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Regioselective Hydroxylation of Prostaglandins by Constitutive Forms of Cytochrome *P*-450 from Rat Liver: Formation of a Novel Metabolite by a Female-Specific *P*-450

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Previous studies demonstrated that liver microsomes from untreated rats catalyze the ω, ω-1, and ω-2 hydroxylation of prostaglandins [K. A. Holm, R. J. Engell, and D. Kupfer (1985) *Arch. Biochem. Biophys.* **237**, 477-489]. The current study examined the regioselectivity of hydroxylation of PGE_1 and PGE_2 by purified forms of *P*-450 from untreated male and female rat liver microsomes. PGE_1 was incubated with a reconstituted system containing cytochrome *P*-450 RLM 2, 3, 5, 5a, 5b, 6, or f4, NADPH-*P*-450 reductase, and dilauroylphosphatidylcholine in the presence or absence of cytochrome b_5. Among the *P*-450 forms examined, only RLM 5 (male specific), 5a (present in both sexes), and f4 (female specific) yielded high levels of PGE hydroxylation. With PGE_1, RLM 5 catalyzed solely the ω-1 hydroxylation and 5a catalyzed primarily the ω-1 and little ω and ω-2 hydroxylation. By contrast, f4 effectively hydroxylated PGE_1 and PGE_2 at the ω-1 and at a novel site. Based on retention on HPLC and on limited mass fragmentation, we speculate that this site is ω-3 (i.e., 17-hydroxylation). Kinetic analysis of PGE_1 hydroxylation demonstrated that the affinity of f4 for PGE_1 is approximately 100-fold higher than that of RLM 5; the K_m values for f4, monitoring 19- and 17-hydroxylation of PGE_1, were about 10 μM. Surprisingly, cytochrome b_5 stimulated the activity of RLM 5a and f4, but not that of RLM 5. Hydroxylation of PGE_2 by RLM 5 was at the ω, ω-1, and ω-2 sites, demonstrating a lesser regioselectivity than with PGE_1. These findings show that the constitutive *P*-450s differ dramatically in their ability to hydroxylate PGs, in their regioselectivity of hydroxylation, and in their cytochrome b_5 requirement. © 1988 Academic Press, Inc.

Prostaglandins (PGs)[2] are metabolized in mammals *in vivo* by a variety of enzymatic reactions. The excreted metabolites are primarily dinor and tetranor ω carboxy and ω-1 hydroxy products (1-5). Previous investigations suggested that the ω-carboxyl of the PG metabolites is generated by an initial ω hydroxylation followed by oxidation of the hydroxyl (1, 6-9). Studies to characterize the enzymes involved in the hydroxylation of PGs, revealed that microsomal cytochrome *P*-450 monooxygenases in a variety of tissues from several mammalian species catalyze the hydroxylation of PGs at the ω and at other sites of the alkyl chain (9-14). Subsequently, using a reconstituted system with different *P*-450 forms, primarily from rabbit liver but also from other tissues, it

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[2] Abbreviations used: PGs, prostaglandins; PGE_1, prostaglandin E_1; PGE_2, prostaglandin E_2; PGEs, prostaglandins E_1 and E_2; PGB_1, prostaglandin B_1; RLM, rat liver microsome; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DLPC, dilauroylphosphatidylcholine.

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was shown that there is a considerable variation in activity and regioselectivity of hydroxylation of PGs among the P-450 forms (9, 15–21). Whereas, rabbit liver P-450 forms 2, 4, 6, and 7 demonstrated high hydroxylase activity, forms 3a, 3b, and 3c exhibited little or no PGE hydroxylase activity (16–18). Additionally, regioselectivity of hydroxylation of PGEs has been demonstrated: form 2 hydroxylated PGE_1 and PGE_2, primarily at ω-1 and minimally at ω position, form 4 hydroxylated at ω-1, form 6 at both ω-1 and ω-2, and form 7 hydroxylated PGEs solely at the ω site (16–18). These findings led to speculations that PGs serve as endogenous substrates of certain forms of P-450 (9).

Although treatment with phenobarbital and 3-methylcholanthrene, respectively, stimulate ω-1 and ω-2 hydroxylation of PGEs in male rats (22, 23), recent observations from this laboratory reveal that liver microsomes from untreated male rats can catalyze ω, ω-1, and ω-2 hydroxylation of PGEs (22). Sexual dimorphism of hepatic monooxygenases in rats is well documented (24, 25). More recently, the isolation of developmentally controlled female-specific and male-specific P-450 forms from rat liver (26–32) provided an explanation for sexual dimorphism observed in microsomal monooxygenase activity. These findings indicate that using individual forms of the hemoprotein, microsomal metabolites can be identified as to their source of formation. The current study was designed to determine whether purified forms of P-450 from livers of untreated male and female rats (referred to as constitutive P-450s) catalyze the hydroxylation of PGs and to establish which P-450s in microsomes are responsible for catalyzing hydroxylation at the various sites of the PGE molecule. Particular emphasis was placed on possible differences between the site-specific activity of the female-specific and male-specific P-450s and whether these differences are also expressed in liver microsomes from male and female rats.[3]

[3] A preliminary account of this investigation was previously presented as an abstract [Kupfer, D., Jansson, I., and Schenkman, J. B. (1987) *Fed. Proc.* **46**(3), 379, Abstr. 363].

