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Oxidation of prostaglandin H_2 and prostaglandin H_2 analogues by human cytochromes P450: analysis of ω-side chain hydroxy metabolites and four steroisomers of 5-hydroxyprostaglandin I_1 by mass spectrometry

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Abstract

The objective was to examine the NADPH-dependent oxygenation of prostaglandin H_2 (PGH_2) and three PGH_2 analogues, 9,11-diazo-15-deoxy-PGH_2 (U51605), 9,11-epoxymethano-PGH_2 (U44069), and 11,9-epoxymethano-PGH_2 (U46619), by cytochromes P450, and to characterize the metabolites by mass spectrometry. CYP2C19, CYP4A11, CYP4F8, and liver and renal cortical microsomes oxidized the ω-side chain of U44069, U46619, and U51605, whereas only CYP4F8 oxidized the ω-side chain of PGH_2. PGH_2 was transformed to four stereoisomers of 5-hydroxy-PGI_1 by recombinant cytochromes P450. CYP4F8 formed the 5-hydroxy-PGI_1 isomers in small amounts compared to the 19-hydroxy metabolites of PGH_2. Isomers of 5-hydroxy-PGI_1 and 6-keto-$PGF_{1\alpha}$ were detectable when PGH_2 decomposed in the presence of hemin, hemoglobin, or heat-inactivated microsomes. 5-Hydroxy-PGI_1 is likely formed from PGH_2 in a pseudo-enzymatic reaction involving homolytic scission of the endoperoxide and formation of an ether between C-9 and C-6 and a carbon-centered radical at C-5, which reacts with molecular oxygen. CYP4F8 catalyzes 19-hydroxylation of PGH_2, but the absolute configuration of the 19-hydroxy group is unknown, whereas human seminal fluid contains (19*R*)-hydroxy-PGE_2. CYP4F8 was found to metabolize U51605 to 90% of the (19*R*)-hydroxy metabolite, providing further evidence in favor of a role of CYP4F8 in biosynthesis of (19*R*)-hydroxy PGE in human seminal vesicles. We conclude that ω-side chain hydroxylation of PGH_2 analogues may be catalyzed by many different cytochromes P450, but only CYP4F8 oxidizes the ω-side chain of PGH_2 efficiently.

Keywords: CYP2C19; CYP4A11; CYP4F8; 5-hydroxy-PGI_1; Mass spectrometry; PGH_2 analogues

1. Introduction

PGH synthases are heme-containing dioxygenases of polyunsaturated fatty acids [1,2]. They catalyze *bis*-oxygenation and cyclization of arachidonic acid into the hydroperoxy PG endoperoxide, PGG_2, and the peroxidase-catalyzed reduction of PGG_2 to PGH_2. PG endoperoxides are key intermediates in the biosynthesis of PGs, TXA_2, and PGI_2. PGs and TXA_2 activate prostanoid receptors, which are known to elicit important biological effects in inflammation, fever, and reproduction, as revealed by gene target disruption of prostanoid receptors in mice and by the pharmacological actions of cyclooxygenase inhibitors [3,4].

Biosynthesis of PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2 from PGH_2 is catalyzed by specific synthases, but PGH_2 may also decompose to all these products by non-enzymatic mechanisms [5]. PGH_2 is isomerized to PGE_2 by microsomal and soluble GSH-dependent PGE synthases [6–9], to $PGF_{2\alpha}$ by reducing agents and PGF synthases [10], and to PGD_2 by albumin and PGD synthases [11–13]. PGH_2 can also be metabolized by three specific cytochromes P450, i.e. CYP5A, CYP8A, and CYP4F8. CYP5A of platelets transforms PGH_2 to TXA_2 [14,15], whereas CYP8A of the vascular endothelium catalyzes biosynthesis of PGI_2 [15]. Both

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Abbreviations: CYP, cytochrome P450; HHT, (12*S*)-hydroxy-5*Z*,8*Z*,10*E*-heptadecatrienoic acid; MDA, malondialdehyde; MS/MS, tandem mass spectrometry; PG, prostaglandin; PGI_2, prostacyclin; and TX, thromboxane.

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cytochromes P450 catalyze homolytic scission of the endoperoxide group, and do not require NADPH for catalysis. CYP4F8 is present in human seminal vesicles, and both recombinant CYP4F8 and microsomes of human seminal vesicles can catalyze NADPH-dependent oxidation of PGH_2 to 19- and 18-hydroxy-PGH_2 [16,17].

PGH_2 is unstable and decomposes with a half-life of ~5 min in aqueous media to PGE_2 and to smaller amounts of PGD_2, $PGF_{2\alpha}$, HHT and MDA [5]. In buffer and organic solvents containing hemin, PGH_2 also decomposes to TXB_2 and 6-keto-$PGF_{1\alpha}$ [15]. Rat liver microsomes and a bacterial cytochrome P450, CYP101, metabolize PGH_2 to HHT and MDA both in the presence and absence of NADPH [15]. Human liver microsomes, CYP3A4, CYP2E1, and CYP1A2 have recently been reported to metabolize PGH_2 to HHT and MDA [18]. PGH synthases are located on the luminal side of the endoplasmic reticulum [19] and cytochromes P450 at the cytosolic side. The substrate channel of PGH synthases faces the lipid bilayer and PGH_2 may diffuse to cytochromes P450 and other enzymes through the endoplasmic reticulum.

