for the monkey 2E1 isoform cannot be assumed, because interspecies differences in 2E1 activities toward the same substrate have been reported (Mikalsen et al., 1991; Puccini et al., 1992). In general, the modification of a few key amino acids in the sequences of orthologous P450 isoforms may result in different substrate specificities in different species. The case of mouse P450 2A4 and 2A5, for example, is well known; the replacement of only one amino acid brings about a change of substrate specificity (Lindberg and Negishi, 1989).

Besides DMN demethylation, other reactions, such as the hydroxylation of aniline, pNP, and the muscle relaxant CLZ, have been

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frequently used as tools to screen for P450 2E1 activity in rats (Reinke et al., 1985; Carriere et al., 1993), rabbits (Raucy et al., 1987), and humans (Peter et al., 1990; Girre et al., 1994; Tassaneeyakul et al., 1993); however, their relevance for 2E1 in monkeys has not been assessed. The aim of the present study was to identify, by using a large number of untreated male and female cynomolgus monkeys, the interindividual variability of 2E1 expression in liver and to establish which of the aforementioned oxidations are suitable for evaluation of P450 2E1 in this species.

Materials and Methods

Chemicals. Nitrocellulose filters (0.45 μm), 4-chloro-1-naphthol, corticosterone, 16β-hydroxytestosterone, 4-androstene-3,17-dione, α-naphthoflavone, triacetyloleanandromycin, metyrapone, erythromycin, DMN, 4MPy, and 8MP were purchased from Sigma Chemical Co. (St Louis, MO). 15β-, 6β-, 16α-, and 2β-hydroxy metabolites of testosterone were obtained from the Steroids Reference Collection (St. Louis, MO). Goat anti-rabbit IgG was purchased from Dako (Copenhagen, Denmark). Enzymes and coenzymes were obtained from Boehringer (Mannheim, Germany). Ethoxyresorufin was synthesized from resorufin by ethylation with ethyl iodide (Klotz et al., 1984). CLZ, its 6-hydroxy metabolite, and benzoxazolone were kindly provided by Prof. F. P. Guengerich (Vanderbilt University, Nashville, TN). All other chemicals and solvents were of analytical grade and were obtained from commercial sources.

Animals and Preparation of Microsomes. Male and female cynomolgus monkeys (Macaca fascicularis), 2–3 years old (Inverserse Research, Tranent, Scotland), were housed individually in stainless steel cages in a temperature-, humidity-, and light-controlled facility (70–72°F, 48–52% humidity, 12-hr light/dark cycle). The animals were allowed free access to water and food. They were killed by CO2 asphyxia; the livers were collected and microsomes were prepared from single animals, as described previously (Longo et al., 1986). The washed microsomal pellets were resuspended in 100 mM phosphate buffer, 1 mM EDTA (pH 7.4), and stored at −80°C. Protein content was determined according to the method of Lowry et al. (1951), using bovine serum albumin as the standard.

Enzyme Assays. Hepatic P450 levels were measured according to the method of Omura and Sato (1964). Microsomal AnH was determined by measuring the formation of p-aminophenol, as described by Ko et al. (1987). Aminopyrine, DMN, and erythromycin demethylase activities were assayed by measuring the formation of formaldehyde (Tu and Yang, 1983). Ethoxyresorufin O-deethylation activity was determined by measuring the formation of resorufin, with a Perkin-Elmer spectrofluorometer (Krijghsheld and Gram, 1984). pNPH activity was determined by measuring the formation of 4-nitrophenolate according to the method of Reinke and Moyer (1985). Testosterone hydroxylase was determined as reported previously (Longo et al., 1991), using a HPLC method described by Platt et al. (1989). CLZ6H was assayed by a HPLC method described by Peter et al. (1990), using benzoxazolone as the internal standard. All enzymatic activities were assayed under conditions of linearity with respect to protein and time.

Gel Electrophoresis and Immunoblotting. Sodium dodecyl sulfate-gel electrophoresis was performed using the discontinuous system of Laemmli (1970), with a 1.5-mm-thick gel and 3% and 7.5% acrylamide in the stacking and separation gels, respectively. Proteins were transferred from the slab gel to nitrocellulose filters using the method of Towbin et al. (1979). Immunodetection was performed using rabbit anti-rat P450 2E1 as the primary antibody and anti-rabbit IgG conjugated to horseradish peroxidase as the secondary antibody. The peroxidase activity was detected with chloro-1-naphthol and H2O2 as previously described (Puccini et al., 1992). The bands on the nitrocellulose membranes were quantified with a laser densitometer (Ultrascan 2202; LKB).