EXPERIMENTAL PROCEDURES

Compounds. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49), D-glucose 6-phosphate monosodium salt, NADP, and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO). [5,6-^{3}H]PGE_1 (50 Ci/mmol) was purchased from Amersham (Arlington Heights, IL) and [5,6,8,11,12,14,15-^{3}H]PGE_2 (200 Ci/mmol) was purchased from NEN (Boston, MA). Prostaglandins (unlabeled) were obtained from Upjohn Co. (Kalamazoo, MI) and from Ono Pharmaceutical Co. (Japan). Acetonitrile for HPLC, a Burdick and Jackson product, was obtained from American Scientific Products (McGaw Park, IL).

Animals and hepatic microsomes. Sprague–Dawley CD male and female rats were obtained from Charles River Breeding Labs (Wilmington, MA). Liver microsomes were prepared as previously described (33). The microsomal pellet was resuspended in ice-cold 1.15% aqueous KCl solutions and centrifuged again for 1 h at 105,000*g*. The microsomal pellet was covered with fresh 1.15% KCl solution and stored frozen at −70°C. Prior to use, the microsomes were thawed and resuspended in fresh ice-cold KCl solution. Protein determinations were made by a modification (34) of the Lowry procedure (35), using BSA as standard.

Purification of microsomal enzymes. The purification of RLM 2 from untreated male rats has been described (36). Forms RLM 3 and RLM 5 from untreated male rats were purified according to the method of Cheng and Schenkman (30). The purification of RLM 5a and RLM f4 has recently been described (37). These forms were isolated from female rats, although RLM 5a also can be purified from male rats. RLM 5b and RLM 6 were purified from diabetic male rats as described (38). The specific contents of these purified forms of cytochrome P-450 are 16 (RLM 2), 16 (RLM 3), 16 (RLM f4), 12 (RLM 5), 14 (RLM 5a), 12 (RLM 5b), and 14 (RLM 6) nmol/mg of protein. The isolated RLM P-450s exhibited homogeneity on SDS-PAGE (Fig. 1). NADPH-cytochrome P-450 reductase was purified from liver microsomes of untreated rats according to the method of Yasukochi and Masters (39). The reductase preparation catalyzed the reduction of cytochrome *c* at a rate of 1.9 units/nmol of reductase (1 unit = 1 μmol of cytochrome *c* reduced/min at 22°C in 0.1 M Tris-Cl buffer, pH 7.5) and exhibited a specific content of 12 nmol/mg of protein. Cytochrome b_5 was purified from liver microsomes of untreated rats as previously described (40). The specific content of this preparation was 42 nmol/mg of protein.

Incubation. [^{3}H]PGE_1 (0.1 or 0.5 mM, 0.05 μCi) or [^{3}H]PGE_2 (0.5 mM, 0.05 μCi) was added in ethanol to glass scintillation vials, the ethanol was evaporated to dryness at room temperature under a stream of nitrogen, and the incubation with microsomes or reconstituted system was carried out as described below:

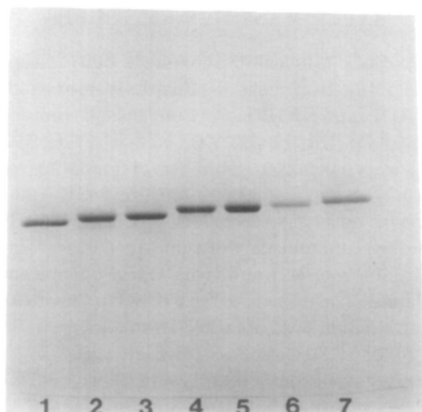

FIG. 1. SDS-PAGE analysis of constitutive RLM forms of cytochrome *P*-450 isolated from male and female rats. Tracks from left to right contained RLM 2 (1). RLM 3 (2), RLM f4 (3), RLM 5 (4), RLM 5a (5), RLM 5b (6), and RLM 6 (7). One microgram of each protein was used.

Microsomes. The incubation contained potassium phosphate (50 mM, pH 7.4), $MgCl_2$ (10 mM), and EDTA (1 mM). The microsomal suspension (for protein concentrations see tables) was added and the vials were preincubated for 2 min at 37°C. Reaction were initiated with NADPH (1.2 mM) and NADPH-regenerating system (38.8 mM glucose 6-phosphate and 3 IU glucose-6-phosphate dehydrogenase) for 30 min at 37°C, in 1 ml final volume, as previously described (13); linearity of product formation for at least 30 min was previously established. Reactions were terminated as described below.

Reconstituted cytochrome P-450 metabolizing system. The cytochrome *P*-450 metabolizing system was reconstituted into dilauroylphosphatidylcholine (DLPC) vesicles as previously described (36). The final concentrations of assay components in 1 ml were as follows: $[^3H]PGE_1$ or $[^3H]PGE_2$ (0.5 mM, 0.05 μCi), 50 mM sodium phosphate buffer, pH 7.25, 10 mM $MgCl_2$, 5 mM glucose-6-phosphate, 2 units of glucose-6-phosphate dehydrogenase, and 25 μM DLPC vesicles containing 0.15 μM cytochrome *P*-450, 0.15 μM NADPH-cytochrome *P*-450 reductase, and with or without 0.15 μM cytochrome b_5. The metabolism studies were performed at 37°C. The reaction was initiated by the addition of NADPH to a final concentration of 0.5 mM. The reaction, in incubations with microsomes or reconstituted system, was terminated by addition of 10 ml ethanol and the PG metabolites were isolated as previously described (17). In the course of the workup, PGEs and their respective metabolites were converted by base-treatment to the corresponding PGB derivatives to facilitate their detection by uv spectroscopy.

