Characterization of the Cytochrome P450 CYP234: Expression in Rat Small Intestine and Role in Retinoic Acid Biotransformation from Retinal*

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Abstract:

The sites of expression in the small intestine and the function of CYP2J4, a recently identified rat cytochrome (P450) isoform found to be predominantly expressed in the small intestine, were characterized. Immunoblot analysis with a polyclonal antibody to heterologously expressed CYP2J4 revealed that expression of CYP2J4 was at the highest level in the distal duodenum and jejunum and decreased toward the ileum. Villous cells expressed higher levels of CYP2J4 than crypt cells. Isoform-specific RNA polymerase chain reaction indicated that a related P450 isoform, CYP2J3, was only a minor form in rat small intestine. Since the intestinal mucosa is exposed to high levels of dietary nutrients, we hypothesized that CYP2J4 may be active toward diet-derived factors. We determined that purified, heterologously expressed CYP2J4 is active toward all-*trans*- and 9-*cis*-retinal in reconstituted systems, producing the corresponding retinoic acids as the major products. Apparent K_m values for the formation of retinoic acids were 54 and 49 μM, respectively, and apparent V_{max} values were 20 and 21 nmol/min/nmol P450, respectively. These activities were readily inhibited by a polyclonal anti-CYP2J4 antibody. Rat enterocyte microsomes were also active with all-*trans*-retinal to produce all-*trans*-retinoic acid in the pres ence of NADPH, and the majority of retinoic acid synthesis activity was inhibited by the polyclonal anti-CYP2J4 antibody. These findings suggest that CYP2J4 plays a major role in intestinal microsomal metabolism of retinal to retinoic acid and may be involved in the maintenance of retinoid homeostasis in the small intestine in vivo.

Key Words:

CYP2J4: retinoic acid; retinal; small intestine.

Article:

Intestinal cytochromes P450 (P450) are involved in the biotransformation of dietary nutrients as well as orally ingested toxicants, procarcinogens, and other xenobiotics (1). Previous studies from one of these and other laboratories indicated that multiple forms of P450 are expressed in rat small intestinal epithelial cells or enterocytes (1, 2). In a recent study, a previously unidentified rat P450, designated CYP2J4, was identified in the small intestine (3). RNA blot and immunoblot analyses indicated that CYP2J4 is predominantly expressed in the small intestine and to a lesser extent in the liver. Heterologously expressed CYP2J4 protein has been purified to near electrophoretic homogeneity and found to be active toward arachidonic acid in a reconstituted system with NADPH–P450 reductase and phospholipid, producing both hydroxyeicosatetraenoic and epoxyeicosatrienoic acids (3). However, CYP2J4 was not active toward testosterone and progesterone, as well as several xenobiotic compounds.

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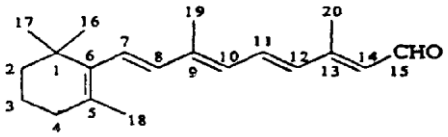
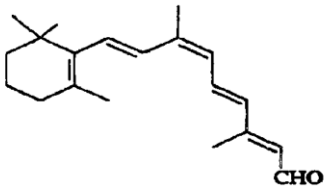
All-*trans* -Retinal



9-*cis* -Retinal



13-*cis* -Retinal

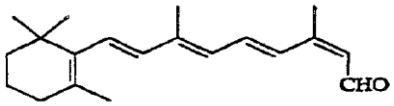


FIG. 1. Structures of the retinoids.

Since the small intestinal mucosa is exposed to high levels of dietary nutrients, CYP2J4 may be active toward other diet-derived factors. Potentially important examples are vitamin A or all-*trans*-retinol, its metabolites all-*trans*-retinal and all-*trans*-retinoic acid, and the corresponding 9-*cis* and 13-cis-isomers (Fig. 1). These compounds elicit pleiotropic biological responses by binding to the retinoid receptors (4). The functions of retinoids include regulation of growth, differentiation, development, epithelial maintenance, and cancer prevention and anticancer therapy. The diversity of their physiological, pathological, therapeutic, and developmental roles imparts great importance to the enzymes and the metabolic pathways involved in their biotransformation.

A recent review by Duester (5) has provided an overview of the pathways of retinol biotransformation. The biotransformation of retinol to retinal is catalyzed by alcohol dehydrogenase and the reverse reaction by retinol dehydrogenase. The conversion of all-*trans*-retinal to all-*trans*-retinoic acid is catalyzed by aldehyde dehydrogenase and cytochromes P450. While the formation of the 9-*cis* and 13-cis isomers of these various retinoids is well documented (6, 7), the specific metabolite precursors for the isomerizations have not been clearly defined.