PGH_2 exerts a wide spectrum of biological effects. PGH_2 contracts the rabbit aorta, increases airway resistance, and induces human platelet aggregation [20]. Stable analogues of PGH_2 have been developed. The endoperoxide group has been replaced with an epoxymethano group (U44069, U46619) or a diazo group, and these analogues mimic biological effects of PGH_2 [21–24]. Other analogues have been developed as enzyme inhibitors. The 9,11-diazo-15-deoxy analogue of PGH_2 (U51605) blocks the conversion of PGH_2 to TXA_2 and the effect of PGH_2 on platelet aggregation [25]. In contrast, little is known about the biological effects of 18- and 19-hydroxy-PGH_2, whereas 20-hydroxy-PGH_2 has been reported to contract rat aortic rings [26].

Recent observations suggest that (19*R*)-hydroxy-PGE compounds, which are present in large amounts in human seminal fluid [27,28], are formed by 19-hydroxylation of PGH_2 catalyzed by CYP4F8 of the seminal vesicles [17]. This hypothesis is based on the observation that PGE_2 is a poor substrate for CYP4F8 in comparison with PGH_2 and PGH_2 analogues. It remains to be determined whether CYP4F8 metabolizes PGH_2 and PGH_2 analogues to (19*R*)-hydroxy metabolites.

The first objective of the present study was to determine the structure of a series of novel metabolites of PGH_2, which were apparently formed by many different recombinant cytochromes P450. The second aim was to determine whether CYP4F8 catalyzes hydroxylation of the PGH_2 analogue U51605 at C-19 with *R* stereospecificity. The third object was to assess whether other recombinant cytochromes P450 than CYP4F8 can metabolize PGH_2 and its analogues in a NADPH-dependent manner, and determine the structure of the metabolites by mass spectrometry. We chose to investigate CYP4F8, CYP2C19, and CYP4A11, as these enzymes are prominent ω3-, ω2-, and ω1-hydroxylases of arachidonic acid, respectively [17,29,30]. Finally, we also assessed the NADPH-dependent metabolism of PGH_2 analogues by microsomes of human and rodent tissues.

2. Materials and methods

2.1. Materials

PGH_2 was prepared as described [17], stored in acetone at −80°, and brought to room temperature in a desiccator before use. 15(*S*)-Hydroxy-[9α,11α-epoxymethano]prosta-5,13(*Z*,*E*)-dienoic acid (U44069), 15(*S*)-hydroxy-[11,9-epoxymethano]prosta-5,13(*Z*,*E*)-dienoic acid (U46619), [9α,11α-diazo]prosta-(5*Z*,13*E*)-dienoic acid (U51605), the (5*R*,6*R*), (5*R*,6*S*), and (5*S*,6*R*) stereoisomers of 5-hydroxy-PGI_1 methyl ester and PGs were from the former Upjohn Co. and from Cayman Chemical Co. Unlabelled and deuterium-labelled (5*R*,6*R*)- and (5*S*,6*S*)-5-hydroxy-PGI_1 were obtained by chemical synthesis from $PGF_{2\alpha}$ and [3,3,4,4-$^2H_4$]$PGF_{2\alpha}$, respectively [31,32]. Methyl esters were hydrolyzed by 0.5 M KOH in 90% methanol (70°, 1 hr) and purified by RP–HPLC.

bis(Trimethylsilyl)trifluoroacetamide, 6-oxoheptanoic acid, and (2*S*)-phenylpropionic acid (97%), hemin (ferriprotoporphyrin chloride) were from the Sigma Aldrich Co. (2*S*)-Phenylpropionyl chloride was prepared as described [33]. Recombinant CYP4F8 was expressed in yeast as described [17]. Microsomes of insect cells with recombinant human CYP2C19 (with recombinant cytochrome b_5) and CYP4A11 (Supersomes, baculovirus expression of cytochromes P450 and cytochrome P450 reductase) and control microsomes without recombinant cytochromes P450 were obtained from Gentest Corp. Human kidney tissue was obtained from Uppsala University Hospital. Human liver microsomes were obtained from the liver bank of Huddinge University Hospital. Microsomes of rat tissues were prepared as described [17].

2.2. Experimental

Yeast microsomes (50 μg, ~2 pmol CYP4F8) and insect microsomes (10–25 pmol CYP2C19, and CYP4A11) were incubated with 1 mM NADPH and 10–100 μM substrate in a total volume of 0.1–0.25 mL 0.1 M $KHPO_4$/2 mM EDTA (pH 7.4) for 5–30 min at +4° or 37° for LC–MS analysis. For GC–MS analysis, we used up to 10 times larger incubations. Microsomes of human and rodent tissues were incubated as described [17]. Substrates were added in a small volume (<0.5%) of ethanol or acetone. The reactions were terminated by four volumes of ethanol or buffered $SnCl_2$ in ethanol in order to trap PGH_2 metabolites [5]. Precipitated proteins were removed by centrifugation. The

metabolites were extracted on a SepPak/C_{18} cartridge [34]. (5*S*,6*S*)- and (5*R*,6*R*)-[3,3,4,4-$^2H_4$]5-hydroxy-PGI_1 (0.1–0.5 μg) were added after termination as internal standards in some experiments.

2.3. Steric analysis of 19-hydroxy-U51605

6-Oxoheptanoic acid methyl ester was reduced with $NaBH_4$ to 6-hydroxyheptanoic acid methyl ester. Derivatization of methyl 6-hydroxyhepanoate (2 mg) with (2*S*)-phenylpropionyl chloride in toluene/pyridine afforded two diastereomers, which were separated by GC–MS. 19-Hydroxy-U51605 (9.2 μg) was obtained by biosynthesis (see below) and treated with 100 μL acetic anhydride/pyridine, 1/1, at room temperature overnight. The acetylated material was subjected to oxidative ozonolysis and the products were saponified by treatment with 0.5 M NaOH in methanol/water, 3/1, at 50° for 2 hr. After extraction with diethyl ether and evaporation of the solvent at 23° under vacuum, the residue was methyl-esterified and derivatized with (2*S*)-phenylpropionyl chloride. The phenylpropionyl derivative was isolated by TLC (solvent system ethyl acetate/hexane, 7/93) and analyzed by GC–MS.