Preparation of Antibodies. Female New Zealand white rabbits were immunized, as described previously (Kaminsky et al., 1981), with the purified rat P450 2E1 antigen. Preimmune serum was collected before the injections. For the first immunization, about 0.2 mg of antigen was mixed with complete Freund’s adjuvant. After 6 and 10 weeks, injections of 0.1 mg of antigen in incomplete Freund’s adjuvant were administered. The immune serum was collected 10 days after the last injection. IgG fractions from preimmune serum and immune serum were purified by precipitation of non-IgG proteins with caprylic acid at pH 4.5, followed by precipitation of the IgG fraction with ammonium sulfate at pH 7.4 (McKinney and Parkinson, 1987). These antibodies recognized, in microsomal samples of rat or human liver, a single, electrophoretically distinct protein. The IgG, at a concentration of 10 mg/ml, was able to inhibit by approximately 80% the AnH activity in hepatic microsomes from rats pretreated with acetone, a potent inducer of 2E1 (Menicagli et al., 1994).

Chemical Inhibition Assay. All of the inhibitors were dissolved in methanol, except metyrapone and 4MPy, which were dissolved in water. Because methanol could have an inhibitory effect, it was evaporated under N2. To facilitate the redissolution of inhibitors, the residue was resuspended in assay buffer by sonication and then microsomes were added and vigorously vortex-mixed before addition of the other components.

Immunoprecipitation Assay. Microsomes (0.75 mg/ml) were mixed with different amounts of anti-2E1 antibodies (IgG fraction) or preimmune rabbit IgG in 100 mM potassium phosphate buffer (pH 7.4) and were preincubated for 20 min at 4°C. Cofactors and substrate were then added and incubated for 30 min at 37°C, under the conditions previously described.

Analysis of Data. The Michaelis-Menten parameters K_M and V_max were obtained by fitting kinetic data to one- or two-enzyme models by using a simple computer program (devised by Dr. R. Ambrosi, Istituto di Chimica Quantistica, Consiglio Nazionale delle Ricerche, Pisa, Italy) designed for nonlinear, least-squares, regression analyses; the data were depicted as Eadie-Hofstee plots. Correlation coefficients were calculated by least-squares regression analysis of the raw data. Student’s t test was used, and correlations were considered to be statistically significant at p < 0.05.

Results

Immunodetection of P450 2E1 in Cynomolgus Monkey Liver Microsomes. The anti-rat 2E1 antibodies were used to probe immunoblots of cynomolgus liver microsomal preparations. As shown in fig. 1, the antibodies cross-reacted with two proteins in monkey liver, one of which had a lower molecular weight than purified rat 2E1. This staining pattern was observed for all of the 13 male and 12 female monkey microsomal samples examined. The staining intensity of the protein with the higher molecular weight varied considerably among the individual liver samples, whereas that of the protein with the lower molecular weight was very weak and did not exhibit appreciable
variability. Thus, the immunoreactive protein with a molecular weight similar to that of rat 2E1 was believed to be the cynomolgus monkey orthologue of P450 2E1.

Kinetics of CLZ, pNP, and Aniline Hydroxylation and DMN Demethylation by Monkey Liver Microsomes. Microsomal preparations from five male and five female monkey liver samples, expressing the lowest and highest immunodetectable 2E1 levels, were used to obtain the apparent kinetic parameters $V_{\text{max}}$ and $K_M$ for the oxidation of possible 2E1 substrates, i.e. CLZ, aniline, pNP, and DMN. In the case of CLZ 6-hydroxylation, microsomes displayed monophasic Michaelis-Menten kinetics over the range of substrate concentrations examined (5–750 μM) (fig. 2A) and the activity was linear up to 30 min and microsomal protein concentrations of 0.5 mg/ml. As calculated from the least-squares regression analyses, the apparent $K_M$ value was $77 \pm 35$ μM, whereas $V_{\text{max}}$ varied between 1.3 and 5.8 nmol/min/mg of protein. No gender differences were observed in the $K_M$.

The hydroxylation of pNP also displayed simple enzyme kinetic properties with one component, as was clearly apparent from the Eadie-Hofstee plot (fig. 2B). For both male and female monkey liver samples, the apparent $K_M$ was $14 \pm 7$ μM (without gender dependence) and the $V_{\text{max}}$ values were in the range of 0.28–1.1 nmol/min/mg of protein.

