

In Vivo Cimetidine Inhibits Hepatic CYP2C6 and CYP2C11 but Not CYP1A1 in Adult Male Rats^{1,2}

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ABSTRACT

We previously reported that *in vivo* cimetidine inhibits hepatic microsomal enzyme activities mediated by cytochrome P450 (CYP)2C11 and at least one other CYP enzyme but does not inhibit CYP2A1-, CYP2B- or CYP3A-mediated activities in adult male rats. To investigate the effects of *in vivo* cimetidine on CYP1A1, cimetidine (150 mg/kg i.p.) or saline was administered to β -naphthoflavone-induced (40 mg/kg i.p. once daily for 3 consecutive days) or uninduced adult male Wistar rats, and hepatic microsomes were prepared 90 min after the cimetidine injection. Cimetidine had no effect on either methoxyresorufin O-dealkylase (MROD) or ethoxyresorufin O-dealkylase (EROD) activity in microsomes from β -naphthoflavone-induced rats. In these same microsomes, polyclonal anti-CYP1A1 IgG inhibited both MROD and EROD activities by >90%, whereas monoclonal anti-CYP1A1 IgG inhibited MROD and EROD activities by 60% and 80%, respectively. In contrast, cimetidine inhibited MROD and EROD activities in microsomes from uninduced rats by 50% and 65%, respectively ($P < .05$). Immunoinhibition studies with polyspecific and monospecific anti-CYP2C11 IgG

indicated that MROD and EROD activities are mediated by a CYP2C enzyme or enzymes other than CYP2C11 in these microsomes. To investigate the possibility that the drug affected EROD activity in uninduced rats by inhibiting CYP2C6, cimetidine was administered as described to rats that had been pretreated with phenobarbital (80 mg/kg i.p. once daily for 4 consecutive days). In hepatic microsomes from these rats, cimetidine inhibited progesterone 21-hydroxylase activity (mediated by CYP2C6) by 62% and progesterone 2 α -hydroxylase activity (mediated by CYP2C11) by 39% but had no effect on progesterone 6 β -hydroxylase activity (mediated by CYP3A). Taken together, the results indicate that *in vivo* cimetidine has no effect on CYP1A1 but inhibits CYP2C6 in addition to CYP2C11. Preincubation of microsomes from uninduced rats with cimetidine and NADPH *in vitro* increased the potency of inhibition of EROD activity by 20-fold, suggesting that cimetidine inhibits CYP2C6, as it does CYP2C11: by forming a metabolite/intermediate complex.

Cimetidine, a substituted imidazole, is an H₂ receptor antagonist used in the treatment of peptic ulcer disease and related disorders. Early case reports of drug/drug interactions indicated that cimetidine potentiates the anticoagulant effect of warfarin, and this led to the discovery that cimetidine is an effective inhibitor of CYP (Somogyi and Gugler, 1982). Investigators also found that cimetidine inhibits the metabolism of a large number of compounds *in vitro* and *in vivo* in both rats (Adedoyin *et al.*, 1987; Galbraith and Jellinck, 1989; Knodell *et al.*, 1982; Pelkonen and Puurunen, 1980; Rendic *et al.*, 1979; Speeg *et al.*, 1982) and humans (Hoensch *et al.*, 1985; Knodell *et al.*, 1982, 1991; Somogyi and Muirhead, 1987). Binding of cimetidine to CYP from unin-

duced and PB- and 3-MC-induced rats is characterized by a type II difference spectrum (Rendic *et al.*, 1979). In experiments performed *in vitro*, the inhibition of CYP by cimetidine exhibits either competitive or mixed competitive/noncompetitive enzyme kinetics (Speeg *et al.*, 1982; Winzor *et al.*, 1986).

As a consequence of these early studies, cimetidine has been considered to be a reversible and nonselective inhibitor of CYP enzymes (Leclercq *et al.*, 1989; Reilly *et al.*, 1988). Galbraith and Jellinck (1989) administered multiple doses of cimetidine to rats *in vivo* and then measured hepatic microsomal enzyme activities. In male rats, they found that cimetidine treatment led to a reduction in hepatic microsomal estradiol 2-hydroxylase, estradiol 16 α -hydroxylase, ethylmorphine N-demethylase, aniline hydroxylase and benzo[a]pyrene hydroxylase activities but had no effect on 7-ethoxycoumarin O-deethylase activity. However, in female rats, none of these activities was affected by cimetidine. The results of their study suggested that the inhibition of CYP by

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ABBREVIATIONS: CYP, cytochrome P450; PB, sodium phenobarbital; 3-MC, 3-methylcholanthrene; BNF, β -naphthoflavone; MROD, methoxyresorufin O-dealkylase; EROD, ethoxyresorufin O-dealkylase; DMSO, dimethylsulfoxide, HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

cimetidine *in vivo* is more selective than was previously thought. In a more recent study with male rats, we found that cimetidine inhibits CYP2C11 but has no effect on enzyme activities mediated by CYP2A1, CYP2B or CYP3A (Chang *et al.*, 1992a). In a related study, we observed that inhibition of CYP2C11 enzyme activity by cimetidine is competitive *in vitro* but noncompetitive in microsomes from rats treated with cimetidine *in vivo* (Chang *et al.*, 1992b). Preincubation of hepatic microsomes with NADPH and low concentrations of cimetidine *in vitro* before the initiation of substrate oxidation resulted in noncompetitive inhibition of CYP2C11 but had no effect on CYP2A1-, CYP2B- or CYP3A-mediated activities (Chang *et al.*, 1992b). This was the same pattern of inhibition that was seen after the treatment of rats with cimetidine *in vivo*, suggesting that cimetidine inhibits CYP *in vivo* by formation of a metabolite/intermediate complex. Spectral evidence in support of this has been reported recently (Levine and Bellward, 1995).

