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Oxidation of Midazolam and Triazolam by Human Liver Cytochrome P450IIIA4

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SUMMARY

The metabolism of midazolam and triazolam to their 1′-hydroxy and 4-hydroxy metabolites was studied in microsomes of 15 human livers. The formation of both metabolites was inhibited by more than 90% by an antiserum directed against a pregnenolone 16α-carbonitrile-inducible cytochrome P450 (P450PCN1) of rat liver. Moreover, midazolam hydroxylase activity was immunoprecipitated from solubilized human microsomes with polyclonal antibodies against rat P450PCN1 and the closely related human isozyme P450NF. A close correlation was observed between the amount of protein detected in immunoblots with these antibodies and the midazolam or triazolam hydroxylase activity. The formation of both metabolites of midazolam was inhibited by triacetyloleandomycin, a known inhibitor of cytochromes P450 of the IIIA family. Direct evidence that P450IIIA4 catalyzes the metabolism of midazolam was provided through the use of cDNA-directed expression. Monkey COS cells transfected with human P450PCN1 cDNA were able to catalyze both the 1′- and the 4-hydroxylation of midazolam. We conclude that the metabolism of midazolam and triazolam in human liver is predominantly mediated by cytochrome P450IIIA4. Two of 15 human livers expressed a second immunoreactive microsomal protein of higher apparent M_r and were more active in midazolam 1′-hydroxylation. Our data also provide evidence that the marked interindividual variation in the response to these widely used benzodiazepine drugs is due to variable hepatic metabolism.

P450[2] is the collective term for a group of hemoprotein isozymes responsible for the oxidative metabolism of a large number of endogenous and exogenous compounds (1). The amounts and structures of the metabolites formed from a specific substrate are determined by numerous factors regulating the expression and function of P450 isozymes. Genetic factors, hormones, exogenous and possibly endogenous inducers, inhibitors, and allosteric activators of P450 are such regulators. Working in concert, they determine the often large interindividual differences in P450-catalyzed reactions.

Midazolam is widely used as a benzodiazepine tranquilizer with a short duration of action and it shows considerable interindividual variation in its plasma elimination half-life. The involvement of a genetic polymorphism of oxidation as one of the causes of this variability has recently been proposed (2). Triazolam, a drug closely related to midazolam in structure and metabolism (Fig. 1) (3), also shows pronounced interindividual variation of pharmacokinetic parameters after standard doses (4).

The present investigation is based on the assumption that variable metabolism of these two drugs may be the major cause of variation in *in vivo* kinetics and response to these drugs. We studied the metabolism of midazolam and triazolam in microsomes from different human livers. Antibody probes were used for initial information on the P450 enzyme family metabolizing these drugs and this was followed by direct identification by cDNA expression of the P450 isozyme catalyzing midazolam hydroxylation.

Experimental Procedures

Materials

Midazolam, triazolam, and their metabolites (Fig. 1), as well as bufuralol, were generous gifts of Hoffmann La-Roche (Basel, Switzerland). Cyclosporine was kindly supplied by Sandoz (Basel, Switzerland); triacetyloleandomycin was a gift of Pfizer (Brussels, Belgium).

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[2] In this paper we apply the nomenclature of cytochrome P450 based on the name given in the original paper referred to. If appropriate, the nomenclature based on nucleotide or amino acid sequence, as proposed in Ref. 1, is given in parenthesis. Human P450PCN1 (23) and P450NF (15) are designated P450IIIA4, to which P450 HLp (29), designated P450IIIA3, is closely related. Rat hepatic cytochrome P450PCN1 corresponds to P450IIIA1 (5).

ABBREVIATIONS: P450, cytochrome P450, CHAPS, 3-(3′-cholamidopropyl)dimethylammonio-1-propane sulfonate; TAO, triacetyloleandomycin; SDS, sodium dodecyl sulfate; NaP_i, 0.1 M sodium phosphate buffer, pH 7.4; HPLC, high performance liquid chromatography; KDL, kidney transplant donor liver.

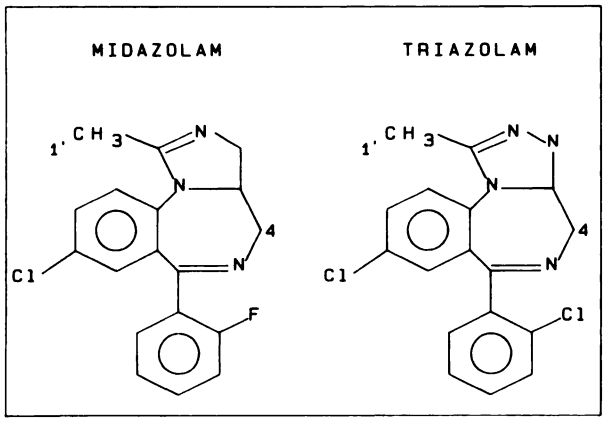

Fig. 1. Structures of midazolam and triazolam. The sites of metabolism at the 1′ and 4 position are indicated.

Acetonitrile and methanol (both chromatography grade) were obtained from Merck (Darmstadt, FRG). Protein A-Sepharose was from Pharmacia (Uppsala, Sweden) and CHAPS was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of highest commercially available purity. Rabbit antiserum against a pregnenolone-16α-carbonitrile-inducible rat hepatic P450 (P450PCN1, P450IIIA1) (5)[2] was provided by Dr. James P. Hardwick (Biomedical Division, Argonne National Laboratories, Argonne, IL). The monoclonal antibody 13-7-10 and a polyclonal rabbit antiserum directed against the same human antigen (P4505) (6) were gifts from Drs. P. Beaune (Hôpital Necker, Paris, France) and P. Kremers (University of Liège, Belgium). Human autoantibodies recognizing P450bufI (anti-LKM1) (7) and P450meph (anti-LKM2) (8) were kindly supplied Dr. J. C. Homberg (Hôpital St. Antoine, Paris, France). The expression vector p91023(B) was provided by Dr. Randall J. Kaufman (Genetics Institute, Cambridge, MA).