Despite the importance of the retinol metabolic pathways, the roles of P450s in catalyzing the conversion of retinals to retinoic acids have not been clearly delineated. Characterization of these P450-mediated pathways has been most extensively undertaken in rabbits. In these studies eight purified rabbit liver and nasal P450s were reconstituted with NADPH–P450 reductase and cytochrome b_5 and tested for their capacity to metabolize retinals to retinoic acids (8). Only forms CYP1A2 and CYP3A6 catalyzed the oxidation of all-*trans*-retinal to all-*trans*-retinoic acid, and cytochrome b_5 did not facilitate the metabolism. In a subsequent extension of these studies (9), rabbit CYP1A1 was demonstrated to be the most effective form of P450 in the synthesis of retinoic acids from retinals in decreasing order of 9-*cis* > 13-cis > all-*trans*-retinal, based on V_{max}/K_m values. Purified rat liver CYP1A1 also catalyzes the formation of all-*trans*-retinoic acid from all-*trans*-retinal with a Km of 11.6 μM (10), although rat hepatic microsomal metabolism occurred with an apparent K_m of 52 μM (11).

In this paper, we have examined the activities of purified, heterologously expressed CYP2J4 toward different isomers of retinal in a reconstituted system. We now report the characterization of the sites of expression of

CYP2J4 in the rat small intestine and that CYP2J4 is active with both all-*trans*- and 9-*cis*-retinal, producing retinoic acids as the major products. In addition, rat enterocyte microsomes are active with alltrans-retinal to produce all-*trans*-retinoic acid in the presence of NADPH.

MATERIALS AND METHODS

Preparation and analysis of RNA.

Total RNA was prepared from frozen small intestinal and liver tissue of male Wistar rats (approximately 200 g body wt) according to the method of Chomczynski (12), with use of TRI Reagent obtained from the Molecular Research Center (Cincinnati, OH), RNA concentrations were determined spectrally with a Cary 3E UV-visible spectrophotometer (Varian), and the integrity of the RNA samples was assessed by ethidium bromide staining following agarose gel electrophoresis.

RNA PCR was performed using 0,5 µg of total RNA from small intestine or liver as described recently (2), PCR primers used for amplifying CYP2J3 and CYP2J4 fragments (5'-ACAACATCCACTA-CACTGCG-3' and 5'-ACCTGGCCAGTTGTTCTCC-3') were designed to amplify the region corresponding to nucleotides 987–1410 in CYP2J4 cDNA (3), For restriction digestion of PCR products with HincII or KpnI (obtained from Boehringer-Mannheim), the restriction enzyme and corresponding reaction buffer stock were directly added to PCR mixtures and incubated at 37°C for at least 2 h to ensure complete digestion, The DNA fragments were analyzed by agarose gel electrophoresis.

Production of polyclonal anti-CYP2J4 antibodies and immunoblot analysis.

Polyclonal antibodies were prepared in Flemish giant chinchilla rabbits using purified, heterologously expressed CYP2J4 as the antigen, as described previously for the production of antiCYP2J4 anti-peptide antibodies (3), The IgG fraction, which was used in immunoinhibition studies, was purified from antiserum as previously described (13).

For immunoblotting, small intestinal microsomal fractions and purified CYP2J4 were electrophoresed in 10% sodium dodecyl sulfate–polyacrylamide gels and the resolved proteins were transferred electrophoretically onto nitrocellulose membranes, Anti-CYP2J4 antiserum was used at 1:2000 dilution, The immunoblots were developed with a goat anti-rabbit IgG conjugated to horseradish peroxidase (Sigma) as secondary antibody and the immunoreactive proteins were visualized with the enhanced chemiluminescence Western blotting kit from Amersham (Arlington Heights, IL), Densitometric analysis of immunoreactive bands was performed with a LKB ImageMaster DTS densitometer (Pharmacia).

All-*trans*- and 9-*cis*-retinal metabolism.

All metabolism studies involving all-*trans*- or 9-*cis*-retinal as the substrate were carried out as described by Raner et al. (9), with several minor variations, An appropriate amount of 1 mM stock all-*trans*- or 9-*cis*-retinal solutions, in methanol, and 30 µg of 1,2-dilauroyl-sn-glycero-3-phosphorylcholine in chloroform were added to a glass test tube and the solvent was evaporated under a stream of N_2, L-Ascorbic acid (25 µl of a 20 mM stock solution) was added to each tube, to prevent nonenzymatic reactions, along with 25 µl of 1.0 M potassium phosphate buffer, pH 7.4, and deionized water in an amount to bring the final volume to 0.5 ml, and the solution was mixed using a vortex mixer for 30 s. In a separate tube, CYP2J4 and NADPH–P450 reductase were combined in a 1:4 molar ratio and stored on ice for 20 min prior to the addition of an aliquot to the all-*trans*- or 9-*cis*-retinal solution to give a final concentration of 0.1 µM CYP2J4 and 0.4 µM reductase. For rat enterocyte microsomal studies, an aliquot of 1 mg microsomal protein was added to the all-*trans*- or 9-*cis*-retinal solution. Control experiments were performed in which either NADPH or the reductase was omitted. All mixtures were preincubated at 37°C for 1.0 min before the reaction was initiated with 20 µl of a 25 mM NADPH stock solution. Reactions were carried out for 15 min at 37°C and terminated by quenching with 2.0 ml of ethyl acetate containing 50 µg/ml butylated hydroxytoluene, In studies to determine kinetic parameters, reactions were run for 5 min. After the ethyl acetate extract was removed, the remaining aqueous solution was acidified with 10 µl of 88% formic acid and extracted with an additional 2.0 ml of ethyl acetate. The extracts were

combined, the solvent was evaporated, and the residue was dissolved in 0.10 ml of methanol for analysis by HPLC. Extraction efficiency for the retinoic acids was greater than 95%. All reactions and other procedures were carried out in the absence of overhead light.