In addition to CYP2C11, cimetidine inhibits at least one other CYP in uninduced male rats, although the identity of this enzyme or enzymes remains to be established (Chang *et al.*, 1992a). The possibility that cimetidine inhibits CYP1A1 was suggested by results of an earlier *in vivo* study by Drew *et al.*, (1981) in which 3-MC-induced rats were administered a single dose of cimetidine (150 mg/kg i.p.) and killed 2 hr later. The authors reported that cimetidine inhibited the hepatic microsomal benzo[a]pyrene hydroxylase activity by 89% but had no effect on 7-ethoxycoumarin O-deethylase activity or several other nonspecific enzyme activities. There is an inconsistency in their finding because it was subsequently shown that CYP1A1 accounts for >80% of the benzo[a]pyrene hydroxylase activity (Ryan *et al.*, 1982) and 60% to 70% of the 7-ethoxycoumarin O-deethylase activity in hepatic microsomes from 3-MC-induced rats (Hietanen *et al.*, 1987; Park *et al.*, 1982). The present study was therefore undertaken to investigate the effect of *in vivo* administration of cimetidine on hepatic CYP1A1 in adult male rats with the use of enzyme-selective substrates and inhibitory, enzyme-specific antibodies. The results obtained indicate that *in vivo* cimetidine does not inhibit rat hepatic microsomal CYP1A1. Rather, it is a selective inhibitor of CYP2C6 in addition to CYP2C11. Furthermore, inhibition of CYP2C6, like that of CYP2C11, occurs by the formation of a metabolite/intermediate complex.

Materials and Methods

Chemicals. Cimetidine hydrochloride was a gift from Smith Kline & French Canada (Mississauga, Ontario, Canada). Testosterone, 16-keto-testosterone and BNF were purchased from Sigma Chemical (St. Louis, MO), and 2 α -hydroxytestosterone was obtained from Steraloids (Wilton, NH). [4-¹⁴C]Progesterone (60 mCi/mmol) was purchased from Amersham Canada (Oakville, Ontario, Canada). Resorufin (phenoxazone) was obtained from Aldrich Chemical (Milwaukee, WI), and both methoxyresorufin and ethoxyresorufin were purchased from Molecular Probes (Eugene, OR). PB and NADPH were obtained from British Drug House (Toronto, Ontario, Canada) and Boehringer-Mannheim Canada (Dorval, Quebec, Canada), respectively. Lyophilized bovine serum albumin and BioRad protein assay dye reagent concentrate were purchased from BioRad Laboratories (Mississauga, Ontario, Canada). ChromoPure rabbit IgG (pre-immune IgG) was obtained from Jackson Immunoresearch Lab (West Grove, PA). All other chemicals were of reagent grade.

Animals. Adult male Wistar rats, 10 to 11 weeks old and weighing 225 to 300 g, were obtained from Charles River Canada (Montreal, Quebec, Canada). Animals were allowed to acclimatize in our facility for at least 7 days before treatment. The animal room was maintained at 22°C, and fluorescent lighting was controlled with an automatic timer (8:00 a.m. on/10:00 p.m. off). The animals were housed in polycarbonate cages (two or three animals per cage) containing additive-free corn cob bedding (The Andersons, Maumee, OH) and were allowed free access to Laboratory Rodent Diet 5001 (PMI Feeds, St. Louis, MO) and tap water until the time of death.

Treatment of animals and preparation of hepatic microsomes. To induce CYP1A1, animals were pretreated with intraperitoneal injection of BNF (40 mg/kg/day for 3 days), and to induce CYP2C6, animals were pretreated with PB (80 mg/kg/day i.p. for 4 days). Uninduced rats were those that had not been pretreated. For *in vivo* inhibition studies, animals were treated with a single intraperitoneal dose (150 mg/kg) of cimetidine hydrochloride or 0.9% saline (control) at 24 hours after the last pretreatment dose. The animals were killed 90 min after the cimetidine or saline injection. Hepatic microsomes were then prepared through differential ultracentrifugation as described by Lu and Levin (1972). The microsomal pellet was suspended in 0.25 M sucrose, and aliquots of the suspension were stored at -80°C until use.

Microsomal protein assay and determination of CYP content. Microsomal protein concentration was determined with the BioRad Protein Assay Kit. Absorbance was measured at 595 nm with a Beckman DU-64 spectrophotometer equipped with a protein assay Soft-Pac module (Fullerton, CA). Total microsomal CYP content was determined from the sodium dithionite-reduced carbon monoxide difference spectrum using a molar extinction coefficient of 91 cm⁻¹ mM⁻¹ between 450 and 490 nm (Omura and Sato, 1964; Thomas *et al.*, 1983) with an SLM-Aminco DW-2C spectrophotometer (Urbana, IL).