2.4. LC–MS analysis

Equipment for LC–MS analysis was as described [29]. The columns contained octadecasilane silica (5 μm, 250 × 2 mm or 150 × 2 mm; Chromasil 5 C_{18} 100 A, Phenomenex) and eluted at 0.2 mL/min. The mobile phases were H_2O/CH_3CN/acetic acid, 65/35/0.01 or 68/32/0.01. Nanoelectrospray ionization was performed as described [35].

2.5. GC-MS analysis

Methyl esters and trimethylsilyl ethers were prepared as described [36]. A gas chromatograph (Varian 3100) with a non-polar capillary column (30 m; DB-5, J&W Scientific; film, 0.25 μm; diameter, 0.25 mm) was connected to an ion trap mass spectrometer (ITS40, Finnigan MAT). The gas chromatograph was programmed from 120 to 270° for 5 min, from 270 to 294° for 8 min, and then kept at 294°. C values (number of apparent carbons) were determined from the retention times of fatty acid methyl esters. Phenylpropionyl derivatives were analyzed on a Hewlett-Packard model 5970A mass selective detector connected to a model 5890 gas chromatograph equipped with a capillary column (SPB-1701, Supelco; length, 15 m, film thickness, 0.25 μm), which was programmed from 120 to 210° for 10 min.

2.6. Other analysis

Protein was determined by the Bradford assay [37].

3. Results

3.1. LC–MS analysis of 5-hydroxy-PGI_1

We first recorded the MS/MS spectra of authentic standards of four isomers of 5-hydroxy-PGI_1. The isomers of 5-hydroxy-PGI_1 eluted in the following order from the RP-HPLC column (H_2O/CH_3CN/acetic acid, 68/32/0.01): (5*S*,6*R*) − 8.5 min; (5*R*,6*R*) − 9.5 min; (5*S*,6*S*) − 12.5 min; (5*R*,6*S*) − 13.3 min. The MS/MS spectra of the four stereoisomers appeared to differ only in relative intensities. The MS/MS spectra (*m/z* 369 → full scan) showed signals at *m/z* 351 (369-18), 333 (351-18), 315 (369-44), 269 (369-100, likely loss of OCH-$(CH_2)_4$-CH_3), 251 (351-100), 233 (333-100), 145 (possibly OCH-CH(OH)-$(CH_2)_3$-COO^-), and 127 (145-18). The MS/MS spectrum of (5*R*,6*R*)-5-hydroxy-PGI_1 is shown in Fig. 1A. All these major fragments increased four mass units in the mass spectra of (5*S*,6*S*)- and (5*R*,6*R*)-[3,3,4,4-$^2H_4$]5-hydroxy-PGI_1. The MS/MS spectrum of 5-hydroxy-PGI_1 dissolved in $C^2H_3O^2H$/^2H_2O, 1/1, was recorded by nanoelectrospray and the mass shifts supported the fragmentation mechanism. It is noteworthy that these MS/MS spectra of 5-hydroxy-PGI_1 differ from the MS/MS spectrum (*m/z* 369 → full scan) of 6-keto-$PGF_{1\alpha}$. The latter shows strong signals at *m/z* 351 (369-18), 333 (351-18), 315 (333-18), 307 (351-44), 289 (307-18), 207, and 163 (Fig. 1B).

When PGH_2 was allowed to decompose in a solution of 0.5 mM bovine hemoglobin in buffer or in the presence of 1 mM hemin in organic solvents (CH_3CN, CH_2Cl_2), 6-keto-$PGF_{1\alpha}$ and the four stereoisomers of 5-hydroxy-PGI_1 were formed (Fig. 1C). 6-Keto-$PGF_{1\alpha}$ eluted just after (5*R*,6*R*)-5-hydroxy-PGI_1. A similar profile of products were noticed when PGH_2 decomposed in buffer with heat-inactivated yeast microsomes.

As previously reported, PGH_2 was metabolized by recombinant CYP4F8 by ω2- and ω3-hydroxylation in a ratio of 6:1 [17]. We now report that PGH_2 also was converted to significant amounts of four less polar metabolites as shown in Fig. 2. The formation was strictly NADPH-dependent, as confirmed by analysis with deuterated internal standards. The apparent carboxylate anions of these metabolites yielded signals at *m/z* 369. Their MS/MS spectra (*m/z* 369 → full scan) only differed in relative intensities, and their mass spectra and the retention times on RP-HPLC were identical with the four stereoisomers of 5-hydroxy-PGI_1. The 5-hydroxy-PGI_1 compounds were minor metabolites in comparison with the total 19-hydroxy-PG metabolites (Fig. 2), but were formed by all tested recombinant cytochromes P450. Recombinant CYP4F8 and 1 mM NADH transformed PGH_2 to about 25% of 5-hydroxy-PGI_1 isomers formed in the presence of 1 mM NADPH, and 6-keto-$PGF_{1\alpha}$ was the main product (cf. Fig. 1C). Control microsomes (without recombinant cytochromes P450) and 1 mM NADPH formed only trace amounts of the 5-hydroxy-PGI_1 stereoisomers.