High-performance liquid chromatography and quantitation of metabolites. A small volume of methanol was added to the dried extracts containing the PGB derivatives. To determine recoveries, an aliquot was taken for radioactive determination in a scintillation spectrometer and another aliquot, typically 1-10% of the extract, was subjected to HPLC as previously described (14, 22). The remainder of the extract was kept dry under argon at −20°C to be utilized for isolation and characterization of products. Quantitation of hydroxylated metabolites was achieved from areas under the corresponding HPLC peaks (using standard curves obtained with 19-OH-PGB_1 or 19-OH-PGB_2) and corrected for recoveries (14). Loss of tritium (16–20%) from labile sites during conversion of PGE_2 to PGB_2 was corrected for in calculating recoveries.

Isolation and characterization of metabolites. The residual extract (stored at −20°C) was subjected to HPLC and the metabolites were isolated from the eluate fractions by evaporating the liquid under a stream of nitrogen. Characterization of the metabolites was based on their retention time and by coinjection with authentic compounds on HPLC. The authentic compounds were obtained as follows: 18-OH-PGBs were from incubations of PGE_1 and PGE_2 with liver microsomes from methylcholanthrene-treated rats (22), 19-OH-PGBs were generated from 19-OH-PGE_1 and 19-OH-PGB_2 was isolated from human semen as previously described. 20-OH-PGB_1 was derived from 20-OH-PGE_1, obtained from Ono Pharmaceutical Co., and 20-OH-B_2 was isolated from incubation of PGE_2 with rabbit liver microsomes (14). The isolated metabolites were converted to their respective methyl esters by ethereadiazomethane and analyzed by HPLC. The methyl esters were converted to their respective trimethyl silyl ether derivatives and subjected to GC/MS analysis as previously described (14, 17).

RESULTS

Several forms of rat liver microsomal cytochrome *P*-450 (RLM), from untreated rats, were examined as to their ability to catalyze the hydroxylation of PGE_1. Additionally, in view of previous observations that certain forms of *P*-450 require cytochrome b_5 for PG hydroxylating activity (16, 18), the effect of b_5 on this activity was determined (Table I). Results demonstrate that whereas RLM 2, 3, 5a, 5b, and 6 exhibit low hydroxylating activity, f4 and 5 are highly active, the former being further stimulated by b_5 and the latter unaffected by b_5. The activity of form 2 was lower, but also stimulated by b_5, an observation also made with form 5a. Interest-

TABLE I

EFFECT OF CYTOCHROME b_5 ON HYDROXYLATION OF PGE_1 BY CONSTITUTIVE MALE AND FEMALE RAT LIVER *P*-450s

		Hydroxylated products (nmol product/30 min/nmol *P*-450)				
P-450 RLM	b_5	X_1	18	19	20	Total metabolites
2	−			3.6		3.6
	+			5.9		5.9
3	−			2.5		2.5
	+			2.3		2.3
f4	−	15.7		14.9	Trace	30.6
	+	21.7		21.5	Trace	43.2
5	−			53.3		53.3
	+			50.0		50.0
5a	−		0.7	5.8	0.7[a]	7.2
	+		2.3	12.1	2.1	16.5
5b	−			1.9		1.9
	+			2.6		2.6
6	−			2.1		2.1
	+			2.0		2.0

Note. Cytochrome *P*-450 RLM fractions (0.15 μM) were reconstituted in DLPC (25 μM) vesicles containing 0.15 μM NADPH cytochrome *P*-450 reductase with or without 0.15 μM cytochrome b_5. Incubations were conducted with $[^3H]PGE_1$ (0.5 mM), in the presence of NADPH (0.5 mM) and NADPH regenerating system, for 30 min at 37°C. The prostaglandin products were isolated as PGB derivatives and quantitated by reversed-phase HPLC as described under Experimental Procedures.

[a] Low amounts and poor separation of 20-OH-PGB_1 from the other products permitted only approximate quantitation.

ingly, all of the RLMs exhibited ω-1 hydroxylase activity. This was not surprising, since 19-hydroxylation represents the major activity in liver microsomes from untreated rats (see below, Fig. 2). RLM 5a was the only form which demonstrated significant ω hydroxylation (20-OH) (Table I); this enzyme also showed equivalent ω-2 hydroxylation (18-OH) (Fig. 3), an activity not observed with the other purified forms of *P*-450. A highly interesting observation was the finding that the female-specific form, f4, yielded a novel PGE_1 metabolite, initially referred to as X_1, hitherto not observed;[4] this metabolite is less polar on reversed-phase HPLC than the corresponding 18-hydroxy metabolite (Fig. 4) and was formed in similar amounts to that of the 19-OH metabolite (Table I). We tentatively suggest that this metabolite is 17-OH-PGE_1. Cytochrome b_5 stimulated the formation of both metabolites by about 50%.