Methods

Biochemical procedures. Microsomes were prepared from livers of kidney transplant donors and from Sprague Dawley rat livers, as previously described (9). Protein (10) and P450 content (11) were estimated by standard procedures. Metabolism of (+)-bufuralol (12) and *S*-mephenytoin (13) was determined at a substrate concentration of 200 μM, as recently described. NADPH-P450 reductase was purified from rat liver by published procedures (14). Immunoglobulins were prepared from rabbit serum by chromatography on Protein A-Sepharose (15).

Assay of midazolam metabolism in liver microsomes. Five to 80 μg of microsomal protein were incubated in NaP_i at 37° for 10 min, in a final volume of 100 μl, in the presence of a NADPH-generating system (0.1 units of isocitrate dehydrogenase, 1 mM NADP, 5 mM isocitrate, 5 mM $MgCl_2$). Substrates were added as 10-fold concentrated solutions in 50% acetone. This concentration of acetone slightly activated midazolam metabolism in human liver microsomes. No difference in initial velocity was found whether the reaction was started by addition of substrate, NADPH-regenerating system, or a mixture of both. The formation of metabolites was linear with protein (5–80 μg/assay) and time (0–10 min) and was stopped by addition of 100 μl of cold methanol. Denatured protein was removed by centrifugation (4 min, 10,000 × *g*) and a 10–100-μl aliquot of the supernatant was injected for HPLC analysis.

HPLC. The HPLC system consisted of a pump (model 420; Kontron, Basel, Switzerland), an autosampler (ISS 100), and a variable wavelength UV detector (LC 95) operated at 220 nm (both Perkin-Elmer, Ueberlingen, FRG); the column (4.6 × 125 mm) was packed with Nucleosil 5-C-18 (batch 6071; Machery Nagel, Oensingen, Switzerland) at 600 bar by means of an inbalanced slurry technique. The eluent consisted of 10 mM potassium phosphate, pH 7.4/acetonitrile/methanol (366:280:200, w/w/w) and was delivered at 1 ml/min. Chromatograms were integrated with a CR 3A integrator (Shimadzu, Kyoto, Japan). Sensitivity for the detection of midazolam metabolites is 5 ng/ml. This method was also capable of measuring triazolam metabolites after slight modifications of the eluent composition (366:300:100, w/w/w). Triazolam metabolites were identified by their relative retention in the HPLC system, as compared with the respective midazolam metabolites. Representative chromatograms for midazolam metabolite formation in microsomes of human livers and Sprague Dawley rat livers are reproduced in Fig. 2.

Immunoinhibition. Inhibition was performed by preincubation of 20 μg of microsomal protein in 80 μl of NaP_i with various amounts of antisera or immunoglobulins for 30 min at 22°, in the absence of the NADPH-regenerating system and substrate. The assay was then initiated with the NADPH-regenerating system and substrate and incubated at 37° for 10 min; metabolites were analyzed as described above.

Inhibition by chemicals. Inhibition was tested by addition of a mixture of substrate and inhibitor to the assay. The reaction was started by addition of the NADPH-regenerating system. Inhibition of midazolam hydroxylation by TAO was carried out by incubating 20 μl of microsomal protein and the NADPH-regenerating system in 80 μl of NaP_i for 40 min at 37°, in the presence or absence of 128 μM TAO. The incubation was continued for an additional 10 min after addition of midazolam (64 μM, final concentration) and another 10 μl of NADPH-regenerating system. The reaction was stopped with methanol and analyzed as described above.

Indirect immunoprecipitation of midazolam hydroxylase. Microsomes were solubilized with 20 mM CHAPS and 20% glycerol in 0.1 M sodium phosphate buffer, pH 7.4 (solubilization buffer), at a concentration at 12.5 mg/ml for 30 min at 22°, and were centrifuged for 15 min at 148,000 × *g* (Airfuge; Beckman, Palo Alto, CA). The supernatant was diluted 1:5 with solubilization buffer.

Fifty microliters of a 30% slurry of Protein A-Sepharose, which had been washed three times with 1 ml of 0.2% bovine serum albumin in NaP_i, were incubated for 2 hr at room temperature with 20 μl of either rabbit antiserum against rat P450PCN1 (5), rabbit antiserum against human P450NF (6, 15, 16), or rabbit anti-mouse antiserum, in 1 ml of 0.2% bovine serum albumin in NaP_i. The monoclonal antibody 13-7-10 (ascites fluid corresponding to 20 μg of protein) against human

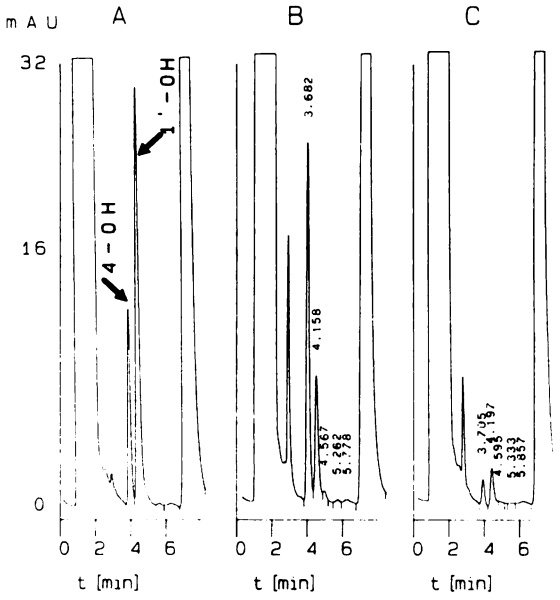

Fig. 2. Chromatograms of midazolam metabolites formed in microsomal incubations of human liver (KDL-27) (A) and of male (B) and female (C) Sprague-Dawley rat liver. *4-OH* and *1′-OH* indicate the midazolam metabolites (see Fig. 1). The peak with a retention time of 3 min corresponds to 1′,4-hydroxy-midazolam. The last peak represents the substrate midazolam.