MROD and EROD O-dealkylase assays. Microsomal MROD and EROD activities were determined by a direct fluorometric method originally described by Burke and Mayer (1974) with some modification. Assay mixtures contained 1.93 ml of 100 mM HEPES/5 mM MgCl₂ (pH 7.8), 50 μ l of microsomes diluted in 0.25 mM sucrose and 10 μ l of MROD or EROD dissolved in DMSO. Final substrate concentrations were 1 μ M for BNF-induced microsomes and 5 μ M for uninduced microsomes. Final protein concentrations were 150 μ g/ml for both activities for uninduced microsomes and 10 and 5 μ g/ml for MROD and EROD activities, respectively, for BNF-induced microsomes to ensure linearity of each activity. Ten microliters of NADPH dissolved in HEPES buffer was added (0.25 mM final concentration) to start the reaction. The total volume of the reaction mixture was 2 ml, and reactions were carried out at 37°C. The increase in fluorescence associated with the formation of resorufin was monitored using a Shimadzu RF-540 spectrofluorometer (Columbia, MO), with excitation and emission wavelength set at 530 and 582 nm, respectively. The amount of resorufin formed was determined from a standard curve of fluorescence *vs.* resorufin concentration.

Testosterone and progesterone oxidation assays. Microsomal testosterone 2 α -hydroxylase activity was quantified with an HPLC method (Wood *et al.*, 1983) as described previously (Chang *et al.*, 1992a). Microsomal progesterone 2 α -hydroxylase, 6 β -hydroxylase and 21-hydroxylase activities were determined with a TLC method (Waxman, 1991). Reactions were carried out for 10 min at 37°C in 200- μ l incubation mixtures containing 100 mM HEPES (pH 7.4), 0.1 mM EDTA, 50 μ M ¹⁴C-labeled progesterone, 30 μ g of microsomal protein and 1 mM NADPH. The reaction products were extracted twice with 1 ml of ethyl acetate and then chromatographed on silica gel TLC plates developed initially in ethyl acetate/*n*-hexane/acetic acid (16:8:1, v/v/v) followed by two developments in benzene/ethyl acetate/acetone (10:1:1, v/v/v) (Chang *et al.*, 1993). Monohydroxyprogesterone metabolites were localized by autoradiography and quantified by liquid scintillation counting.

Purification of CYP. Purification of CYP2C11 was carried out according to the method of Ryan *et al.* (1984), with modifications as described previously (Chang *et al.*, 1992a). CYP1A1 was purified to electrophoretic homogeneity from pooled livers of Aroclor 1254-treated, adult male Long-Evans rats according to the method of Ryan *et al.* (1984).

Preparation of antibodies. Polyclonal antibody directed against rat CYP2C11 (anti-CYP2C11 IgG) was purified from a pool of heat-inactivated sera collected from rabbits immunized with the electrophoretically homogeneous protein. IgG was purified from sera through a combination of caprylic acid precipitation followed by ammonium sulfate precipitation and a final purification on a DEAE-Sephacel column. The purified antibody preparation recognized several other members of the CYP2C subfamily and is referred to as polyspecific anti-CYP2C11 IgG. This polyspecific anti-CYP2C11 IgG was passed repeatedly through a series of columns containing microsomal proteins from adult female untreated rats, partially purified CYP3A1, CYP2C7, CYP2C13 (and purified CYP2C13), and epoxide hydrolase. After removal of the cross-reactive IgG fraction, the specificity of the remaining antibody was tested using noncompetitive enzyme-linked immunosorbent assay and immunoblots with purified cytochromes P450 and microsomal samples from control and induced rats. The back-absorbed antibody preparation did not cross-react with purified CYP1A1, CYP2B1, CYP2C7, CYP2C13, CYP3A1, epoxide hydrolase or any protein in hepatic microsomes from untreated female rats. This antibody preparation is referred to as monospecific anti-CYP2C11 IgG and is different from that used in our previous study (Chang *et al.*, 1992a). The procedure for preparation and purification of polyclonal antibody directed against CYP1A1 was similar to that described for the anti-CYP2C11 IgG except that rabbits were immunized with purified rat CYP1A1 instead of CYP2C11. The specificity of the anti-CYP1A1 preparation was assessed using enzyme-linked immunosorbent assay and immunoblots, and it was found to cross-react with CYP1A1 and CYP1A2. This preparation is referred to as anti-CYP1A1 IgG. Monoclonal antibody directed against rat CYP1A1 (Mab C-8.1), which does not recognize CYP1A2, was a gift from Dr. P. E. Thomas (College of Pharmacy, Rutgers University, Piscataway, NJ). The preparation, specificity and inhibitory activity of this antibody have been described previously (Thomas *et al.*, 1984).

Immunoinhibition studies. The effects of the above antibody preparations on hepatic microsomal enzyme activities were determined as outlined previously (Chang *et al.*, 1992a).

Cimetidine inhibition studies *in vitro*. To determine the effects of preincubation on the *in vitro* inhibition of enzyme activities, conditions were essentially the same as those optimized previously for inhibition of hepatic microsomal testosterone 2 α -hydroxylase activity (Chang *et al.*, 1992b). Microsomes were incubated with NADPH, for 15 min at 37°C, before the initiation of the reaction by the addition of substrate. Cimetidine was dissolved in distilled water and added to the microsomes (final concentration, 0.025 to 10 mM) either (1) at the same time as the substrate (no preincubation) or (2) at the same time as NADPH (preincubation).