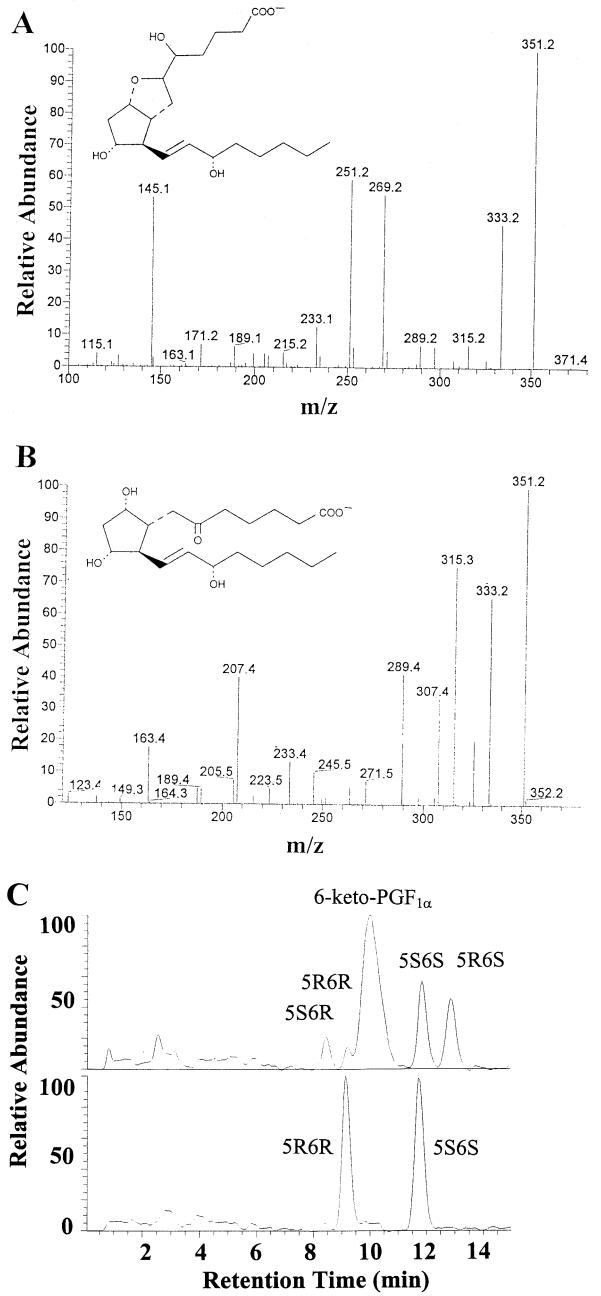

Fig. 1. LC–MS analysis of 5-hydroxy-PGI_1 and 6-keto-$PGF_{1\alpha}$. (A) MS/MS spectrum of (5*R*,6*R*)-5-hydroxy-PGI_1. The inset shows the structure of 5-hydroxy-PGI_1 as the carboxylate anion. (B) MS/MS spectrum of 6-keto-$PGF_{1\alpha}$ and the inset shows the carboxylate anion. (C) LC–MS analysis of 5-hydroxy-PGI_1 and 6-keto-$PGF_{1\alpha}$ formed from PGH_2 by 1 mM hemin in acetonitrile. (Top) The chromatogram shows selective ion monitoring of *m/z* 369 for analysis of 5-hydroxy-PGI_1. (Bottom) The chromatogram shows selective ion monitoring of *m/z* 373 for analysis of the internal standards, (5*R*,6*R*)- and (5*S*,6*S*)-[3,3,4,4-$^2H_4$]5-hydroxy-PGI_1, which elute slightly before the protium forms. All products were identified by MS/MS spectra (*m/z* 369 → full scan). Mobile phase: H_2O/CH_3CN/acetic acid, 68/32/0.01; C_{18} column 250 × 2 mm.

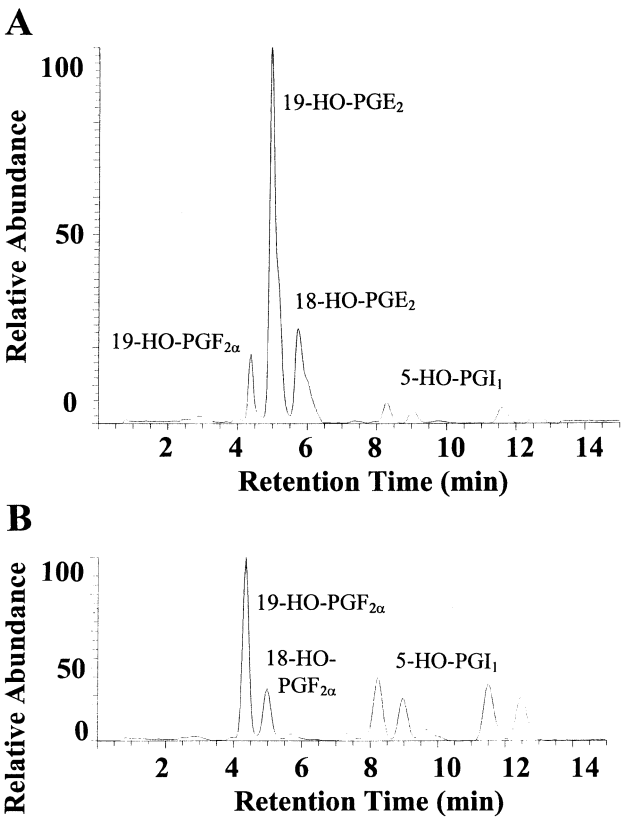

Fig. 2. LC–MS analysis of main metabolites formed from PGH_2 by CYP4F8. (A) Total ion chromatogram. The major components of the peaks are marked. 5-HO-PGI_1 refers to the four stereoisomers of 5-hydroxy-PGI_1 (cf. Fig. 1), which eluted between 8–14 min. (B) Selective ion monitoring (*m/z* 369) of the anions of 18-hydroxy- and 19-hydroxy-$PGF_{2\alpha}$ and the four stereoisomers of 5-hydroxy-PGI_1.