HPLC methods.

A Waters Nova-pak C18 column (8 X 100 mm) preceded by a C18 precolumn cartridge was used for all assays, in conjunction with a Waters HPLC system consisting of a Model 600E controlling unit, a Model 710 solvent delivery system, a Model 712 satellite sample injector, a Model 996 photodiode array detector, and the Millennium 2010 software package. For the analysis of metabolites of 9-*cis*- and all-*trans* retinal, a single solvent system was employed that consisted of solvent A (100% acetonitrile) and solvent B (99.9% H_2O and 0.1% trifluoroacetic acid), An 8-min linear gradient from 45% A and 55% B to 55% A and 45% B was followed by a 12-min linear gradient to 100% A, which was maintained for an additional 6 min (flow rate, 2 ml/min). Retinoic acids were quantified using the peak area at 360 nm, and all standard curves were linear over the concentration range of the retinoic acid products.

Other methods and materials.

Small intestinal enterocyte microsomes were prepared as previously described (14). Preparation of crypt and villous cells and segmentation of small intestine have been described elsewhere (14). Heterologously expressed CYP2J4 was purified to near electrophoretic homogeneity from insect cell microsomes as recently described, with a specific content of 4.3 nmol of P450 per milligram of protein (3). NADPH–P450 reductase was purified from phenobarbital-treated rat liver according to a published procedure (15). The sources of retinoids, metabolite standards, and other reagents have been described previously (9), The source of purified rabbit CYP2B4 has been described in an earlier study (16), Protein concentrations were determined using bicinchoninic acid reagent (Pierce) with bovine serum albumin as the standard.

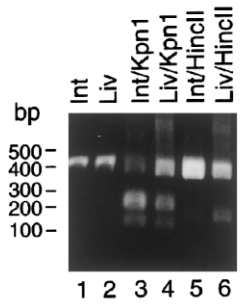


FIG. 2. Differential expression of CYP2J3 and CYP2J4 in rat liver and small intestine. RNA PCR was performed with total RNA preparations from rat small intestine (Int) and liver (Liv) with a primer pair speci®c for both CYP2J3 and CYP2J4 as described under Materials and Methods. PCR products were either untreated (lanes 1 and 2) or digested with restriction enzymes speci®c for CYP2J4 (*Kpn*I, lanes 3 and 4) or CYP2J3 (*Hinc*II, lanes 5 and 6), submitted to electrophoresis on 1.5% agarose gels, and visualized by staining with ethidium bromide. The positions of selected fragments of a 100-bp DNA size marker (BRL) are indicated.

RESULTS

Rat CYP2J4 has previously been identified by immunoblot and RNA blot techniques to be predominantly expressed in the small intestine (3). However, a more recent report suggested that rat CYP2J3, a highly similar isoform, is also expressed in the small intestine (17, 18). Since the anti-CYP2J4 antibodies and CYP2J4 cDNA probes used in our previous study may not distinguish these two isoforms, we have developed an approach to differentiate between the CYP2J isoforms expressed in rat small intestine and liver. A common set of PCR primers was used to amplify cDNA fragments of 424 bp (corresponding to nucleotides 987– 1410 in CYP2J4 cDNA) from either reverse-transcribed CYP2J3 or CYP2J4 mRNA in rat liver and small intestine. PCR

products derived from the two isoforms were distinguished by differential restriction digestion with HincII, which cuts only the amplified CYP2J3 cDNA to produce two almost equal-sized fragments, and KpnI, which cuts only the CYP2J4 cDNA to generate two fragments of 252 and 172 bp, respectively. Thus, the relative amounts of the CYP2J3- and CYP2J4-derived cDNA fragments following HincII or KpnI digestion indicate the relative levels of the two mRNAs in a given tissue. As shown in Fig. 2, rat small intestine expresses mainly CYP2J4, with very little CYP2J3, since HincII produces barely detectable fragments and KpnI yields only minimal levels of the full- length PCR product, whereas rat liver contains substantial amounts of CYP2J3 and CYP2J4.