The incubation of PGE_2 with several RLM *P*-450s yielded qualitatively similar resuls to those obtained with PGE_1 (Table II); however, some differences are apparent. Whereas with PGE_1, form 5 produced almost exclusively the 19-OH product, with PGE_2 in addition to the 19-OH metabolite, significant amounts of the 18- and 20-OH metabolites were also observed.

[4] In the course of isolation of 18-OH-PGB_1 from incubations of PGE_1 with rabbit liver *P*-450 LM6, we detected a small amount of chromatographically less polar PGB_1 derivative (K. A. Holm and D. Kupfer, unpublished); whether that product is identical to the one isolated from incubation of PGE_1 with f4, has not been established.

A decrease in the concentration of PGE_1 from 0.5 to 0.1 mM, did not decrease the extent of formation of metabolites by form f4, while the metabolism by form 5 was substantially diminished. This suggested that there is a considerable difference in affinity between these two forms of *P*-450 for PGE_1. In fact, kinetic studies supported this suggestion (Table III); the apparent K_m value for f4 was about 10 μM, while the value for form 5 was in excess of 1 mM, indicating that f4 had a much higher affinity for PGE_1. The extremely large differences in K_m values indicate that at concentrations lower than 0.5 mM the rates of hydroxylation by forms with low affinity for PGEs would be substantially altered so that their relative activities would markedly differ from those in this study. Incidentally, because of potential solubility problems, the use of concentrations of PGEs higher than 0.5-1 mM is impractical.

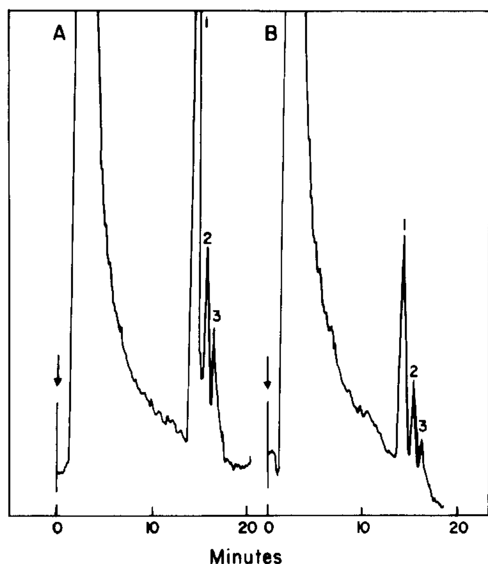

FIG. 2. HPLC analysis of extracts containing prostaglandin metabolites (as PGB derivatives) from incubation of PGE_1 with liver microsomes from mature male (A) and female (B) rats. A C_8 (5 μm) reversed-phase Whatman column was used. The mobile phase was 24% CH_2CN/H_2O containing 0.01% acetic acid, run at 2 ml/min. Monitoring was at 278 nm, using 0.001 absorbance units full scale. Arrow indicates injection point. Peak 1 = 19-OH-PGB_1; Peak 2 = 20-OH-PGB_1; Peak 3 = 18-OH-PGB_1.

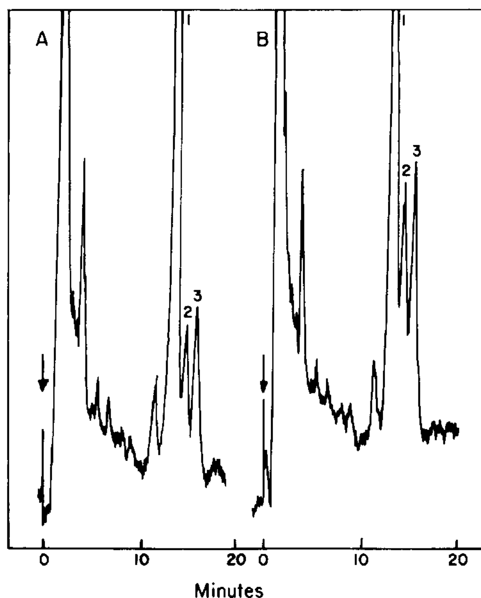

FIG. 3. HPLC analysis of an aliquot of an extract containing prostaglandin metabolites (as PGB derivatives) from an incubation of PGE_1 with RLM 5a (A). Coinjection of an aliquot of an extract with authentic 18-OH-PGB_1 and 20-OH-PGB_1 (B). Conditions were as in Fig. 2. Peak 1 = 19-OH-PGB_1; Peak 2 = 20-OH-PGB_1; Peak 3 = 18-OH-PGB_1.

Based upon immunological data from several laboratories, the content of *P*-450 f4 protein in mature female rat liver microsomes ranges from 0.23 nmol/mg (27, 31) to 0.35 nmol/mg protein (29). The observation of the formation of a novel PGE mtabolite by f4, the female-specific *P*-450, suggested that liver microsomes from mature female rats would also catalyze the formation of significant amounts of this metabolite. Surprisingly, our studies failed to demonstrate the formation of this metabolite by liver microsomes from mature female rats (Table IV). Since f4 is present in minimal amounts in adult male rats (27, 29, 31) and appears to be developmentally controlled in female rats,[5] it was anticipated that microsomes of immature female and mature male rats would not

[5] This assumes that f4 is identical to the female-specific 15β hydroxylase of steroid sulfates (27), and to female-specific *P*-450 described by Waxman (26) and by Kamataki *et al.* (29), and to *P*-450i (41). Its catalytic activity spectrum and NH_2-terminal amino acid sequence are the same (37, 42).