Statistical analysis. Differences between mean enzyme activities were analyzed using two-tailed Student's *t* tests for independent samples with the use of Statistica for Windows (StatSoft, Tulsa, OK). Statistical significance was set at *P* < .05.

Results

Effect of *in vivo* cimetidine on MROD and EROD activities in microsomes from BNF-induced rats. To investigate its effects on CYP1A1, cimetidine was administered to adult male rats that had been induced with BNF. Cimetidine treatment of these animals had no effect on hepatic microsomal MROD or EROD activity, but testosterone 2 α -hydroxylase activity was inhibited by $\approx 60\%$ (fig. 1A).

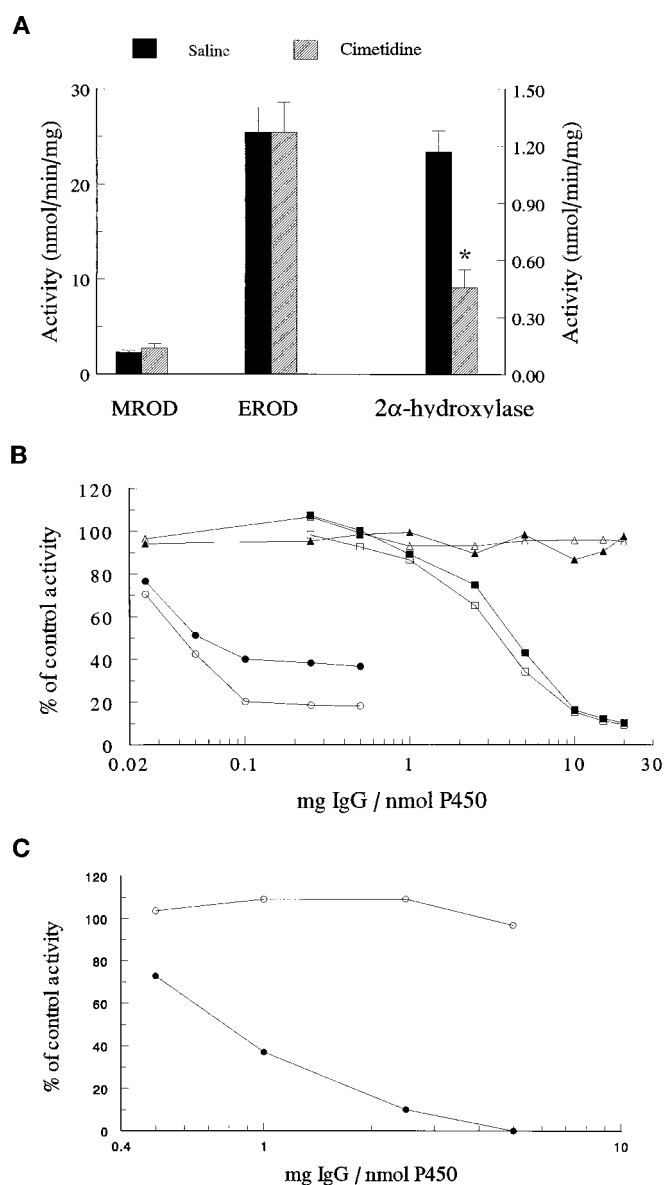


Fig. 1. A, Effect of *in vivo* cimetidine on hepatic microsomal MROD, EROD and testosterone 2 α -hydroxylase activities in BNF-induced rats. Results are expressed as mean \pm S.D. for four rats per group, **P* < .01. Left y-axis, MROD and EROD activities; right y-axis, testosterone 2 α -hydroxylase activity. B, Inhibition of MROD and EROD activities by anti-CYP1A IgG preparations in hepatic microsomes from BNF-induced rats. Open symbols indicate EROD activity, and closed symbols indicate MROD activity. Triangles indicate pre-immune IgG, circles indicate Mab C-8.1 IgG and squares indicate anti-CYP1A IgG. Microsomes were prepared from pooled livers of four BNF-induced rats; control (no IgG) MROD and EROD activities (mean \pm S.D. of three experiments) were 1.58 ± 0.17 and 20.3 ± 1.9 nmol/min/mg of protein, respectively. C, Inhibition of testosterone 2 α -hydroxylase activity by monospecific anti-CYP2C11 IgG in hepatic microsomes from uninduced rats. ○, Preimmune IgG; ●, monospecific anti-CYP2C11 IgG. Microsomes were prepared from pooled livers of four uninduced rats; control (no IgG) testosterone 2 α -hydroxylase activity (mean \pm S.D. of four experiments) was 1.91 ± 0.37 nmol/min/mg of protein.

Because MROD and EROD activities are associated with CYP1A1, we performed immunoinhibition experiments to determine the contribution of CYP1A1 to these activities in microsomes from BNF-induced rats. As shown in figure 1B, polyclonal anti-CYP1A1 IgG inhibited both MROD and EROD activities by $>90\%$ in the microsomes from BNF-

induced rats, whereas monoclonal anti-CYP1A1 IgG inhibited MROD and EROD activities by 60% and 80%, respectively (fig. 1B).