Having established that CYP2J4, but not CYP2J3, is the major CYP2J isoform expressed in the rat small intestine, the level of rat CYP2J4 protein expression along the length of the small intestine was examined with use of a polyclonal anti-CYP2J4 antibody, gener-

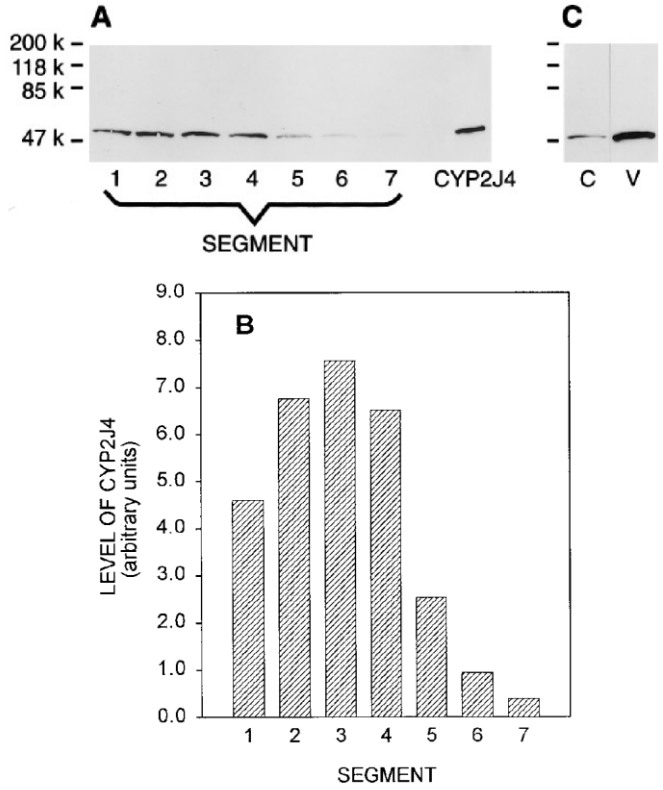


FIG. 3. Immunoblot analysis of CYP2J4 expression along the length of rat small intestine (A and B) and in crypt and villous cells (C). Microsomal proteins from each small intestinal segment (1 through 7; duodenum through ileum) of untreated rats or from crypt (C) and villous (V) cells from the entire length of the small intestine (10 μg/lane) were analyzed on immunoblots with a rabbit anti CYP2J4 antibody. Heterologously expressed CYP2J4 (0.5 μg) was applied as a control (A). The positions of the constituents of a prestained high-molecular-weight protein marker from Bio-Rad are indicated. The intensity of immunoreactive CYP2J4 bands in A was quantified by densitometry to yield the data in B.

ated in rabbits against purified, heterologously expressed CYP2J4 protein. As shown in Fig. 3A, this antibody recognized a single band in intestinal microsomes with the same mobility as that of purified, heterologously expressed CYP2J4. Figure 3A also shows the immunoblot analysis of microsomal preparations from different rat intestinal segments; the entire length of the small intestine was equally divided into seven segments, with segment 1 being adjacent to the pyloric valve and segment 7 nearest to the cecum. Densitometric analysis of the relative intensities of the bands (Fig. 3B) indicated that CYP2J4 expression is at the highest level in the distal duodenum and the jejunum and decreases markedly toward the ileum. The relative expression of CYP2J4 in the mature villous cells and in undifferentiated crypt cells was also examined. As shown in Fig. 3C, villous cells

express about a seven times higher level of CYP2J4 protein than do crypt cells on the basis of equal quantities of microsomal protein.

The capability of purified CYP2J4 to biotransform all-*trans*- and 9-*cis*-retinals to the corresponding retinoic acids was determined in a reconstituted system with NADPH–P450 reductase and dilauroylphosphatidylcholine. The chromatograms of the reaction products of all-*trans*- and 9-*cis*-retinal, detected by absorbance at 360 nm, are shown in Fig. 4. The major NADPH-dependent product in both reactions is the relevant retinoic acid isomer, resulting from oxidation of the isomeric substrate aldehydes (Figs. 4A and 4C). Product identification is based on comigration with authentic standard compounds and comparisons of ultraviolet spectra of the constituents of the identified peaks with those of authentic standards, recorded with a photodiode array detector (not shown). NADPH was omitted in control reactions (Figs. 4B and 4D). Nonenzymatic isomerization of the substrate was noted in all reactions. Additional, minor NADPH-dependent metabolites were also formed with both substrates and were designated X_1 and X_2 (from all-*trans*-retinal) and X_3, X_4, and X_5 (from 9-*cis*-retinal).

To determine whether any of the minor metabolites represent the 4-hydroxy derivative of all-*trans*- and 9-*cis*-retinal or all-*trans*- and 9-*cis*-retinoic acids, their retention times were compared with those of the metabolites produced by purified and reconstituted rabbit CYP2B4, which is known to metabolize all-*trans*-retinal and all-*trans*-retinoic acid primarily to the corresponding 4-hydroxy compounds (8) and presumably will also metabolize the 9-*cis* isomers in the same fashion. Under the same HPLC conditions used for analyzing CYP2J4 metabolites, the major NADPH-dependent metabolites generated by CYP2B4, 4-hydroxy-alltrans-retinal, 4-hydroxy-all-*trans*-retinoic acid, 4-hydroxy-9-*cis*-retinal, and 4-hydroxy-9-*cis*-retinoic acid from the corresponding aldehyde and acids, eluted at -15.2, 11.7, 15.8, and 13.3 min, respectively (data not shown). Thus, based on retention times and spectra, X_1 and X_3 are the 4-hydroxy-all-*trans*- and 4-hydroxy-9-*cis*-retinoic acids, respectively, and X_4 may be 4-hydroxy-9-*cis*-retinal. Peak X_2 corresponds to 13-*cis*-retinoic acid, and the other peak, X_5, which was less prominent, did not comigrate with any known metabolites.