3.2. Steric analysis of CYP4F8-catalyzed 19-hydroxylation of U51605

Human seminal fluid contains (19*R*)-hydroxy-PGEs, which likely are formed by CYP4F8-catalyzed 19-hydroxylation of PGH_2 in the seminal vesicles [17,28]. This proposal would be strengthened if CYP4F8 mainly formed (19*R*)-hydroxy metabolites. To address this question, we chose the PGH_2 analogue U51605, which is suitable for steric analysis with established methods [33].

CYP4F8 was found by LC–MS to metabolize U51605 to two monohydroxy metabolites (marked I and II in Fig. 3). Metabolite I was identified as 19-hydroxy-U51605 by LC–MS and by ozonolysis, whereas metabolite II was identified by LC–MS as 18-hydroxy-U51605. The LC–MS analysis is described below.

The position and absolute configuration of the hydroxyl group of metabolite I was determined by ozonolysis of the 13–14 double bond. Ozonolysis and methylation yielded 6-hydroxyheptanoic acid methyl ester, which was converted to the 6-phenylpropionyl derivative. The phenylpropionyl derivative of authentic (6*R*,*S*)-hydroxyheptanoic acid methyl ester yielded two peaks on GC–MS, which eluted after 8.7 (*S*-stereoisomer) and 9.0 min (*R*-stereoisomer) [33], respectively (Fig. 3B). The mass spectra showed strong signals, *inter alia*, at *m/z* 105 (base peak) and *m/z* 132 (20% intensity). The correspond-

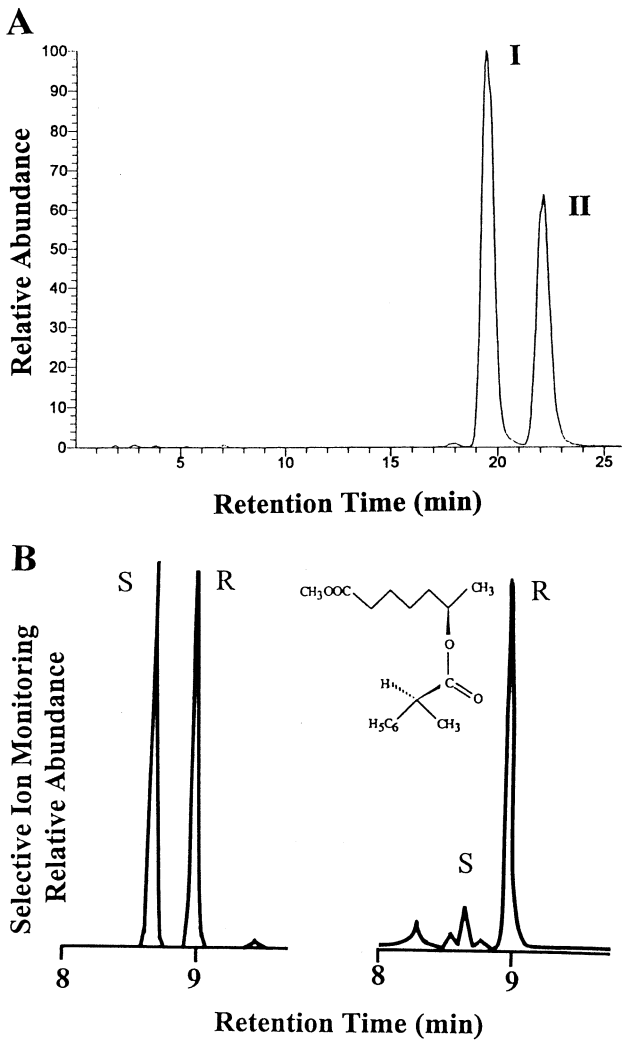

Fig. 3. LC–MS and steric analysis by GC–MS of 19-hydroxy-U51605 formed by CYP4F8. (A) LC–MS analysis of the metabolites. Peaks I and II contain the 19- and 18-hydroxy metabolites of U51605. These hydroxy metabolites were analyzed by selective ion monitoring (*m/z* 365) of the carboxylate anions and the ion intensity is shown. Mobile phase: H_2O/CH_3CN/acetic acid, 65/35/0.01. Column: C_{18} 150 × 2 mm. (B) Capillary GC–MS analysis by selective ion monitoring (*m/z* 105) of the *R* and *S* stereoisomers of the phenylpropionyl derivative of methyl 6-hydroxyheptanoate. (Left) Analysis of authentic (6*R*,*S*)-hydroxyheptanoic acid methyl ester. (Right) Analysis of 6-hydroxyheptanoic acid methyl ester obtained by ozonolysis of 19-hydroxy-U51605, which thus consisted of 90% of the 19*R* stereoisomer.

ing GC–MS analysis of 6-hydroxyheptanoic acid, which was derived from metabolite I by ozonolysis, showed that (6*R*)-hydroxyheptanoic acid was the main product (Fig. 3B). Quantification with selective ion monitoring of two fragments (*m/z* 105 and *m/z* 132) yielded similar results, 89–90% of the 6*R* and 10–11% of the 6*S* stereoisomers were obtained. We conclude that metabolite I consisted of ~90% (19*R*)-hydroxy- and ~10% (19*S*)-hydroxy-U51605.