P450NF (6, 16) was coupled to the rabbit anti-mouse-Protein A-Sepharose by incubation in 1 ml of 0.2% bovine serum albumin in NaP_i. Sepharose beads were washed twice with the same buffer and once in solubilization buffer. Then, 100 μl of solubilized microsomes were incubated with each aliquot of these beads for 2 hr at 22°, the samples were centrifuged (1 min, 10,000 × *g*), and 10 μl (≈25 μg of protein) of supernatant were reconstituted with 0.3 units of NADPH-P450 reductase (20 μl), 50 μl of NaP_i, 10 μl of NADPH-regenerating system, and 10 μl of 3 mM midazolam in 50% acetone and incubated at 37°. The reaction was stopped after 10 min and processed as described for microsomal incubations. Under these conditions, activities between 50 and 90% of those of unsolubilized microsomes were obtained.

Immunopurification. Fifty milligrams of microsomal protein were heated to 100° for 3 min in 5 ml of NaP_i that contained 2% SDS and were diluted 10-fold with 190 mM sodium chloride, 50 mM Tris·HCl, pH 7.4, 1 mM EDTA, 2.5% (w/v) Triton X-100 (immune buffer) (17). Ascites fluid containing the monoclonal antibody 13-7-10 was coupled to cyanogen bromide-activated Sepharose at a concentration of 5 mg/ml, according to the manufacturers instructions (18) (Pharmacia). Then, 1.6 ml of this Sepharose was packed into an Econo-column (Bio-Rad, Richmond, CA) and washed with (a) 5 ml of 10 mM potassium phosphate, pH 7.4, 0.2% Lubrol PX; (b) 8 ml of 0.1 M glycine-HCl, pH 3.0, 0.2% Lubrol PX; and (c) 8 ml of 0.1 M sodium phosphate, pH 7.4. The column was then equilibrated with 8 ml of immune buffer. SDS-solubilized microsomes were applied and the column was subsequently washed with 20 ml of immune buffer and 10 ml of 10 mM potassium phosphate, pH 7.4. The protein was eluted with 0.1 M glycine-HCl, pH 3.0, 0.2% Lubrol PX. Fractions of 1.8 ml were collected into tubes containing 180 μl 1 M Tris·HCl buffer, pH 8.0.

Immunoquantitation. Microsomal protein (15μg) was separated on 8% polyacrylamide SDS-gels (19), transferred to nitrocellulose (20) at 30 V for 2 hr, and incubated with the monoclonal antibody 13-7-10. The immunoreaction was visualized with the peroxidase anti-peroxidase technique (21). The stained bands were quantified by reflectance scanning at 562 nm (TLC scanner II and integrator SP 4290; Camag, Muttenz, Switzerland).

Analysis of kinetic data. Enzyme kinetics were calculated by nonlinear least squares fitting, using a commercially available program, assuming Michaelis-Menten kinetics (22).

Expression of human P450IIIA4 cDNA. The human P450PCN1 (P450IIIA4) cDNA has recently been described (23). The cDNA was inserted into the vector p91023(B) and transfected into COS cells as detailed earlier (23). Forty-eight hours after transfection, the medium was changed and midazolam, at a concentration of 160 μM, was added to the cells in fresh medium. The medium was collected after 4 hr and analyzed for midazolam metabolites, as described above. Sonicated cell lysates were also analyzed for P450IIIA4 protein by immunoblotting with the polyclonal antibody against rat P450PCN1 (IIIA1).

Results

Kinetics of midazolam metabolite formation. Human liver microsomes metabolized midazolam predominantly to 1′-hydroxy- and to a lesser extent to 4-hydroxy-midazolam (Fig. 2A). Formation of 1′, 4-dihydroxy midazolam was not observed under the described incubation conditions but was demonstrable in rat liver microsomes (Fig. 2, B and C). We also observed considerable differences in midazolam metabolism between male and female Sprague-Dawley rats. Microsomes from male rats converted midazolam predominantly to the 4-hydroxy metabolite and to a lesser extent to 1-hydroxy-midazolam (Fig. 2B). Microsomes from female rat livers showed generally lower activities of midazolam metabolism, most evident for the formation of the 4-hydroxy metabolite (Fig. 2C). Formation of all metabolites was dependent on NADPH and was inhibited by carbon monoxide. The kinetics of substrate-dependent metabolite formation in human liver microsomes are shown in Fig. 3. These data indicate typical characteristics of Michaelis-Menten kinetics with uncompetitive substrate inhibition (Fig. 3). In microsomes from KDL-26, the calculated maximal velocity (V_{max}) for 1′-hydroxymidazolam formation was 191 ± 10 nmol × mg^{-1} × hr^{-1} (estimate ± SE), the apparent affinity constant (K_m) was 3.6 ± 0.6 μM, and the inhibition constant for substrate inhibition (K_i) was 350 ± 60 μM. For 4-hydroxymidazolam formation the V_{max} was 108 ± 7 nmol × mg^{-1} × hr^{-1}, K_m was 40 ± 6 μM, and K_i was 2050 ± 600 μM. These parameters were not affected by the presence of 1 mM NADH (data not shown). The different kinetic parameters for the formation of the two metabolites may indicate that more than one enzyme is involved or that midazolam may bind in two different ways to the enzyme, causing the formation of different products.