The kinetics of retinal oxidation was examined with concentrations of all-*trans*- and 9-*cis* retinal varying between 10 and 120 μM (Fig. 5). The reconstituted system containing CYP2J4 catalyzed the metabolism of 9-*cis*- and all-*trans*-retinal to the corresponding retinoic acids with apparent K_m values of 49 and 54 μM, respectively, and apparent V_{max} values of 21 and 20 nmol/min/nmol CYP2J4, respectively, indicating that CYP2J4 has comparable efficiencies for all-*trans*- and 9-*cis*-retinal oxidations.

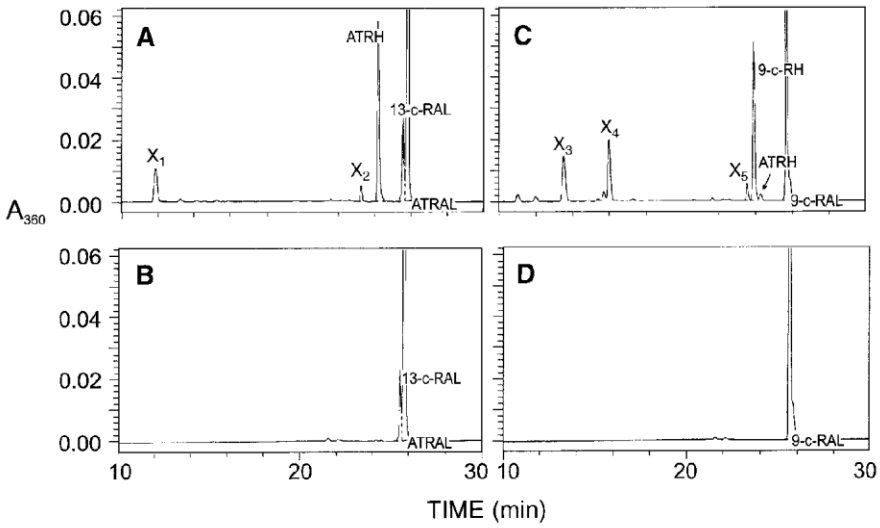


FIG. 4. Metabolites of 9-*cis*- and all-*trans*-retinal generated by puri®ed and reconstituted CYP2J4. The retinal isomer substrates and their metabolites were resolved by HPLC as described under Materials and Methods. Reaction mixtures contained 50 mM phosphate buffer, pH 7.4, 1 mM ascorbic acid, a reconstituted system containing 0.1 μM CYP2J4, 0.4 μM P450 reductase, and 30 μg phospholipid, and 20 μM all-*trans*- (A and B) or 9-*cis*-retinal (C and D). Reactions were performed at 37°C for 15 min in the presence (A and C) or absence (B and D) of NADPH (1 mM). Extraction and HPLC analysis of metabolites were carried out as described under Materials and Methods. The substrates, all-*trans*-retinal (ATRAL) and 9-*cis*-retinal (9-c-RAL), and the major metabolites, all-*trans*-retinoic acid (ATRH) and 9-*cis*-retinoic acid (9-c-RH), respectively, as well as ®ve minor peaks, X_1±X_5, are indicated. X_1 and X_3 are 4-hydroxy all-*trans*- and 4-hydroxy 9-*cis*-retinoic acids, respectively; X_4 is 4-hydroxy 9-*cis*-retinal; X_2 is 13-*cis*-retinoic acid; and X_5 is unknown.

Rat small intestinal microsomes were also active in retinal oxidation. As shown in Fig. 6, the major oxidative product of all-*trans*-retinal in the microsomal reactions was all-*trans*-retinoic acid, formed at a rate of 29 pmol/min/mg protein with substrate at 100 μM. The microsomal reactions were carried out in the presence