3.3. LC– and GC–MS analysis of hydroxy metabolites of PGH_2 analogues

The epoxymethano analogues U44069 and U46619 have been used to study pharmacological effects of PGH_2 [21–23], but their metabolism has attracted little attention except for a single report on metabolism by TXA_2 synthase [38]. We therefore systematically examined the mass spectra of the ω1-, ω2-, and ω3-hydroxy metabolites of these analogues and U51605. The metabolites were generated by recombinant CYP4F8, CYP2C19, and CYP4A11. U51605 and its metabolites were too unstable for conventional GC–MS analysis.

3.3.1. LC-MS analysis

The hydroxyl group at C-20, C-19, or C-18 of the metabolites could be expected to give rise to α-cleavage with losses of 30 (OCH_2), 44 (OCH-CH_3), and 58 (OCH-CH_2-CH_3), respectively, from the carboxylate anion (A^-) or fragments thereof. The 18-hydroxy metabolites of all three analogues and the 19- and 20-hydroxy metabolites of U51605 could be identified in this way. U51605 and its metabolites readily lost N_2 during MS/MS analysis. 19- and 20-hydroxy-U51605 were identified by signals at *m/z* 245 (A^--28-30-44) and *m/z* 231 (A^--28-44-44), respectively. MS/MS spectra (*m/z* 365 → full scan) of the 20- and 19-hydroxy metabolites of U44069 and U46619 showed strong signals at *m/z* 335 (A^--30) due to loss of OCH_2 mainly from the epoxymethano group. Loss of 44 (CO_2) from the carboxylate group complicated the analysis of 19-hydroxy metabolites. The 20- and 19-hydroxy metabolites of the epoxymethano analogues were readily identified by GC–MS analysis (see below).

The MS/MS spectrum (*m/z* 365 → full scan) of 18-hydroxy-U44069 is shown in Fig. 4A. Strong signals were noted at *m/z* 347 (A^--18), 335 (A^--30), 317 (A^--30-18, base peak), 299 (317-18), 289 (weak, 347-58), 273 (317-44), 259 (317-58, loss of OCH-CH_2-CH_3), 249 (A^--116, loss of OCH-$(CH_2)_2$-CHOH-CH_2-CH_3), 219 (335-116), and 205 (249-44). The corresponding MS/MS spectra of 19- and 20-hydroxy-U44069 and 19- and 20-hydroxy-U46619 differed in relative intensities, and lacked the characteristic signal at *m/z* 259 of the 18-hydroxy metabolites.

The MS/MS spectra (*m/z* 347 → full scan) of 18-, 19-, and 20-hydroxy-U51605 showed an intense signal at *m/z* 319 (A^--28, loss of N_2; 100%) and other signals were weak (<3%), but most of them were also found in the MS/MS/MS spectra (*m/z* 347 → 319 → full scan) of these metabolites. Many fragments were formed by formal loss of N_2, H_2O, CO_2, and $N_2C_3H_4$. These were *m/z* 319 (A^--28), 301 (319-18), 279 (A^--68, loss of $N_2C_3H_4$), 275 (319-44, loss of CO_2). The MS/MS spectrum of 18-hydroxy-U51605 showed characteristic signals at *m/z* 261 (319-58) and *m/z* 217 (319-58-44) with a weak signal at *m/z* 275 (319-44), as shown in Fig. 4B. The MS/MS spectrum of 19-hydroxy-U51605 showed a characteristically strong signal at *m/z* 275