Interindividual variation in midazolam and triazolam metabolism. Formation of midazolam metabolites was determined in microsomes of 15 different human livers, at a substrate concentration of 60 μM. A considerable variation between individual livers was observed, which affected both metabolites in parallel (Fig. 4A). Triazolam is highly similar to midazolam in terms of structure and metabolism (Fig. 1) (3) and yielded a similar variation of metabolites in these 15 individual livers (Fig. 4B). Formation of both midazolam metabolites correlated significantly with the formation of both triazolam metabolites, as judged by Spearman rank correlation ($r_s > 0.895$; $p < 0.001$; $n = 15$). Two livers (KDL-12 and KDL-24; Fig. 4) had consistently enhanced activity for midazolam 1′-hydroxylase on repeated experiments but were indistinguishable from the other livers in regard to triazolam metabolite formation.

Immunoinhibition. We used two different human autoantibodies that are potent inhibitors of (+)-bufuralol-1′-hydroxylation (LKM-1) (7) or *S*-mephenytoin 4-hydroxylation (LKM-2) (8) and found no inhibition of midazolam metabolism under conditions (0–1 mg of IgG/nmol of P450) at which more than 80% of the activity of the polymorphically controlled cytochromes P450db1 and P450meph are inhibited (data not shown).

Of a panel of monoclonal and polyclonal antibodies raised against various rat and human P450 isozymes midazolam and triazolam metabolism was potently and uniquely inhibited by an antiserum directed against rat P450PCN1 (P450IIIA) (5). This inhibition was dose dependent and is exemplified for midazolam hydroxylation in Fig. 5. Further dose-dependent inhibition experiments with immunoglobulins prepared from the above serum confirmed these results. At a ratio of 2 mg of IgG/nmol of P450, metabolite formation from midazolam and triazolam was inhibited by 70%. Another antiserum that strongly inhibited midazolam metabolism (92% inhibition) at 0.5 μl/μg of microsomal protein was an antiserum raised against human P450-5 (6), later characterized to correspond to P450NF (P450IIIA4) (15, 16).

Indirect immunoprecipitation. We coupled the monoclonal antibody 13-7-10 (6) via a rabbit anti-mouse IgG to Protein A-Sepharose. The described inhibitory polyclonal antibodies were coupled directly to Protein A-Sepharose. We then used these coupled antibodies to immunoprecipitate midazolam hydroxylase(s) from CHAPS-solubilized microsomes and we compared the residual enzymatic activity of the reconstituted supernatant with the activity obtained by precipitation with

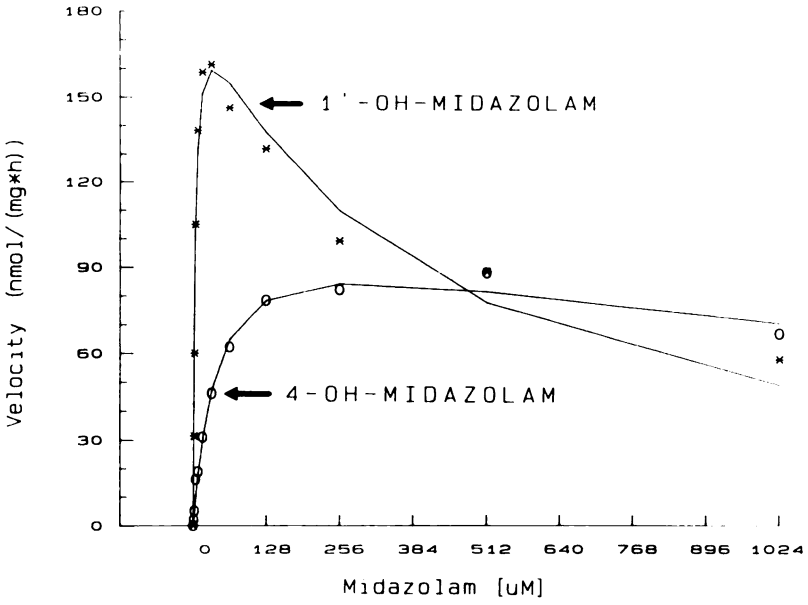

Fig. 3. Kinetics of formation of midazolam metabolites in human liver microsomes (KDL-26). The *points* are the mean of duplicate incubations. The *curves* represent the best fit obtained under assumption of Michaelis-Mentin kinetics with noncompetitive substrate inhibition. The apparent affinity constant, K_m, was 3.5 μM for midazolam 1′-hydroxylation and 40 μM for the formation of 4-hydroxy-midazolam. Further kinetic details are given in Results.