Testosterone 2 α -hydroxylase is mediated by CYP2C11 in microsomes prepared from uninduced rats (Waxman, 1984), but the enzyme responsible for this activity in microsomes from BNF-induced rats has not previously been determined. The results in figure 1C indicate that monospecific anti-CYP2C11 IgG completely inhibited testosterone 2 α -hydroxylase activity in these microsomes. Overall, these observations in BNF-induced rats indicate that *in vivo* cimetidine does not affect CYP1A1 but that it does inhibit CYP2C11.

Effect of *in vivo* cimetidine on MROD and EROD activities in microsomes from uninduced rats. The administration of cimetidine to uninduced adult male rats resulted in a 50% and 65% decrease in hepatic microsomal MROD and EROD activities, respectively (fig. 2A). There has been some uncertainty in the literature regarding the CYP enzymes responsible for EROD activity in microsomes from uninduced rats. Although Kelley *et al.* (1987) suggested that this activity is mediated in large part by CYP1A2, other observations have implicated CYP2C11 (Nakajima *et al.*, 1990) and CYP2C6 (Burke *et al.*, 1994; Nakajima *et al.*, 1990). It is not known at present which enzymes mediate hepatic MROD activity in uninduced rats. To investigate the role of CYP2C11 in the O-dealkylation of ethoxyresorufin and

methoxyresorufin in microsomes from uninduced rats, we performed immunoinhibition studies with the polyspecific and monospecific anti-CYP2C11 IgG preparations. The polyspecific anti-CYP2C11 IgG inhibited EROD activity completely in microsomes from uninduced rats and inhibited MROD activity by $\approx 85\%$, although the inhibition curve was shifted somewhat to the right. In contrast, the monospecific anti-CYP2C11 IgG had no effect on either EROD or MROD activity in these microsomes (fig. 2B). This suggested that, in microsomes from uninduced rats, these activities are catalyzed in large part by one or more CYP2C enzymes other than CYP2C11.

Effect of *in vivo* cimetidine on progesterone hydroxylase activities. In hepatic microsomes from PB-induced rats, progesterone 21-hydroxylase is predominantly mediated by CYP2C6 (Swinney *et al.*, 1987). Therefore, to investigate the effect of cimetidine on this enzyme, progesterone 21-hydroxylase activity was determined in microsomes from PB-induced rats that had been treated with cimetidine. The results in figure 3 show that *in vivo* cimetidine resulted in 62% inhibition of progesterone 21-hydroxylase activity in microsomes from these animals. Cimetidine treatment also inhibited progesterone 2 α -hydroxylase (mediated by CYP2C11) by 39% but had no effect on progesterone 6 β -hydroxylase (mediated by CYP3A) activity in the same microsomes. These results indicate that *in vivo* cimetidine inhibits CYP2C6 and CYP2C11 but does not affect CYP3A.

Effect of preincubation on potency of inhibition of EROD activity by cimetidine *in vitro*. Cimetidine is a nonselective inhibitor of cytochromes P450 *in vitro* under standard assay conditions in which both substrate and inhibitor are added at the same time. However, when cimetidine is preincubated with hepatic microsomes in the presence of NADPH before the addition of substrate, the potency of inhibition is increased, and the selectivity of the inhibition reflects that seen *in vivo* (Chang *et al.*, 1992b). The results in figure 4A demonstrate that preincubation of microsomes from uninduced rats with cimetidine in the presence of NADPH increased the potency (*i.e.*, reduction in IC₅₀) of inhibition of EROD activity by ≈ 20 -fold. This observation was consistent with the inhibition of EROD activity by *in vivo* cimetidine in microsomes from uninduced rats (fig. 2A) and suggests that cimetidine forms a metabolite/intermediate complex with CYP2C6. In contrast, preincubation of microsomes from BNF-induced rats with cimetidine and

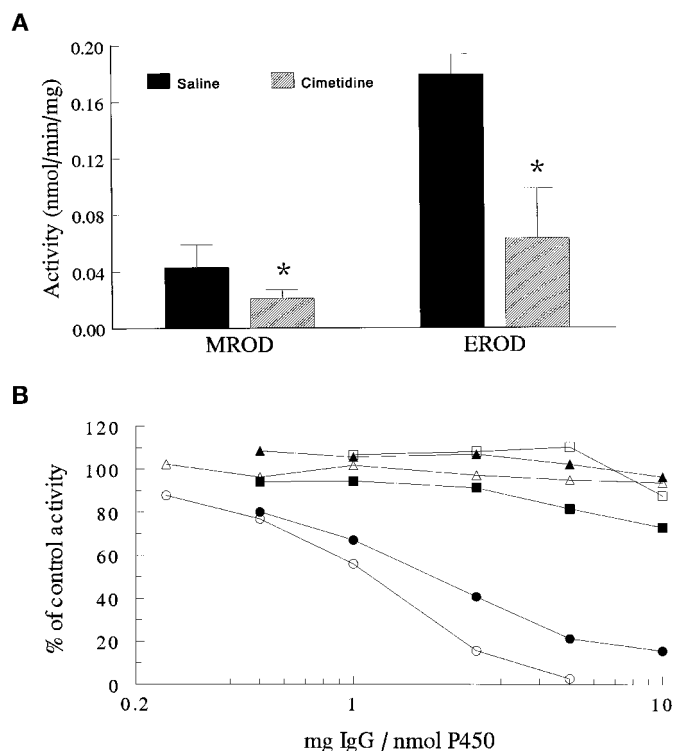


Fig. 2. A, Effect of *in vivo* cimetidine on hepatic microsomal MROD and EROD activities in uninduced rats. Results are expressed as mean \pm S.D. for eight or nine rats per group, * $P < .002$. B, Inhibition of MROD and EROD activities by anti-CYP2C11 IgG preparations in hepatic microsomes from uninduced rats. Open symbols indicate EROD, and closed symbols indicate MROD activity. Triangles indicate preimmune IgG, circles indicate polyspecific anti-CYP2C11 IgG and squares indicate monospecific anti-CYP2C11 IgG. Microsomes were prepared from pooled livers of four uninduced rats; control (no IgG) MROD and EROD activities (mean \pm S.D. of four experiments) were 0.07 ± 0.002 and 0.35 ± 0.01 nmol/min/mg of protein, respectively.