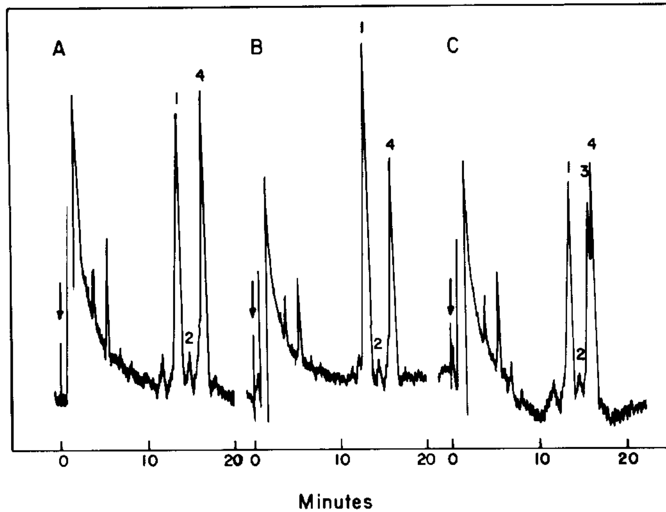

FIG 4. HPLC analysis of an aliquot of an extract containing prostaglandin metabolites (as PGB derivatives) from an incubation of PGE_1 with RLM f4 (A). Coinjection of an aliquot of the extract with 19-OH-PGB_1 (B) and 18-OH-PGB_1 (C). Conditions were as in Fig. 2. Peak 1 = 19-OH-PGB_1; Peak 2 = 20-OH-PGB_1 (?); Peak 3 = 18-OH-PGB_1; Peak 4 = PGB derivative of X_1.

produce detectable amounts of this PGE metabolite. As anticipated, liver microsomes from mature male or immature female rats did not form this metabolite (Table V). The possibility that this metabolite was indeed formed by liver microsomes from mature females but remained undetected by being chromatographically indistinguishable from the corresponding 18-OH metabolite, was ruled out: (a) Based on K_m determinations with f4, the novel metabolite should have been formed by a high affinity enzyme; however, the metabolite with retention time corresponding to 18-OH-PGB_1 appeared to be formed by low affinity enzyme(s), as evident by a marked decrease in its formation when the concentration of PGE_1 was de-

TABLE II

EFFECT OF CYTOCHROME b_5 ON HYDROXYLATION OF PGE_2 BY CONSTITUTIVE MALE AND FEMALE RAT LIVER *P*-450s

		Hydroxylated products (nmol product/30 min/nmol *P*-450)				
P-450 RLM	b_5	X_2	18	19	20	Total metabolites
5	−		8.3	52.1	2.0	62.4
	+		13.1	49.9	2.9	65.9
5a	−		3.1	14.9	0.5[a]	18.5
	+		15.7	60.3	3.4	79.4
f4	−	3.7		6.6	1.3	11.6
	+	3.7		14.3	1.8	19.8

[a] Because of low amounts of this product, the quantitation was only approximate. Conditions were the same as in Table I, except that [^{3}H]PGE_2 (0.5 mM) was used.

TABLE III

KINETIC CONSTANTS FOR PGE_1 HYDROXYLATION BY P-450 RLM f4 AND 5

		Hydroxylated PGE_1			
		X_1		19-OH	
P-450	Sex specific	K_m (μM)	V_{max}[a]	K_m (μM)	V_{max}[a]
f4	Female	9.6	0.34	10.6	0.40
5	Male			>1000[b]	~10

Note. Conditions were the same as in Table I, except that [^{3}H]PGE_1 concentration was varied from 0.005 to 0.1 mM and incubations were conducted for 10 min. The kinetic parameters were obtained by a Lineweaver-Burk double reciprocal plot of velocity of product formation versus concentration of PGE_1.

[a] nmol/min/nmol P-450.

[b] Values between 1 and 2 mM.

creased from 0.5 to 0.1 mM (Table IV) and (b) Separation of the 18-OH metabolite from the novel metabolite was demonstrated by coinjection of the extract, from microsomal incubation of PGE_1, with metabolite X_1 (Fig. 5). Although not formed in significant amounts, X_1 could be demonstrated, albeit in very low amounts. This was determined by combining extracts from several incubations and subjecting a larger portion of the extract to HPLC analysis (not shown).

TABLE IV

EFFECT OF SUBSTRATE CONCENTRATION ON PGE_1 HYDROXYLATION BY LIVER MICROSOMES FROM MATURE MALE AND FEMALE RATS

		PGE_1 hydroxylation[a] (nmol/30 min/mg protein)		
Sex	PGE_1 (mM)	18	19	20
Male	0.5	1.54	6.34	1.87
Male	0.1	0.58	2.88	0.83
Female	0.5	0.58	2.20	0.80
Female	0.1	0.25	1.00	0.42

Note. Incubations were conducted in 1 ml volume containing 1-1.2 mg microsomal protein (pooled from four rats) and 0.5 or 0.1 mM [^{3}H]PGE_1 (0.05 μCi) for 30 min at 37°C in the presence of NADPH (1.2 mM) and NADPH regenerating system (see Experimental Procedures). Incubations were terminated by the addition of 10 ml ethanol and the products were isolated and quantitated by reversed-phase HPLC, as described under Experimental Procedures. Mature male rats were 10-11 weeks old and mature female rats were 9 weeks old.