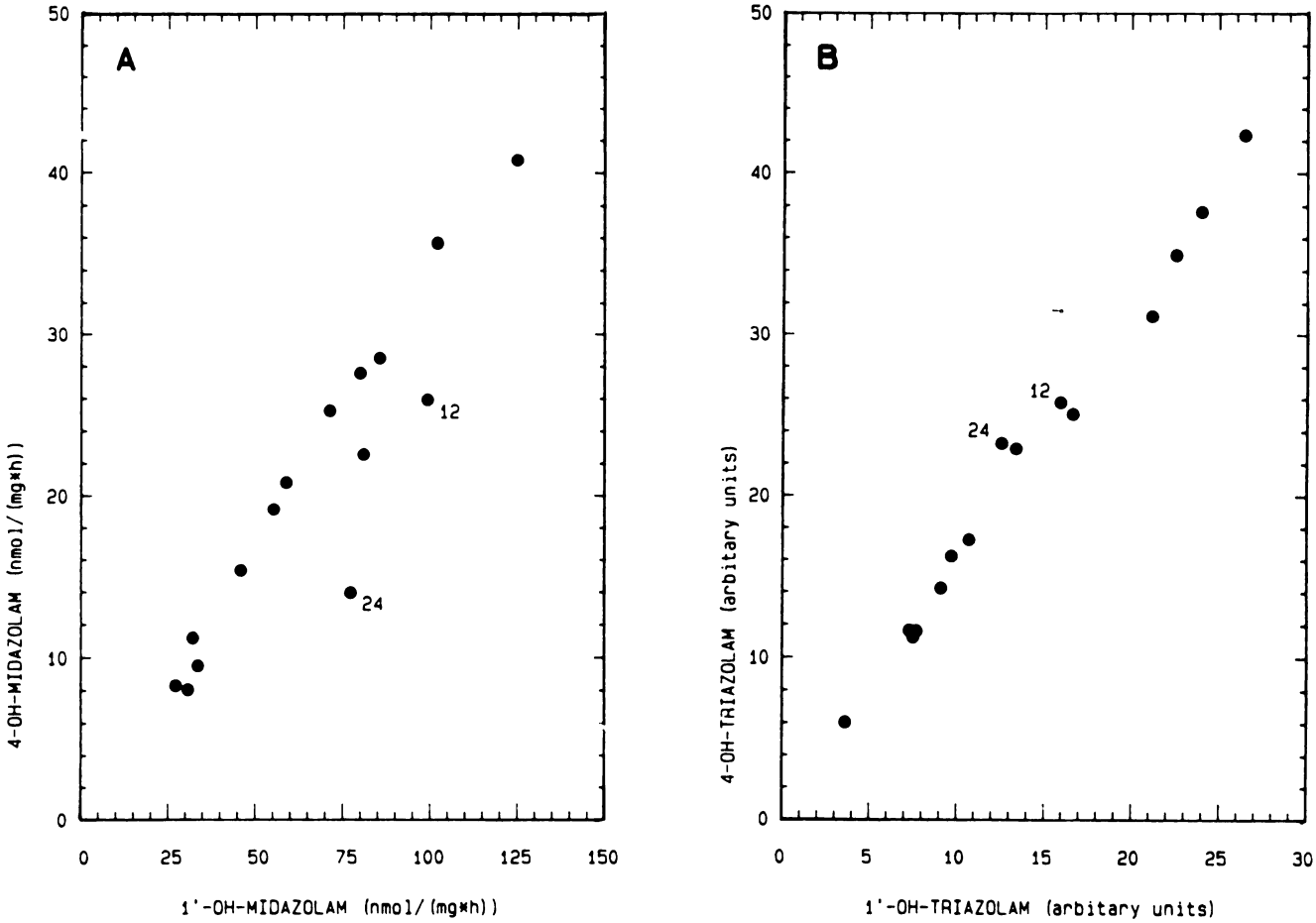

Fig. 4. Comparison of the 1′- and 4-hydroxy metabolites formed *in vitro* in microsomes of 15 different human livers from midazolam (60 μM) (A) or triazolam (60 μM) (B). The Spearman rank correlation coefficient (r_s) is 0.929 ($p < 0.0005$; $n = 15$) for the two midazolam metabolites, and 0.982 ($p < 0.005$; $n = 15$) for the two triazolam metabolites. An r_s of 0.895 ($p < 0.001$; $n = 15$ was calculated for the relationship between midazolam and triazolam metabolites. Microsomes from livers with an additional immunoreactive band on Western blots (see Fig. 6) are identified as KDL-12 (*12*) and KDL-24 (*24*).