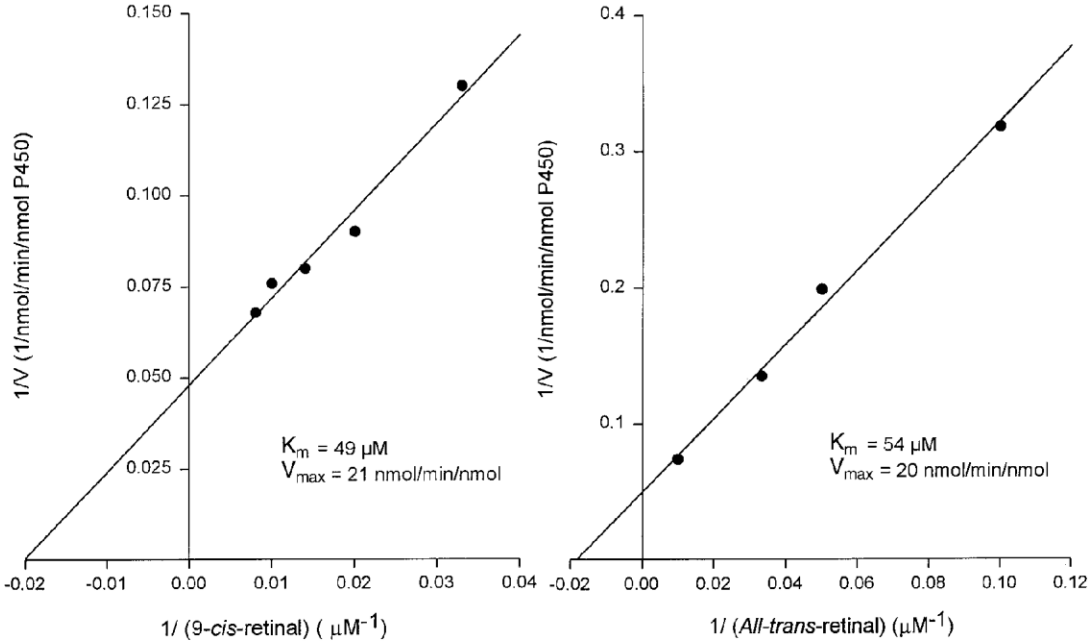


FIG. 5. Lineweaver±Burk plots for the oxidation of 9-*cis*- and all-*trans*-retinal to the corresponding retinoic acids by puri®ed and reconstituted CYP2J4. Reaction conditions are as described in the legend to Fig. 4, with substrate concentrations in the range of 10±120 μM. Reactions were carried out at 37°C for 5 min in duplicate and initial rates were measured.

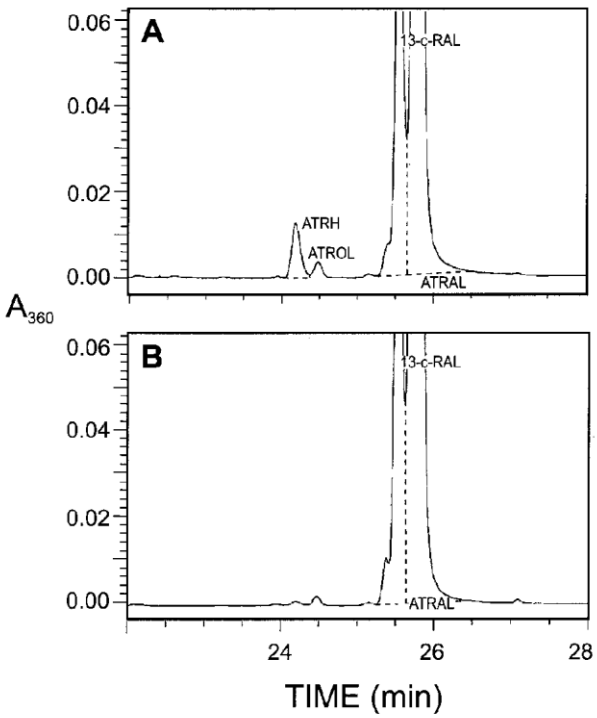


FIG. 6. Metabolism of all-*trans*-retinal by small intestinal microsomes. All-*trans*-retinal metabolism was performed as described in the legend to Fig. 4, except that reaction mixtures contained 100 μM all-*trans*-retinal (ATRAL), 2 mg/ml small intestinal microsomal protein (0.07 nmol P450/mg protein), and 50 μM disul®ram to inhibit microsomal aldehyde reductase, which converts the substrate to all-*trans*-retinol (ATROL). NADPH was omitted in control reactions (B).

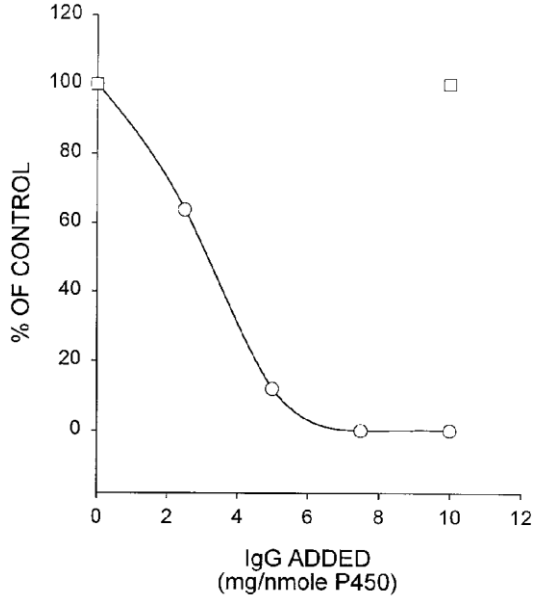


FIG. 7. Inhibition of the activity of puri®ed CYP2J4 in a reconstituted system in the metabolism of all-*trans*-retinal to all-*trans*-retinoic acid by a rabbit anti-CYP2J4 antibody. Retinal metabolism was carried out as described in the legend to Fig. 4 with the substrate at 20 μM. IgG fractions were added in the amounts indicated: ○, anti-CYP2J4 IgG; □, preimmune IgG. Activities are shown as percentages of control reactions.

of disulfiram, an aldehyde reductase inhibitor. The reductive product all-trans-retinol migrates close to all-trans-retinoic acid (Fig. 6). Addition of disulfiram (50 μM) did not affect the rate of all-trans-retinoic acid formation (not shown).