[a] The amounts of X_1 formed, detected as a shoulder to the 18-OH peaks, were too low for accurate quantitation.

TABLE V

COMPARISON OF PGE_1 HYDROXYLATION BY LIVER MICROSOMES FROM IMMATURE AND MATURE MALE AND FEMALE RATS

		PGE_1 hydroxylation[b] (nmol/30 min/mg protein)		
Sex	Development[a]	18	19	20
Male	Immature	0.58	1.15	0.62
Male	Mature	1.54	6.34	1.87
Female	Immature	0.36	0.95	0.49
Female	Mature	0.58	2.20	0.80

Note. Microsomal preparations were obtained from a pool of livers from four (mature) and nine (immature) rats. Incubations were conducted as described in Table IV, except that the concentrations of microsomal proteins were between 1.0 and 1.4 mg per incubation.

[a] Immature, 19 days old; mature males, 10-11 weeks old; mature females, 9 weeks old.

[b] The amounts of X_1 detected were too low for quantitation.

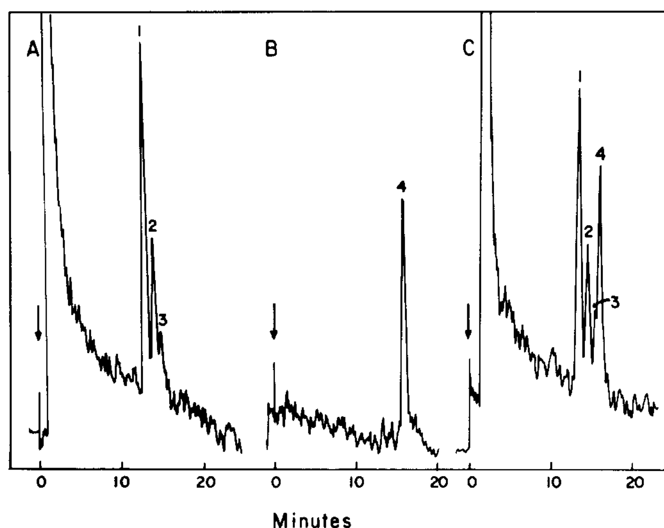

FIG. 5. HPLC analysis of an aliquot of an extract containing prostaglandin metabolites from an incubation of liver microsomes from mature female rats with PGE_1 (A). Chromatogram of PGB derivative of compound X_1 isolated from incubation of PGE_1 with RLM f4 (B). HPLC of a coinjection of extract from (A) with compound X_1 (C). Conditions were as in Fig. 2. Peak 1 = 19-OH-PGB_1; Peak 2 = 20-OH-PGB_1; Peak 3 = 18-OH-PGB_1; Peak 4 = PGB derivative of X_1.

Maturation of both male and female rats resulted in a marked increase in microsomal PGE_1 hydroxylation (Table V). In the female, the increase in hydroxylation due to maturation was similar at the various sites, being about twofold at C-18, C-19, C-20. By contrast, in the male, maturation caused a much larger increase in hydroxylation at C-19 (sixfold) than at other sites (threefold). This increase in 19-hydroxylation in the male is probably due to the appearance of several developmentally controlled male-specific P-450s, such as RLM 2, 3, and 5 (27–30, 34), all of which catalyze the 19-hydroxylation of PGE_1 (Table I).

DISCUSSION

The current findings demonstrate a diversity of regioselectivity of hydroxylation of PGEs (Fig. 6) by constitutive forms of rat liver P-450s. A most interesting finding is the observation that female-specific P-450 form f4 catalyzed the formation of 19-hydroxy metabolites plus novel metabolites of PGE_1 and PGE_2, X_1 and X_2, respectively. Based on the longer retention time on reversed-phase HPLC of X_1 and X_2 than of the corresponding 18-OH, 19-OH, and 20-OH metabolites, we speculate that these novel metabolites are the corresponding 17-OH metabolites formed by ω-3 hydroxylation. A possibility was considered that the metabolites may be ketones resulting from subsequent oxidation of monohydroxylated metabolites; the latter pathway was observed in the conversion of testosterone to androstenedione by rat liver monooxygenases (43, 44). However, the speculation that X_1 is the 17-hydroxylated metabolite of PGE_1 is supported by preliminary mass fragmentation studies of the methyl ester trimethylsilyl ether of PGB_1 derivative of X_1, yielding an M+ at m/z = 510, corresponding to the molecular ion of the derivatized monohydroxylated metabolite and a significant ion at m/z = 364 $[M-(146)]^+$; the latter possibly representing a loss of $[CH_2CH_2CH_2CHOTMS + 1]$. Previously, we observed that mass fragments (m/z) 117 and 131 were diagnostic ions of 19- and 18-hydroxylation, respectively (14, 17, 24); therefore, we expected a mass fragment at m/z = 145 $[CH_2CH_2CH_2CHOTMS]$ if X_1 an X_2 were indeed the 17-hydroxylated metabolites. However, the presence of this ion or other ions in that portion of the