In contrast, the Eadie-Hofstee plots for aniline hydroxylation showed at least two components (fig. 2C). The nature of these enzyme kinetics suggested that more than one P450 enzyme was capable of hydroxylating aniline. The kinetic parameters were determined by nonlinear regression of untransformed data derived from microsomes from five male and five female monkey livers. In each case, high- and low-$K_M$ components were apparent. The relatively low-$K_M$ component had a $K_M,1$ of $28 \pm 13$ μM, with a $V_{\text{max},1}$ of $0.23 \pm 0.16$ nmol/min/mg of protein, and the relatively high-$K_M$ component had a $K_M,2$ of $540 \pm 290$ μM, with a $V_{\text{max},2}$ of $0.63 \pm 0.31$ nmol/min/mg of protein.

The demethylation of DMN, also assayed at substrate concentrations between 50 and 1000 μM, using the same male and female monkey samples, showed nonlinear kinetics (data not shown). Although it was not possible to calculate definite multiple $K_M$ values, because of the low activities measured, the multiphasic kinetic pattern suggested that, at substrate concentrations of 1 mM, more than one P450 isoform was involved in DMN demethylation.

Determination of P450-Linked Monooxygenase Activities and Comparison with CLZ6H in Liver Microsomes from Male and Female Monkeys. Table 1 shows findings for total P450 estimations and the oxidative metabolism of various P450 substrates in 13 male and 12 female monkey liver microsomal samples. AnH, DMNd, pNPH, and CLZ6H activities were determined, because they are known to be 2E1-dependent activities in rodents or humans (Raucy et al., 1987; Peter et al., 1990; Tassaneeyakul et al., 1993). The P450 content and DMNd activity level were similar to published data (including the lack of gender differences) (Sharer et al., 1995; Bullock et al., 1995; Longo et al., 1992; Weaver et al., 1994); no comparison was possible for the AnH, CLZ6H, and pNPH activities, because they had not been previously determined.
To examine the role of 2E1 and other P450 isoforms in the oxidation of aniline, pNP, DMN, and CLZ, we calculated the correlation coefficients (table 2) for correlation of the monoxygenase activities (table 1) with each other and with the 2E1 contents determined by Western blotting. The immunoblots were carried out with 20 μg of protein; in the range of 1–20 μg of microsomal protein, the intensity of 2E1 staining increased linearly. Graphs of some individual comparisons are shown in fig. 3. The level of expression of P450 2E1 was strongly correlated with the hydroxylation of CLZ (r = 0.77) and weakly correlated with the hydroxylation of aniline but was not correlated with the demethylation of DMN. In keeping with this, CLZ6H was strongly correlated with pNPH and weakly correlated with AnH but was not correlated with DMNd; pNPH was also correlated with AnH and not with DMNd. When the correlation coefficients for correlation among the activities of table 1 were calculated separately for male and female monkey samples, values similar to those in table 2 were found (data not shown). A lack of significant correlation, for monkey liver microsomes, was found between the immunodetectable 2E1 contents and the activities of ethoxyresorufin O-deethylase, aminopyrine N-demethylase, coumarin 7-hydroxylase, and testosterone hydroxylase, which are known to be catalyzed by several isoforms, including P450 1A, 2A, 2B, and 3A (Ohmori et al., 1993a; Dalet-Beluche et al., 1992; Shaper et al., 1995; Bullock et al., 1995) (data not shown). pNPH also was not correlated with any of the aforementioned monoxygenases, whereas, unexpectedly, CLZ6H was correlated significantly (r = 0.55) with 6β-hydroxytestosterone hydroxylase activity, which is known to be dependent on members of the P450 3A subfamily in many species, including monkeys (Ohmori et al., 1993a).