The role of CYP2J4 in intestinal microsomal metabolism of retinal was examined with a polyclonal antibody to CYP2J4. Anti-CYP2J4 IgG, when added at amounts greater than 7.5 mg/nmol P450, completely inhibited the activity of purified CYP2J4 in the metabolism of all-*trans*-retinal to all-*trans*-retinoic acid in a reconstituted system, while a high concentration of preimmune IgG did not affect this activity (Fig. 7). In microsomal reactions, addition of anti-CYP2J4 IgG at 0.5 mg/mg microsomal protein resulted in an inhibition of about 50% of this activity (Fig. 8). As shown, addition of α-naphthoflavone to the microsomal reactions resulted in a comparable inhibition of the activity. The microsomal activity was virtually completely blocked by ketoconazole, suggesting that the overall activity may be contributed to by other P450s present in rat small intestine, such as CYP1A1 and CYP3A1. The specificity of this anti-CYP2J4 antibody has been demonstrated in the experiments depicted in Fig. 3, in which only a single band was detected in intestinal microsomes. Furthermore, this antibody did not inhibit CYP1A1-catalyzed warfarin hydroxylation in a reconstituted system with purified rat CYP1A1 (data

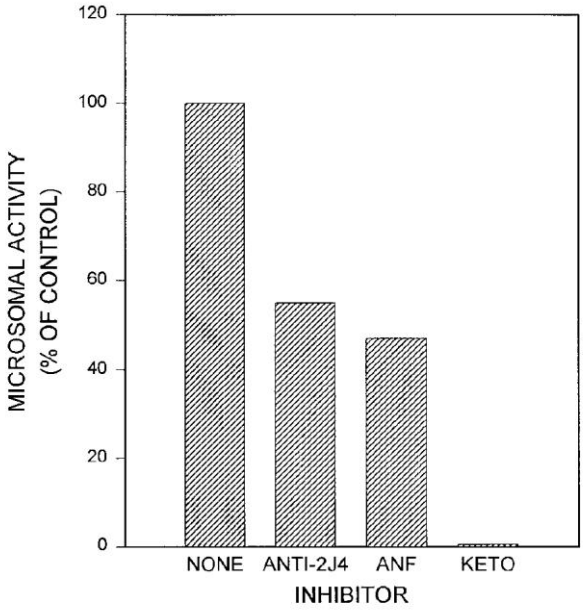


FIG. 8. Inhibition of the activity of intestinal microsomes in the metabolism of all-*trans*-retinal to all-*trans*-retinoic acid. Retinal metabolism was performed as described in the legend to Fig. 6. Anti-CYP 2J4 IgG (0.5 mg), α-naphthoflavone (ANF) (10 μM), and ketoconazole (KETO) (10 μM) were used as inhibitors.

not shown). In additional immunoblot experiments not presented, this antibody did not react with CYP1A, 2B, and 3A isoforms, which are the major P450s known to be present and inducible in rat small intestine (2), in intestinal microsomes from rats treated with β-naphthoflavone, phenobarbital, or dexamethasone, respectively.

DISCUSSION

These studies have served to characterize the location of expression of CYP2J4 in rat small intestine and have determined activities of the purified and microsome-bound enzyme using a dietary-derived substrate, all-*trans*-retinal and its isomer, 9-*cis*-retinal.

Initial observations (3) led to the conclusion that rat CYP2J4 was expressed predominantly in the small intestine based on Northern blot and immunoblot studies, the latter with the use of an anti-CYP2J4 peptide antibody. Subsequent reports indicated that a rat P450 from the same subfamily, CYP2J3, is also expressed at low levels in the small intestine (17, 18) and raised a question as to whether the original observation is accurate. The current study using RNA PCR and differential restriction digestion indicates that CYP2J3 occurs at very low levels, relative to those of CYP2J4, in rat small intestine. These studies thus confirm the observation that CYP2J4 is expressed predominantly in the small intestine.

Previous reports have indicated that total P450 expression levels in rat small intestine decrease as a function of distance along the small intestine from the duodenum to the ileum (1). Subsequently, we determined that both β-naphthoflavone-induced CYP1A1 protein levels and activity also decreased along the length of the small intestine (2). A possible explanation in the latter case is that levels of the aryl hydrocarbon receptor, which participates in CYP1A1 induction, were decreased in concert with CYP1A1 levels. We can offer no explanation for the pattern of CYP2J4 levels along the length of the rat small intestine. No inducers of CYP2J4 have been identified, and its mechanism of regulation is unknown.