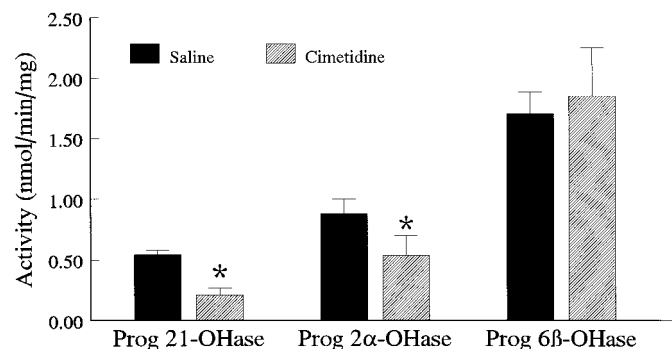


Fig. 3. Effect of *in vivo* cimetidine on hepatic microsomal progesterone (Prog) 21-, 2 α - and 6 β -hydroxylase (OHase) activities in microsomes from PB-induced rats. Results are expressed as mean \pm S.D. for four rats per group, * $P < .001$.

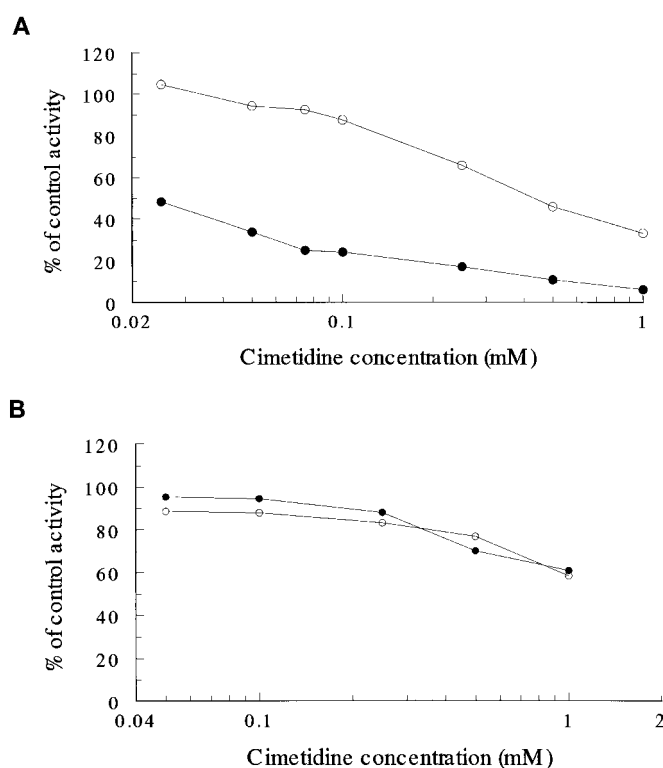


Fig. 4. A, Effect of preincubation on *in vitro* inhibition by cimetidine of EROD activity in hepatic microsomes from uninduced rats. ○, Microsomes that had cimetidine added with ethoxyresorufin, after incubation of microsomes with NADPH (no preincubation). ●, Microsomes that were preincubated with cimetidine in the presence of NADPH before the addition of ethoxyresorufin. Microsomes were prepared from pooled livers of four uninduced rats; control EROD activity (*i.e.*, in the absence of cimetidine) was 0.22 nmol/min/mg of protein. B, Effect of preincubation on *in vitro* inhibition by cimetidine of EROD activity in hepatic microsomes from BNF-induced rats. ○, Microsomes that had cimetidine added with ethoxyresorufin, after incubation of microsomes with NADPH (no preincubation). ●, Microsomes that were preincubated with cimetidine in the presence of NADPH before the addition of ethoxyresorufin. Microsomes were prepared from pooled livers of four BNF-induced rats; control EROD activity (*i.e.*, in the absence of cimetidine) was 16.9 nmol/min/mg of protein.

NADPH had no effect on the inhibition of EROD activity (fig. 4B). This observation was consistent with the lack of effect of *in vivo* cimetidine on EROD activity in microsomes from BNF-induced rats (fig. 1B).

Discussion

Our previous study indicated that cimetidine administration to adult male rats resulted in inhibition of hepatic microsomal CYP2C11 but had no effect on CYP2A1-, CYP2B- or CYP3A-mediated activities (Chang *et al.*, 1992a). The present study confirms the effect of cimetidine on CYP2C11 and provides the first demonstration that this drug also inhibits CYP2C6, whereas it does not affect CYP1A1 activity. Therefore, cimetidine is a selective inhibitor not only of rat hepatic CYP2C11 but also of CYP2C6. In addition, preincubation experiments suggested that cimetidine inhibits CYP2C6 activity through metabolite/intermediate complexation, a mechanism by which this drug inhibits CYP2C11 (Chang *et al.*, 1992b, Levine and Bellward, 1995).