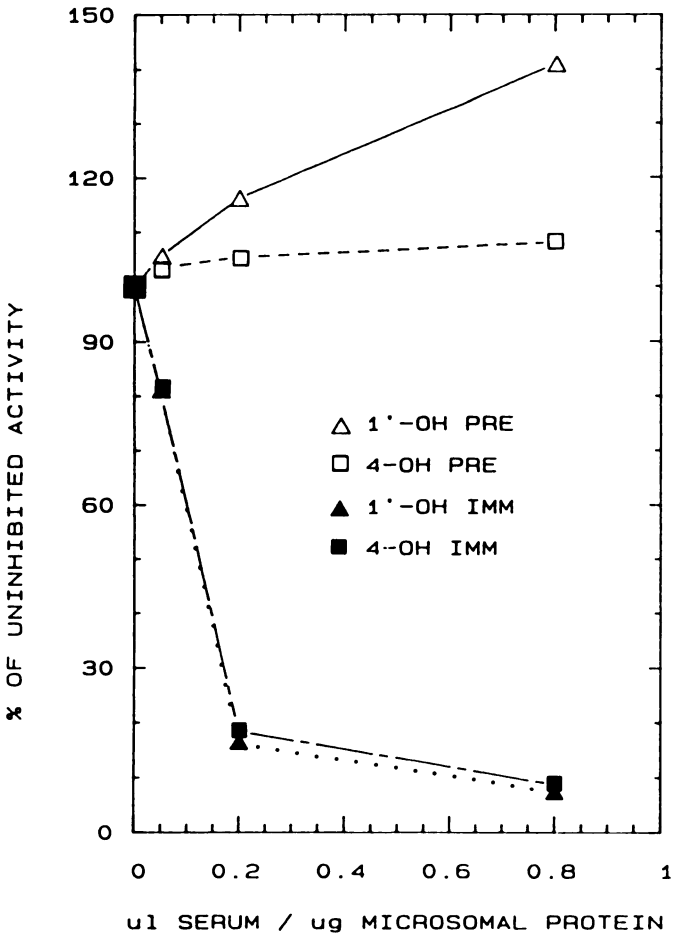

Fig. 5. Dose-dependent inhibition of midazolam 1′-hydroxylation (*triangles*) and 4-hydroxylation (*squares*) in human liver microsomes (KDL-20) with a polyclonal rabbit antiserum directed against rat P450PCN1 (*closed symbols*) or a preimmune serum (*open symbols*). The control activities were 27 and 8 nmol · mg^{-1} · hr^{-1} for midazolam 1′- and 4-hydroxylation, respectively.

rabbit anti-mouse IgGs coupled to Protein-A Sepharose. The polyclonal antibody against the human P450NF precipitated 18% of midazolam 1′- and 31% of 4-hydroxylase activity. The antibody against rat P450PCN1 precipitated 69% of midazolam 1′- and 71% of 4-hydroxylase, and the monoclonal antibody did not precipitate any of these activities. No attempts were made to optimize the conditions for complete immunoprecipitation of the enzymatic activities.

Immunoquantitation and correlation to metabolic activities. Antibodies also were used to immunoquantify the recognized protein in microsomes of 15 livers by Western blotting and subsequent reflectance scanning. Both the inhibitory and immunoprecipitating polyclonal anti-rat P450PCN1 antibody and the monoclonal antibody 13-7-10 were employed. The polyclonal antibody reacted with a 50-kDa band and with a band of 52 kDa (Fig. 6). We attribute the cross-reactivity with the 50-kDa protein to the presence of epoxide hydrolase in the preparation used for immunization, rather than to cross-reactivity with a protein immunologically related to P450PCN1. This interpretation was supported by the finding that only the 52-kDa band was recognized by the monoclonal antibody. The monoclonal antibody recognized similar amounts of 52-kDa protein as the polyclonal antibody against rat P450PCN1 (r_s for the two densities, 0.764; $n = 15$; $p < 0.005$; Fig. 6). In KDL-12 and KDL-14, an additional band of apparently higher molecular weight was observed (Fig. 6, A and B). The two bands could not be resolved by our densitometric system. We, therefore, used the total area of both bands for the correlations between density area and catalytic activity. Comparison of the amount of protein detected by the polyclonal antiserum with midazolam 1′- and 4-hydroxylation activity yielded Spearman rank correlation coefficients of 0.793 and 0.789 ($n = 15$; $p < 0.005$), respectively. The amount of protein detected with the monoclonal antibody was more closely correlated with midazolam hydroxylation; the r_s values were 0.911 ($n = 15$; $p < 0.001$) and 0.936 ($n = 15$; $p < 0.0005$) for 1′-hydroxy- and 4-hydroxy-midazolam, respectively. Fig. 7 exemplifies the correlation between immunoreactivity and catalytic activity for the monoclonal antibody 13-7-10 and the formation of 4-hydroxy-midazolam. Formation of 1′-hydroxy-triazolam ($r_s = 0.843$; $n = 15$; $p < 0.005$) and 4-hydroxy-triazolam ($r_s = 0.832$; $n = 15$; $p < 0.005$) also was highly correlated with the Western blot signal of the monoclonal antibody 13-7-10. No correlation was observed between the density area on blots and (+)-bufuralol hydroxylation, *S*-mephenytoin 4-hydroxylation, or spectrally determined microsomal P450 content (data not shown).

Immunoisolation. The polyclonal antibody against rat P450PCN1 was covalently linked to the matrix of Protein A-Sepharose with glutardialdehyde, and CHAPS-solubilized microsomes were incubated with these "immunobeads," as described in Methods. A protein fraction could be eluted from these beads, which was subjected to SDS-polyacrylamide gel analysis and Western blotting with the monoclonal antibody 13-7-10. One band was recognized, which had the same apparent molecular weight as the band stained in the original microsomal fraction, suggesting that both antibody preparations must recognize common epitopes or the same protein (data not shown).

Immunopurification with the covalently coupled monoclonal antibody from SDS-treated microsomes, as described in detail in Methods, resulted in the enrichment of a 52-kDa protein when performed with microsomes of KDL-12 but two proteins were seen in this area in the eluates from KDL-24, in accordance with the Western blot results (Fig. 6C).

Inhibition of midazolam hydroxylation by TAO complex formation and by cyclosporine. TAO is a macrolide antibiotic that forms inactive P450-Fe(II)-metabolite complexes with human P450HLp (24, 25). Incubation of human liver microsomes with midazolam in the presence of 0.06 to 512 μM TAO did not affect midazolam hydroxylation. However, preincubation of microsomes with TAO in the presence of a NADPH-regenerating system resulted in a decrease of 77 ± 6% and 79 ± 5% (mean ± SD) for midazolam 1′- and 4-hydroxylation, respectively, in all of the 15 human livers. Under the same conditions, microsomal midazolam 1′- and 4-hydroxylation was inhibited by only 6 and 12% in male and 29 and 58% in female Sprague Dawley rats, respectively. We recently have shown that a P450 isozyme of the same human P450III gene family is the major human hepatic cyclosporine-metabolizing enzyme (26). At a midazolam concentration of 8 μM, cyclosporine inhibited more than 70% of midazolam metabolite formation in a liver with high activity (KDL-26). In the microsomal sample with two immunoreactive proteins (KDL-24; Fig. 6),