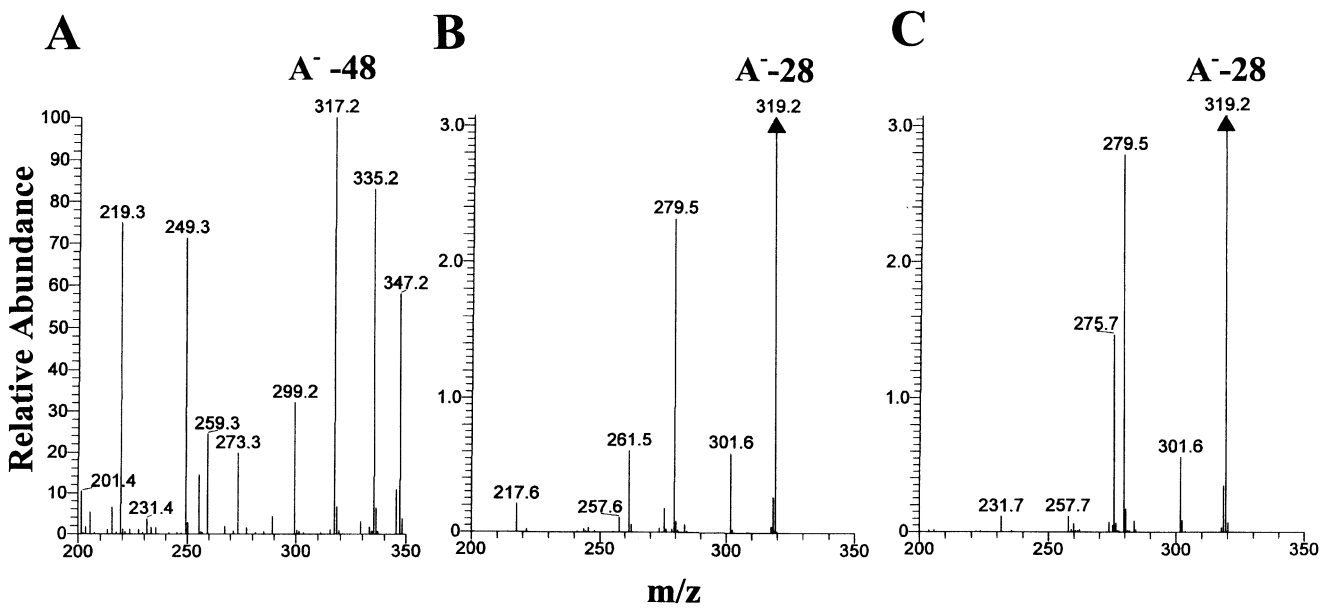

Fig. 4. MS/MS spectra in the upper mass range of 18-hydroxy-U44069, 18-hydroxy-U51605, and 19-hydroxy-U51605. (A) MS/MS spectrum of 18-hydroxy-U44069 (*m/z* 365 → full scan) with a structurally important signal at *m/z* 259 (A^--18-30-58). The base peak was *m/z* 317 (A^--18-30) (B) MS/MS spectrum of 18-hydroxy-U51605 (*m/z* 347 → full scan) with important signals due to α-cleavage at *m/z* 261 (A^--28-58) and *m/z* 217 (261-44). The base-peak was *m/z* 319 (A^--28, loss of N_2). (C) MS/MS spectrum of 19-hydroxy-U51605 (*m/z* 347 → full scan) with important signals at *m/z* 275 (A^--28-44) and 231 (275-44) due to α-cleavage. The base peak was *m/z* 319. See text for the fragmentation.

(319-44, presumably due to loss of CO_2 or OCH-CH_3) and an important signal at *m/z* 231 (319-44-44, loss of CO_2 and OCH-CH_3) as shown in Fig. 4C. Finally, 20-hydroxy-U51605 yielded a characteristic signal at *m/z* 245 (319-30-44, loss of OCH_2 and CO_2).

3.3.2. GC–MS analysis

The 20-, 19-, and 18-hydroxy metabolites of U44069 and U46619 were analyzed as trimethylsilyl ether methyl ester derivatives by electron impact ionization. The ω1-, ω2-, and ω3-hydroxy metabolites were identified by their C values and characteristic fragments formed by α-cleavage [39]. The mass spectra of the ω2- and ω3-hydroxy metabolites yield strong signals at *m/z* 117 and *m/z* 131, respectively, whereas the ω1-hydroxy metabolites have characteristic C values. The C values for 20-, 19-, and 18-hydroxy metabolites of U44069 were 26.0, 25.1, and 24.9, and for the same metabolites of U46619 the C values were 26.6, 25.6, and 25.4, respectively. The mass spectra of 19-hydroxy-U44069 and 18-hydroxy-U46619 are shown in Fig. 5. Important signals were noted at *m/z* 524 (M^+), 507 (MH^+-18), 434 (M^+-90), 417 (507-90), 365 (M^+-159, loss of $(CH_2)_3$-CH($O^+Si(CH_3)_3$)-CH_3), 345, 327, 275 (365-90), 225, 197, and 73 (base peak). The mass spectra of the two epoxymethano analogues differed in ion intensities, particularly in the upper mass range with relatively weak signals of U44069 and the hydroxy metabolites of U44069.

3.4. Metabolism of PGH_2 and PGH_2 analogues by CYP2C19 and CYP4A11

CYP2C19 metabolised PGH_2 to four stereoisomers of 5-hydroxy-PGI_1 and to 19- and 18-hydroxy-PGE_2, but the latter were likely formed by hydroxylation of PGE_2 rather than PGH_2 for two reasons. First, 18- and 19-hydroxy-PGH_2 could not be trapped and converted to 18- and 19-hydroxy-$PGF_{2\alpha}$ with $SnCl_2$ in spite of short time incubation with CYP2C19. Second, CYP2C19 converted PGE_2 to 18- and 19-hydroxy-PGE_2 (in a ratio of ~4:1).

CYP2C19 transformed U51605 to 19-hydroxy-U51605 (~85%) and to 18-hydroxy-U51605 (~15%) according to LC–MS. U44069 appeared to be oxygenated to almost equal amounts of 19/20-hydroxy-U44069 (55%) and 18-hydroxy-U44069 (45%).

CYP4A11 formed few polar metabolites of PGH_2 other than 5-hydroxy-PGI_1 stereoisomers (data not shown). On the other hand, CYP4A11 transformed U44069 to its 20-hydroxy metabolite, which was identified by GC–MS. LC–MS analysis showed that CYP4A11 oxidized U51605 to 20-hydroxy-U51605 (data not shown) as the main product.

3.5. Metabolism of PGH_2 analogues by microsomes of human and kidney cortex and liver and by microsomes of rat tissues

Microsomes of human kidney cortex (from two subjects) and liver (from one subject) and NADPH metabolized