Studies with purified CYP2J4 in reconstituted systems indicate that it catalyzes the formation of the corresponding retinoic acids from all-*trans*- and 9-*cis*-retinal. Kinetic analyses of these reactions revealed that the two isomeric substrates are metabolized with similar efficiencies by CYP2J4 and yield products at essentially the same rates. The two retinal isomers have very different overall molecular shapes, and thus the similarity of the kinetic parameters for their oxidation by CYP2J4 suggests that their interaction with the substrate binding site of CYP2J4 is determined primarily by their dienal moieties, from C11 through C15. Three factors could confound the kinetic data for the conversion of all-*trans*- and 9-*cis*-retinal to the corresponding retinoic acids: isomerization of the substrate retinals, further metabolism of the retinoic acid products to 4-hydroxy retinoic acids, and 4-hydroxylation of the substrates. In the case of reactions with all-*trans*-retinal as substrate, isomerization to 13-*cis*-retinal was detected to a variable extent, but maximally at 8% of the added substrate. The 4-hydroxy all-*trans*-retinoic acid metabolite was also formed and could have arisen from 4-hydroxylation of the substrate with its subsequent oxidation to 4-hydroxy all-*trans*-retinoic acid or by 4-hydroxylation of the product all-*trans*-retinoic acid. In studies with 20 μM all-*trans*-retinoic acid as substrate (data not included), 4-hydroxy all-*trans*-retinoic acid was formed at a rate of 0.2 nmol/min/nmol CYP2J4, compared with a rate of 1.4 nmol/min/nmol CYP2J4 when the substrate was 20 μM all-*trans*-retinal. Thus, the detected 4-hydroxy all-*trans*-retinoic metabolite produced with all-*trans*-retinal as substrate must arise predominantly via 4-hydroxylation of the substrate. Any influence of this metabolic step on the kinetics of metabolism of all-*trans*-retinal to all-*trans*-retinoic acid would thus arise from a decrease in the concentration of the substrate. For the 5-min incubations used, this decrease would be <3.5% of the initial substrate and would, together with the maximal 8% increase in substrate concentration through isomerization, produce a total decrease of approximately 11.5% in the substrate concentration. This decrease applies at the end of the 5-min reaction and represents the maximum possible decrease in substrate concentration. Actual decreases relevant to the kinetic parameters would probably be much lower. In the case of reactions with 9-*cis*-retinal as substrate, as a consequence of the rigorous exclusion of light, virtually no isomerization was detected. Furthermore, studies with 9-*cis*-retinoic acid as substrate indicated that, as with the reactions described above, the 4-hydroxy 9-*cis*-retinoic acid formed in reactions with this substrate arose primarily by 4-hydroxylation of the substrate followed by oxidation to the retinoic acid. In this case 4-hydroxy 9-*cis*-retinal was actually detected, suggesting that its oxidation to the corresponding retinoic acid was slow relative to its rate of formation or that these reaction steps are not concerted. Based on the sum of 4-hydroxy 9-*cis*-retinal and 4-hydroxy 9-*cis*-retinoic acid concentrations, this 4-hydroxylation reaction produces a maximal decrease of 10% in substrate concentrations. Overall, the decrease in substrate concentrations by 10 to 11.5%, through isomerization and the alternative 4-hydroxylation reaction, will affect the kinetic data reported, but only to a minor extent.

The studies with microsomal preparations from rat small intestine, incorporating inhibitory studies with rabbit anti-CYP2J4 antibody, indicated that small intestine microsome-bound CYP2J4 also catalyzes the metabolism of all-*trans*-retinal to all-*trans*-retinoic acid. Based on the extent of inhibition by the antibody in the small intestine from uninduced rats, CYP2J4 plays the predominant role in this metabolism, although other P450s also participate. Since in rats with induced levels of small intestinal CYP 1A1 by β-naphthoflavone conversion of all-*trans*-retinal to all-*trans*-retinoic acid is greatly enhanced relative to that in untreated rats (data not presented), CYP 1A1 must also be a participant in this metabolism in untreated rats. Since CYP1A1 is normally present at very low levels in untreated rats, its contribution to retinal metabolism in the intestines of such animals is probably low. Dexamethasone, which induces CYP3 in rat small intestine, did not affect rates of

microsomal metabolism of retinal to retinoic acid (data not presented). Other P450s which possibly contribute to small intestine microsomal metabolism of retinal to retinoic acid are currently unknown.

In view of the known participation of other pathways, the extent of the contribution of P450s to the overall physiological conversion of retinals to retinoic acids is currently unknown. Cytosolic enzymes such as NAD-dependent dehydrogenases also contribute to retinoic acid formation in reactions with K_m values which are reported to range from <1 to 250 μM (19, 20). Clearly further studies are required to resolve physiological relevance.

In summary, the recently identified CYP2J4 is predominantly expressed in rat small intestine. It could play a physiological role in retinoic acid synthesis and further details of its role in retinoid homeostasis are under investigation.

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