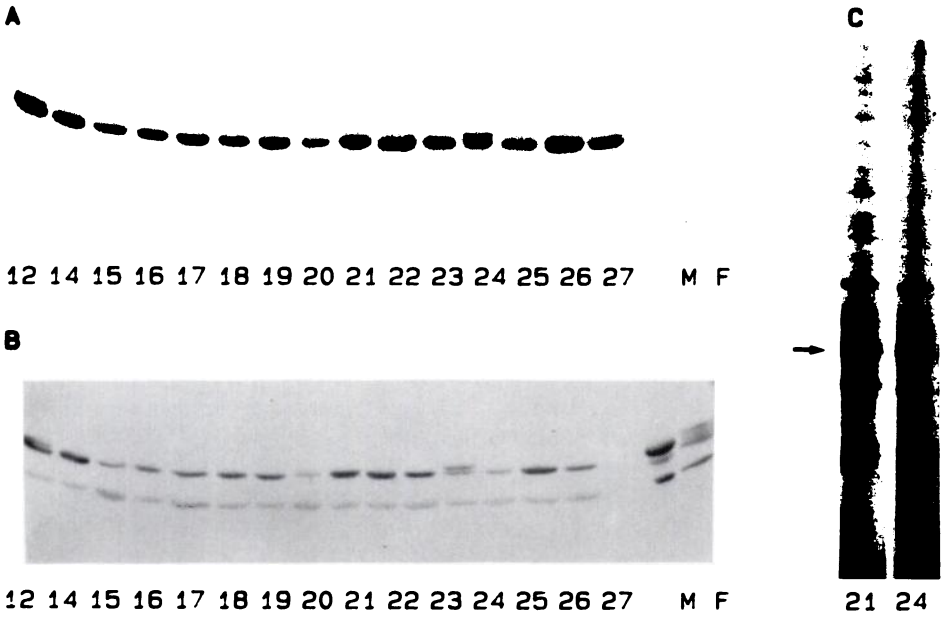

Fig. 6. Western blots of microsomes of 15 human livers (*numbers*) and male (*M*) and female (*F*) Sprague-Dawley rat livers with the monoclonal antibody 13-7-10 directed against human P450NF (A) or with the polyclonal antiserum directed against rat P450PCN1 (B). This latter antiserum also cross-reacts with a band of lower molecular weight, which does not correlate with midazolam or triazolam hydroxylation. C, Immunoisolation. Shown is a silver-stained SDS-polyacrylamide gel electrophoresis of the proteins isolated with monoclonal antibody 13-7-10 from KDL-21 and KDL-24 after SDS-denaturation of microsomes. The *arrow* indicates the apparent molecular weight (52,000) that corresponds to the Western blot signal. KDL-12 and KDL-24 reveal two immunoreactive bands in the 52-kDa region (B) and two 52-kDa proteins are immunopurified from KDL-24 as compared to KDL-21 (C).

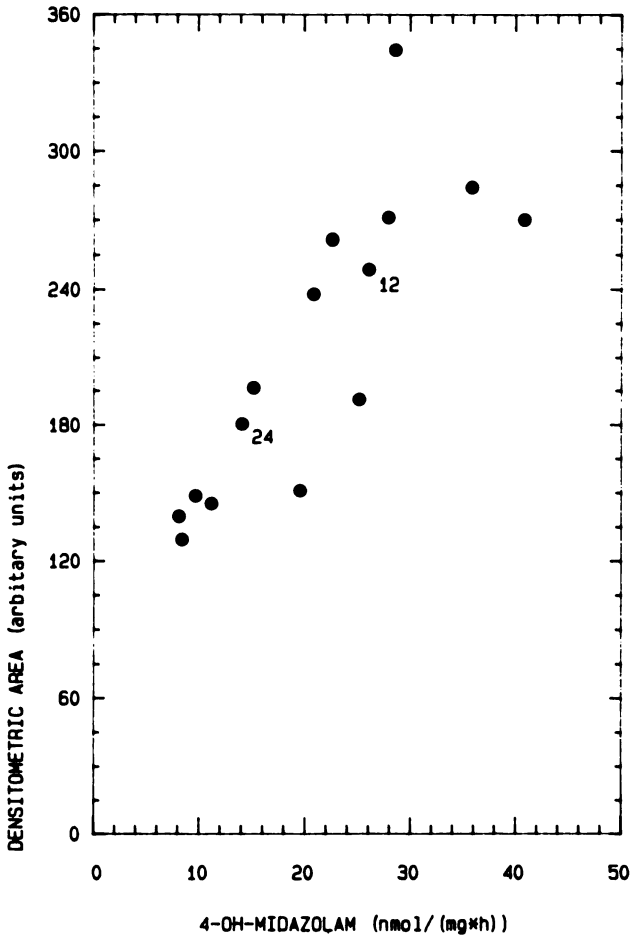

Fig. 7. Comparison of the densitometric area obtained by quantitation of Western blots with the monoclonal antibody 13-7-10 (Fig. 6) and the formation of 4-hydroxy-midazolam in microsomes of 15 human livers. The Spearman rank correlation coefficient is 0.936 ($p < 0.0005$; $n = 15$). Further details are given in the text.

only 29% of midazolam 1′-hydroxylase and 43% of midazolam 4-hydroxylase was inhibited under the same experimental conditions. The type of inhibition was not further characterized.