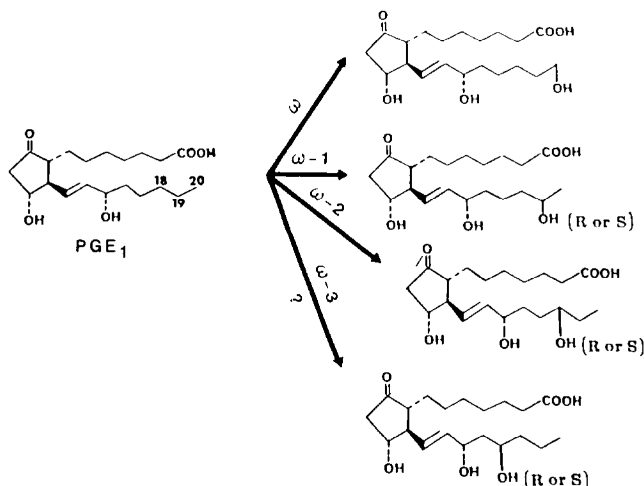

FIG. 6. Pathways of metabolism of PGE_1 by various RLM *P*-450 forms from liver microsomes of untreated male and female rats; ω-hydroxylation by RLM 5a, ω-1 by RLM 2, 3, f4, 5, 5a, 5b, and 6; ω-2 by RLM 5a; and ω-3 by RLM f4.

mass spectrum was not detected because of low mass contamination, thereby precluding a more conclusive identification of X_1.

It is of interest that whereas catalysis of PGE_1 hydroxylation by f4 occurs with high affinity, hydroxylations at ω, ω-1, and ω-2 by other rat liver *P*-450s exhibit low affinity catalysis. By contrast in rabbit liver reconstituted systems and microsomes, only the ω hydroxylation is catalyzed by a high affinity enzyme (14, 16–18). The minimal production of metabolite X_1 by microsomes of the mature female rat is puzzling in view of the high activity of the RLM f4 for PGE_1 and PGE_2 (this paper) and its fairly high levels in these microsomes (27, 29, 31). Perhaps in microsomes this enzyme competes poorly for reductase; however, the possibility exists that enzyme configurations in reconstituted systems may differ sufficiently from those in microsomes as to generate unusual products. Alternatively, there is a possibility that X_1 is formed in significant quantities by microsomes, but undergoes further metabolism into products which are not detected by our methods. The resolution of this dilemma would require further investigation.

The effects of cytochrome b_5 on the hydroxylation of PGs by RLMs were quantitatively and qualitatively different from those previously obtained with *p*-nitroanisole or benzphetamine as substrates (45). Cytochrome b_5 strongly stimulated demethylation of *p*-nitroanisole by both RLM 5 and 5a, but had little or no effect on benzphetamine demethylation (45); by contrast, only the 5a-catalyzed hydroxylation of PGEs was stimulated by b_5 and the stimulation was not as pronounced as in the case of the demethylation of *p*-nitroanisole. Similarly, strikingly different effects by b_5 have been previously observed with rabbit liver LM 2; there was almost an absolute requirement for b_5 in PGE_1 hydroxylation (16), but not for benzphetamine demethylation (46). Currently we have no explanation for these differences in b_5 effects.