### Effects of Anti-P450 2E1 Antibodies and P450 Chemical Inhibitors on the Hydroxylation of CLZ and pNP

Immuno inhibiton experiments demonstrated that antibodies raised against rat P450 2E1 inhibited both CLZ 6-hydroxylation and pNP hydroxylation catalyzed by monkey liver microsomes (fig. 4). Preimmune IgG had no effect on these reactions. At the highest concentration of antibodies (10 mg IgG/nmol P450), the rates of hydroxylation of CLZ and pNP were reduced by 68 and 63%, respectively. When a liver microsomal sample with high CLZ6H activity (5.8 nmol/min/mg of protein) was used, inhibition of about 80% of the CLZ6H activity by anti-rat 2E1 (10 mg IgG/nmol P450) was observed. Although greater inhibition occurred in microsomes exhibiting the highest rate of CLZ 6-hydroxylation, there was a basal level of CLZ6H activity that was not inhibitable by these antibodies, possibly reflecting the contribution of other P450 isoforms to the catalysis of this reaction or a limited ability of these antibodies to inhibit the 2E1-dependent activities. To further assess the role of 2E1 in the hydroxylation of CLZ and pNP and the possible participation of other P450 isoforms, these reactions were carried out in the presence of a panel of selective chemical inhibitors (4MPy, 8MP, metyrapone, α-naphthoflavone, and triacetyloleandro- rymycin). 4MPy, which has been reported to be a potent inhibitor of P450 2E1 in rats and humans (Feierman and Cederbaum, 1987), inhibited both CLZ 6-hydroxylation and pNP hydroxylation in monkey liver microsomes, with IC50 values of approximately 1.5 and 13 μM, respectively (fig. 5). Unexpectedly, 8MP, which has been reported to selectively inhibit P450 2A-dependent coumarin hydroxylase activity in mice and humans (Mäenpää et al., 1993), did not inhibit, without preincubation, this activity in monkey liver microsomes (IC50 > 300 μM). In contrast, 8MP inhibited both CLZ6H and pNPH activities, with IC50 values of 65 and 155 μM, respectively (fig. 5). On the other hand, triacetyloleandromycin, metyrapone, and α-naphthoflavone, which are known to be selective inhibitors of the P450 3A, 2B, and 1A subfamilies, respectively (Murray and Reidy, 1990; Halpert et al., 1994; Namkung et al., 1988; Roos et al., 1993), did not affect or weakly affected both CLZ and pNP hydroxylations, having IC50 values of >300 μM. Regarding the inhibition selectivity of 4MPy, it was observed that, even at a concentration of 50 μM, 4MPy was unable to inhibit the monkey liver microsomal hydroxylation of testosterone (data not shown), a compound known to be oxidizable at many positions, in rats, by several P450 isoforms but not 2E1 (Platt et al., 1989). Thus, 4MPy might be a selective inhibitor of 2E1 also in monkeys. These findings suggest that P450 2E1 contributes primarily to CLZ and pNP oxidation.

### Discussion

In the present investigation, we established that a P450 protein immunologically and catalytically similar to rat 2E1 was, as expected (Komori et al., 1992b; Stevens et al., 1993), present and steadily expressed in adult male and female cynomolgus monkeys. Its variability among several individuals, as determined by immunoblotting, was approximately 8-fold, but the 2E1 content did not vary significantly between male and female liver microsomes.

The data also indicated that monkey hepatic P450 2E1 is the enzyme predominantly responsible for the hydroxylation of pNP and CLZ but not for the oxidation of aniline or DMN. Evidence for this conclusion was derived from studies using monkey liver microsomes to assess the metabolism of the aforementioned substrates, correlation of activities and immunodetectable 2E1 contents, inhibition by chemical agents, and immunoinhibition.

Monkey liver microsomes from 10 individuals metabolized monophasically and without gender differences both CLZ and pNP, but not DMN and aniline, the metabolism of which exhibited multiphasic enzyme kinetics. The multiphasic nature of the steady-state kinetics for the latter two compounds was not surprising, because DMN, at substrate concentrations of 4 mM, was found to be dependent mainly on P450 2E1 and P450 2A5 and their levels of expression in mice (Camus et al., 1993) and because aniline has been reported to...
be hydroxylated by various P450 isoforms in rats (Funae and Imaoka, 1993).

Our kinetic study suggested that P450 2E1 alone may be the major enzyme involved in CLZ 6-hydroxylation in monkeys because, despite a 3-fold variation in $V_{\text{max}}$ values among samples, the same $K_M$ values (77 ± 35 µM) were determined. For pNPH as well, although the $V_{\text{max}}$ values varied about 3-fold, the same $K_M$ values (14 ± 7 µM) were observed, suggesting that this reaction may also be catalyzed mostly by 2E1. Of course, the existence of other P450 isoforms having $K_M$ values close to those of 2E1 for CLZ6H and pNPH cannot be excluded. It should also be noted that the $K_M$ values for these monooxygenases in monkeys are quite similar to those reported for humans (Carriere et al., 1993), P450 1A1 is also involved in the 6-hydroxylation of CLZ, although with a $K_M$ higher than that of 2E1.

Some authors have presented compelling evidence that, in rats and humans (Carriere et al., 1993), P450 1A1 is also involved in the 6-hydroxylation of CLZ, although with a $K_M$ higher than that of 2E1.

In untreated cynomolgus monkeys, however, the contribution of this isoform to CLZ6H activity is ruled out because the liver of this species lacks detectable expression of either 1A1 or 1A2 proteins (Komori et al., 1992a; Bullock et al., 1995).

A further strong indication that pNP and CLZ hydroxylations are catalyzed by 2E1 is derived from the activity correlation experiments. The catalytic properties, toward various substrates, of a number of purified P450s (1A1, 2A, 2B, 2C, and 3A) from monkeys have been