Midazolam hydroxylation of expressed P450IIIA4 cDNA. We have recently reported the expression of a cDNA (P450PCN1, P450IIIA4) in COS-1 cells that codes for a protein with nifedipine oxidase activity (23). The cDNA-derived amino acid sequence was identical to that of P450NF (15), with the exception of a single amino acid. To determine whether P450IIIA4 also metabolizes midazolam, we added the drug to the medium of COS-1 cells transfected with the human P450IIIA4 cDNA. Four hours after addition of midazolam, the medium contained 200 ng/ml 1′-OH-midazolam and 90 ng/ml 4-OH-midazolam, whereas no metabolites were detectable in the medium of COS-1 cells transfected with the vector alone. These data provide direct evidence that both 1′-hydroxy- and 4-hydroxy-midazolam can be produced by one enzyme, P450IIIA4. Moreover, the two metabolites are formed in proportions similar to those observed in human liver microsomes. The COS-1 cells also produced a protein that comigrated with the major 52-kDa immunoreactive protein found in human microsomes (Fig. 8). This protein was absent in cell lysates transfected with vector alone.

Discussion

The present data suggest that human hepatic cytochrome P450IIIA4 or an immunochemically and functionally closely related protein is the major enzyme catalyzing 1′- and 4-hydroxylation of midazolam and triazolam. Interindividual variation in the catalytic activities in microsomes of 15 human livers correlated with variable amounts of the protein recognized by polyclonal and monoclonal antibodies against human cytochrome P450IIIA4. The parallel variation of midazolam 1′- and 4-hydroxylation suggests that only one major P450 isozyme is involved in the formation of these two metabolites. Moreover, the high correlation between the formation of metabolites from midazolam and triazolam in this panel of samples indicates that both benzodiazepines are metabolized by the same enzyme.

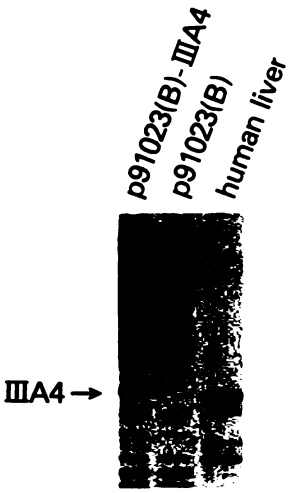

Fig. 8. Immunoblots with anti-rat P450PCN1 antibody of sonicated cell lysates of COS-1 cells transfected with human P450IIIA4 cDNA in vector p91023(B) (*left*) and vector p91023(B) alone (*middle*). For comparison, immunoblots of human liver microsomes with the same antibody are shown (*right*).

Midazolam and triazolam metabolism, however, does not correlate with the microsomal metabolism of bufuralol or mephenytoin, prototype substrates for the genetic polymorphisms of the debrisoquine- and mephenytoin-type. Thus, human inhibitory autoantibodies that recognize the microsomal proteins affected by these polymorphisms did not inhibit midazolam metabolism. This speaks against the possibility suggested by others (2) that these known genetic differences in human drug metabolism could have a major impact on the variability in metabolism of midazolam and triazolam. The findings with bufuralol are also in accordance with a recent study *in vivo*, in which no influence of the debrisoquine polymorphism on midazolam disposition was detected (27).

Immunoinhibition of midazolam and triazolam metabolite formation with an antibody raised against rat P450PCN1 (P450IIIA1) (5) was dose dependent and almost quantitative, indicating that the human P450 catalyzing midazolam and triazolam metabolism is immunochemically related to rat P450PCN1. A recent report by Fabre *et al.* (28) also showed that antibodies against rabbit P4503c inhibit midazolam metabolism in human liver microsomes. The here described immunoinhibition and immunoprecipitation experiments with a polyclonal antibody directed against human P450-5 (6), later identified as P450NF (P450IIIA4), further support the relatedness of the major midazolam hydroxylase with proteins of the P450IIIA subfamily. Expression of the human P450PCN1 cDNA (P450IIIA4) in COS-1 cells provided final and unequivocal evidence that the human P450IIIA4 cDNA codes for a enzyme that catalyzes the hydroxylation of midazolam to both the 1′-hydroxy and 4-hydroxy metabolites. The ratio of the two metabolites formed by the transfected cells was similar to that observed in microsomal incubations. We conclude that midazolam hydroxylation in human liver may be largely or entirely determined by P450IIIA4.

A monoclonal antibody by definition recognizes only a single epitope and, therefore, is less prone to cross-react with related proteins than is a polyclonal antiserum. The monoclonal antibody 13-7-10 raised against purified human P450-5 (6) has been used independently by two laboratories to clone two closely related cDNAs, designated P450NF (15) and P450HLp (29). The fact that midazolam hydroxylation in human liver microsomes was inhibited by preincubation with TAO also points to the functional relatedness of P450 NF and P450 HLp. It is not clear yet whether the two proteins are products of separate genes or represent allelic variants of the same gene. It also is unclear at this time whether and to what extent P450 HLp may contribute to midazolam and triazolam metabolism in human liver.

Southern blot analysis of genomic DNA with a P450HLp (P450IIIA3) cDNA reported by Molowa *et al.* (29) has suggested that multiple genes related to HLp may exist in the human genome. It is not clear how many of these sequences are expressed and how their expression is regulated. Indeed, two of our KDLs contained an additional immunoreactive protein with a higher apparent molecular weight. The functional characteristics of microsomes from these livers indicate that the second protein may be an active P450 with slightly different catalytic properties, as compared with the other livers in our panel. However, this occasionally occurring additional protein remains to be characterized.

The identification of P450IIIA4 as a midazolam and triazolam hydroxylase in human liver *in vitro* suggests that the clinically important variability in the metabolism of midazolam and triazolam observed *in vivo* most likely is due to variable levels of P450IIIA4 and closely related proteins that are expressed in response to endogenous or exogenous inducers.

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