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REFERENCES

1. SAMUELSSON, B., GRANSTROM, E., GREEN, K., AND HAMBERG, M. (1971) *Ann. N.Y. Acad. Sci.* **180,** 138–163.

2. SVANBORG, K., AND BYGDEMAN, M. (1972) *Eur. J. Biochem.* **28,** 127–135.
3. SUN, F. F. (1974) *Biochim. Biophys. Acta* **348,** 249–262.
4. SUN, F. F., AND STAFFORD, J. E. (1974) *Biochim. Biophys. Acta* **369,** 95–110.
5. ELLIS, C. K., SMIGEL, M. D., OATES, J. A., OELZ, O., AND SWEETMAN, B. J. (1979) *J. Biol. Chem.* **254,** 4152–4168.
6. KUPFER, D., NAVARRO, J., AND PICCOLO, D. E. (1978) *J. Biol. Chem.* **253,** 2804–2811.
7. POWELL, W. S. (1978) *J. Biol. Chem.* **253,** 6711–6716.
8. KUPFER, D., NAVARRO, J., MIRANDA, G. K., PICCOLO, D. E., AND, THEOHARIDES, A. D. (1980) *Arch. Biochem. Biophys.* **199,** 228–235.
9. KUPFER, D. (1982) *in* Hepatic Cytochrome P-450 Monooxygenase System (Schenkman, J. B., and Kupfer, D., Eds.), pp. 157–187, Pergamon Press, New York.
10. NAVARRO, J., PICCOLO, D., AND KUPFER, D. (1978) *Arch. Biochem. Biophys.* **191,** 125–133.
11. KUPFER, D., NAVARRO, J., MIRANDA, G. K., AND THEOHARIDES, A. D. (1980) *Arch. Biochem. Biophys.* **205,** 297–307.
12. POWELL, W. S. (1980) *Prostaglandins* **19,** 701–710.
13. KUPFER, D., MIRANDA, G. K., NAVARRO, J., PICCOLO, D. E., AND THEOHARIDES, A. D. (1979) *J. Biol. Chem.* **254,** 10405–10414.
14. THEOHARIDES, A. D., AND KUPFER, D. (1981) *J. Biol. Chem.* **256,** 2168–2175.
15. OKITA, R. T., PARKHILL, L. K., YASUKOCHI, Y., MASTERS, B. S. S., THEOHARIDES, A. D., AND KUPFER, D. (1981) *J. Biol. Chem.* **256,** 5961–5964.
16. VATSIS, K. P., THEOHARIDES, A. D., KUPFER, D., AND COON, M. J. (1982) *J. Biol. Chem.* **257,** 11221–11229.
17. HOLM, K. A., KOOP, D. R., COON, M. J., THEOHARIDES, A. D., AND KUPFER, D. (1985) *Arch. Biochem. Biophys.* **243,** 135–143.
18. HOLM, K. A., AND KUPFER, D. (1985) *J. Biol. Chem.* **260,** 2027–2030.
19. KUSUNOSE, M., KUSUNOSE, E., ICHIHARA, K., OGITA, K., MASATOSHI, K., AND YAMAMOTO, S. (1985) *in* Advances in Prostaglandin Thromboxane and Leukotriene Research (Hayaishi, O., and Yamamoto, S., Eds.), Vol. 15, pp. 155–158, Raven Press, New York.
20. YAMAMOTO, S., KUSUNOSE, E., OGITA, K., MASATOSHI, K., ICHIHARA, K., AND KUSUNOSE, M. (1984) *J. Biochem.* **96,** 593–603.
21. WILLIAMS, D. E., HALE, S. E., OKITA, R. T., AND MASTERS, B. S. S. (1984) *J. Biol. Chem.* **259,** 14600–14608.
22. HOLM, K. A., ENGELL, R. A., AND KUPFER, D. (1985) *Arch. Biochem. Biophys.* **237,** 477–489.
23. THEOHARIDES, A. D., AND KUPFER, D. (1981) *Drug Metab. Dispos.* **9,** 580–581.
24. QUINN, G. P., AXELROD, J., AND BRODIE, B. B. (1958) *Biochem. Pharmacol.* **1,** 152–159.
25. KATO, R. (1982) *in* Hepatic Cytochrome P-450 Monooxygenase System (Schenkman, J. B., and Kupfer, D., Eds.), pp. 99–149, Pergamon, Elmsford, NY.
26. WAXMAN, D. J. (1984) *J. Biol. Chem.* **259,** 15481–15490.
27. MACGEOCH, C., MORGAN, E. T., HALPERT, J., AND GUSTAFSSON, J.-A. (1984). *J. Biol. Chem.* **259,** 15433–15439.
28. MORGAN, E. T., MACGEOCH, C., AND GUSTAFSSON, J.-A. (1985) *Mol. Pharmacol.* **27,** 471–479.
29. KAMATAKI, T., MAEDA, K., YAMAZOE, Y., NAGAI, T., AND KATO, R. (1983) *Arch. Biochem. Biophys.* **275,** 758–770.
30. CHENG, K.-C., AND SCHENKMAN, J. B. (1982) *J. Biol. Chem.* **257,** 2378–2385.
31. WAXMAN, D. J., DANNAN, G. A., AND GUENGERICH, F. P. (1985) *Biochemistry* **24,** 4409–4417.
32. RYAN, D. E., IIDA, S., WOOD, A. W., THOMAS, P. E., LIEBER, C. S., AND LEVIN, W. (1984) *J. Biol. Chem.* **259,** 1239–1250.
33. BURSTEIN, S. H., AND KUPFER, D. (1971) *Ann. N.Y. Acad. Sci.* **191,** 61–67.
34. STAUFFER, C. E. (1975) *Anal. Biochem.* **69,** 646–648.
35. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
36. JANSSON, I., MOLE, J. E., AND SCHENKMAN, J. B. (1985) *J. Biol. Chem.* **260,** 7084–7093.
37. SCHENKMAN, J. B., FAVREAU, L. V., AND JANSSON, I. (1986) *in* Biol. Reactive Intermediates (Kocsis, J. J., Jollow, D. J., Witmer, C., Nelson, S., and Snyder, R., Eds.), Vol. 3, pp. 107–117, Plenum, New York.
38. FAVREAU, L. V., MALCHOFF, D. M., MOLE, J. E., AND SCHENKMAN, J. B. (1987) *J. Biol. Chem.* **262,** 14319–14326.
39. YASUKOCHI, Y., AND MASTERS, B. S. S. (1976) *J. Biol. Chem.* **251,** 5337–5344.
40. JANSSON, I., AND SCHENKMAN, J. B. (1975) *Mol. Pharmacol.* **11,** 450–461.
41. RYAN, D. E., DIXON, R., EVANS, R. H., RAMANATHAN, L., THOMAS, P. E., WOOD, A. W., AND LEVIN, W. (1984) *Arch. Biochem. Biophys.* **233,** 636–642.
42. HANUI, M., RYAN, D. E., IIDA, S., LIEBER, C. S., LEVIN, W., AND SHIVELY, J. E. (1984) *Arch. Biochem. Biophys.* **235,** 304–311.
43. SHIVERICK, K. T., AND NEIMS, A. H. (1979) *Drug Metab. Dispos.* **13,** 453–458.
44. CHENG, K.-C., AND SCHENKMAN, J. B. (1983) *J. Biol. Chem.* **258,** 11738–11744.
45. JANSSON, I., TAMBURINI, P. P., FAVREAU, L. V., AND SCHENKMAN, J. B. (1985) *Drug Metab. Dispos.* **13,** 453–458.
46. CHIANG, J. Y. L. (1981) *Arch. Biochem. Biophys.* **211,** 662–673.