The administration of cimetidine to BNF-induced rats had no effect on hepatic microsomal EROD or MROD activity (fig.

1A). It could be argued that induction of rats with BNF prevented the inhibitory effect of cimetidine by altering its metabolism. However, the fact that the testosterone 2 α -hydroxylase activity was inhibited by cimetidine treatment (fig. 1A) rules out this possibility. Although CYP2C11 contributes extensively to testosterone 2 α -hydroxylase activity in hepatic microsomes from uninduced rats (Waxman, 1984), the CYP enzymes responsible for this activity in microsomes from BNF-induced rats have not been determined previously. In the present study, monospecific anti-CYP2C11 IgG completely inhibited the testosterone 2 α -hydroxylase activity in these microsomes (fig. 1C), indicating that this activity retains its specificity for CYP2C11 after BNF treatment.

In vivo cimetidine did not inhibit rat hepatic microsomal CYP1A1. This conclusion is based on the observation that cimetidine administration to BNF-induced rats did not affect hepatic microsomal MROD or EROD activity. In microsomes from BNF-induced rats, EROD activity was inhibited >90% by polyclonal anti-CYP1A1 IgG and \approx 80% by monoclonal anti-CYP1A1 IgG. This is consistent with previous evidence that CYP1A1 mediates most of the EROD activity in microsomes from BNF-induced rats (Burke *et al.*, 1985). In the same BNF-induced samples, MROD activity was also inhibited >90% by polyclonal anti-CYP1A1 IgG and \approx 60% by monoclonal anti-CYP1A1 IgG (fig. 1B), suggesting that CYP1A1 contributes to a major fraction of MROD activity in microsomes from BNF-induced rats.

BNF is known to be a 3-MC-type inducer of CYP1A1 and CYP1A2 (Burke *et al.*, 1985; Guengerich *et al.*, 1982; Thomas *et al.*, 1983), and in microsomes from 3-MC-induced rats, MROD activity appears to be catalyzed mainly by CYP1A2 (Burke *et al.*, 1994). It is therefore likely that the portion of microsomal MROD activity from BNF-induced rats not affected by monoclonal anti-CYP1A1 IgG was mediated by CYP1A2. If so, the lack of inhibition of this activity by *in vivo* cimetidine in these microsomes provides indirect evidence that cimetidine does not affect CYP1A2.

Nerurkar *et al.* (1993) concluded that MROD activity is specific for CYP1A2 in rodent liver microsomes on the basis of data obtained from rats and mice induced with 3,4,5,3',4',5'-hexachlorobiphenyl. In contrast, the simplest interpretation of the present results and those of Burke *et al.* (1994) is that methoxyresorufin can be metabolized by CYP1A1 and CYP1A2 to varying extents in rat microsomes, depending on the inducer used. MROD activity should therefore not be taken as a specific marker for CYP1A2 activity unless it has been demonstrated to be so under the conditions studied.

In the study by Drew *et al.* (1981), *in vivo* cimetidine inhibited hepatic microsomal benzo[a]pyrene hydroxylase activity but not 7-ethoxycoumarin O-deethylase activity from rats induced with 3-MC. This finding is inconsistent with the fact that both activities are mediated mainly by CYP1A in microsomes from 3-MC-induced rats (Hietanen *et al.*, 1987; Park *et al.*, 1982; Ryan *et al.*, 1982). It has, however, been suggested that there are two substrate binding sites on CYP1A1 (Kao and Wilkinson, 1987; Phillipson *et al.*, 1985). The observation made by Drew *et al.* (1981) may thus indicate that rat hepatic microsomal benzo[a]pyrene hydroxylase and 7-ethoxycoumarin O-deethylase activities arise from different binding sites on CYP1A1 and that cimetidine inhibits the site responsible for the former but not the latter activity.

If this explanation turns out to be correct, then the present results would indicate that MROD and EROD activities are catalyzed by an active site on CYP1A1 that is not susceptible to inhibition by *in vivo* cimetidine.

In the present study, *in vivo* administration of cimetidine inhibited hepatic microsomal EROD activity in uninduced rats. There has been some disagreement regarding the cytochromes P450 responsible for the EROD activity in microsomes from uninduced rats. Kelley *et al.* (1987) reported that a polyclonal anti-CYP1A1 antibody did not affect EROD activity, whereas a polyclonal anti-CYP1A2 antibody inhibited this activity by 78% in microsomes from uninduced rats. Nakajima *et al.* (1990) reported that an anti-CYP2C11 antibody that cross-reacted with CYP2C6 inhibited EROD activity by 74% in microsomes from uninduced rats. More recently, Burke *et al.* (1994) reported that a polyclonal antibody against CYP2C6 that also cross-reacted with CYP2C11 strongly inhibited EROD activity in microsomes from uninduced rats. It can be ruled out that CYP2C11 mediates EROD activity in microsomes from uninduced rats because our monospecific anti-CYP2C11 IgG did not inhibit this activity. The observation that the polyspecific anti-CYP2C11 antibody preparation completely inhibited EROD activity in microsomes from uninduced rats, however, is consistent with the conclusion of Burke *et al.* (1994) that CYP2C6 mediates hepatic EROD activity in these animals.

Cimetidine also inhibited MROD activity in the microsomes from uninduced rats; however, it is not possible at this point to identify the cytochromes P450 responsible for this activity in uninduced rat liver microsomes. Burke *et al.* (1994) concluded that most of the MROD activity in uninduced rat liver is mediated by CYP1A2. This was based on the observation that furafylline, which is a selective inhibitor of human CYP1A2-mediated hepatic microsomal phenacetin O-deethylase activity (Bourrie *et al.*, 1996; Clarke *et al.*, 1994; Newton *et al.*, 1995; Sesardic *et al.*, 1990), decreased MROD activity by >70% in microsomes from uninduced rats. As shown in the present study, polyspecific anti-CYP2C11 IgG inhibited MROD activity in the microsomes from uninduced rats by $\approx 80\%$, and the curve appeared to be shifted somewhat to the right relative to EROD inhibition (fig. 2B), whereas monospecific anti-CYP2C11 IgG had no effect on this activity in same microsome preparation. In addition, polyspecific anti-CYP2C11 IgG did not inhibit EROD or MROD activity in microsomes from BNF-induced rats (results not shown), thereby demonstrating that the antibody preparation does not cross-react with CYP1A1 or CYP1A2. These observations suggest that a CYP2C enzyme other than CYP2C11 is largely responsible for the MROD activity in microsomes from uninduced rats. The explanation most consistent with the results of Burke *et al.* (1994) and our data is that both CYP1A2 and a CYP2C enzyme other than CYP2C11 (possibly CYP2C6) contribute to MROD activity in microsomes from uninduced rats. The inhibition of this activity by *in vivo* cimetidine is likely due to its effect on the component of the activity that is mediated by the CYP2C enzyme.

In vivo cimetidine inhibited hepatic microsomal CYP2C6. This conclusion is based on the finding that CYP2C6 contributes to the majority of progesterone 21-hydroxylase activity in hepatic microsomes isolated from PB-pretreated rats (Swinney *et al.*, 1987) and the present observation that cime-

tidine administration to PB-induced rats resulted in a significant decrease in this microsomal activity (fig. 3). In the same microsome samples, the CYP2C11-mediated progesterone 2 α -hydroxylase activity was decreased, whereas the CYP3A-dependent progesterone 6 β -hydroxylase activity was not altered, indicating that *in vivo* cimetidine also inhibited hepatic microsomal CYP2C11 but not CYP3A. These observations are in agreement with the findings of our previous study with testosterone 2 α - and 6 β -hydroxylase activities as markers for CYP2C11 and CYP3A, respectively (Chang *et al.*, 1992a).

Jensen and Gugler (1985) suggested that cimetidine might form a metabolite/intermediate complex with CYP. On the basis of our previous studies, we concluded that cimetidine *in vivo* inhibits CYP2C11 through metabolite/intermediate complexation (Chang *et al.*, 1992b; Levine and Bellward, 1995). Preincubation of microsomes with cimetidine in the presence of NADPH increases the potency of the inhibitory effect of cimetidine on CYP211 (Chang *et al.*, 1992b), suggesting that the metabolite/intermediate complex can be generated *in vitro*. Given the present observation that cimetidine also inhibits CYP2C6, we postulated that it does so by a similar mechanism and therefore performed *in vitro* inhibition studies using the same preincubation protocol. The results in figure 4A illustrate that preincubation of microsomes from uninduced rats with cimetidine in the presence of NADPH increased the potency of inhibition of EROD activity by >20-fold. This suggests that cimetidine also forms a metabolite/intermediate complex with CYP2C6. In contrast, preincubation had no influence on the inhibition by cimetidine of either MROD or EROD activity in microsomes from BNF-induced rats (fig. 4B). This finding was consistent with the lack of inhibition of these activities by *in vivo* cimetidine treatment (figs. 1A and 2A) and supports the conclusion that neither CYP1A1 nor CYP1A2 is affected by cimetidine.

Taken together, our results indicate that *in vivo* cimetidine has no effect on CYP1A1 but that it does inhibit CYP2C6 in addition to CYP2C11. Also, the effect of cimetidine on CYP2C6 is likely mediated by a mechanism similar to that causing inhibition of CYP2C11. Since our initial report (Chang *et al.*, 1992b), other investigators have begun to perform preincubation studies with cimetidine *in vitro* in rat liver microsomes as a marker for the involvement of CYP2C11 in particular drug oxidation reactions (Roos and Mahnke, 1996; Vage and Svensson, 1994; Wienkers *et al.*, 1995). The present findings indicate that when the potency of inhibition by cimetidine is increased by preincubation of the drug with microsomes and NADPH, both CYP2C6 and CYP2C11 must be considered candidate enzymes that mediate the particular reaction. The enzyme selectivity and mechanism of inhibition of CYP by *in vivo* cimetidine in the rat also have important implications in humans. Cimetidine continues to be used widely as a prescription and nonprescription medication, and large numbers of individuals are therefore exposed to the potential for drug/drug interactions. Our observations in the rat model suggest that cimetidine may be more selective in its inhibitory effect on human CYP than has previously been considered and that members of the human CYP2C subfamily are likely candidates for inhibition by cimetidine. Studies are in progress to investigate this issue.

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