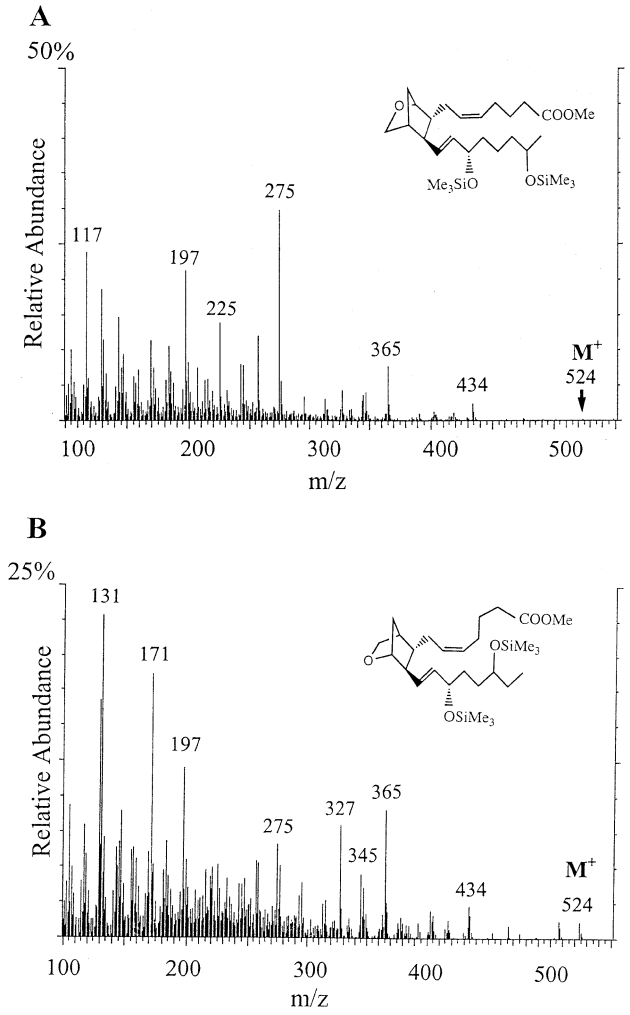

Fig. 5. GC–MS analysis of 19-hydroxy-U44069 and 18-hydroxy-U46619. (A) Mass spectrum of 19-hydroxy-U44069 with electron impact ionization. The molecular ion (at *m/z* 524) was weak but present. (B) Mass spectrum of 18-hydroxy-U46619 with electron impact ionization. Abbreviations: Me, methyl; Me_3Si, trimethylsilyl.

U51605 to one major metabolite with the same retention time and MS/MS spectrum as 20-hydroxy-U51605. These microsomes also oxygenated U44069. LC–MS analysis suggested that 19-hydroxy-U44069 was mainly formed by the renal microsomes and 20-hydroxy-U44069 by liver microsomes.

Microsomes of rodent tissues were initially prepared from a series of rat tissues (liver, kidney cortex, spleen, lung, prostate glands, seminal vesicles, testes) and incubated with U44069 or U46619 in the presence of NADPH. Significant biosynthesis of hydroxy metabolites of these analogues was only noted by microsomes of kidney cortex and liver. Renal cortical microsomes and NADPH metabolized U44069 to 19-hydroxy-U44069 as judged from LC– and GC–MS analysis. U46619 was oxygenated at a much lower rate, about 10%. U51605 was mainly metabolized to 20-hydroxy-U51605 according to LC–MS analysis. We also incubated PGH_2 with microsomes of rat kidney cortex and tried to trap PGH_2 metabolites as $PGF_{2\alpha}$ metabolites with buffered $SnCl_2$, but we could not consistently demonstrate a significant biosynthesis of hydroxy metabolites of PGH_2.

4. Discussion

Human seminal fluid contains large amounts of (19*R*)-hydroxy-PGE with 99% stereo purity [27,40]. CYP4F8 of human seminal vesicles was recently found to catalyze 19- and 18-hydroxylation of PGH_2 and PGH_2 analogues [17]. We now report that CYP4F8 forms 90% of the *R* stereoisomer of 19-hydroxy-U51605. This is consistent with the proposed physiological function of CYP4F8 as an important enzyme in biosynthesis of (19*R*)-hydroxy-PGE compounds of human seminal fluid.

We found that PGH_2 was converted to four stereoisomers of 5-hydroxy-PGI_1 by recombinant cytochromes P450 and NADPH. Two of the isomers, (5*S*,6*S*)- and (5*R*,6*R*)-5-hydroxy-PGI_1 have been described previously [32]. PGH synthase-1 metabolizes 5(6)-epoxyeicosatrienoic acid to 5(6)epoxy-PGs, which explains many biological actions of 5(6)-epoxyeicosatrienoic acid [41]. One of these PGs, 5(6)epoxy-$PGF_{1\alpha}$, forms (5*S*,6*S*)- and (5*R*,6*R*)-5-hydroxy-PGI_1 by intramolecular hydrolysis. The cytochrome P450 catalyzed formation of 5-hydroxy-PGI_1 was NADPH-dependent, and barely detectable when PGH_2 and NADPH were incubated with yeast microsomes without recombinant cytochrome P450. The mechanism of biosynthesis of 5-hydroxy-PGI_1 from PGH_2 by recombinant cytochromes P450 thus differs from the biosynthesis of HHT and MDA, which does not require NADPH [15,18].

The four isomers of 5-hydroxy-PGI_1 could also be formed by non-enzymatic mechanisms. For example, the isomers were detected when PGH_2 was allowed to decompose in organic solvents (CH_3CN; CH_2Cl_2) with 1 mM hemin and in heat-inactivated microsomes, but under these conditions 6-keto-$PGF_{1\alpha}$ was obtained in larger amounts than 5-hydroxy-PGI_1.

We suggest that 5-hydroxy-PGI_1 is formed by recombinant cytochromes P450 in a pseudo-enzymatic reaction, as the reaction was NADPH-dependent and catalyzed by all investigated cytochromes P450 and by many microsomal preparations. It is of interest to compare the pseudo-enzymatic biosynthesis of 5-hydroxy-PGI_1 with the enzymatic biosynthesis of PGI_2 by CYP8A [15]. The ferric iron of CYP8A catalyzes homolytic scission of the PG endoperoxide with formation of a hydroxyl group at C-11, an ether between C-9 and C-6, and a postulated PGI_1 compound with radical at C-5 (Fig. 6). In the next step, hydrogen is abstracted at C-6, a 5,6-double bond is introduced, and PGI_2 is formed. It seems likely that the intermediate with radical at C-5 may also be important in the formation of 5-hydroxy-PGI_1. The radical at C-5 may react with molecular oxygen and form 5-hydroperoxy-PGI_1. The latter can be reduced to 5-hydroxy-PGI_1 by peroxidases. CYP8A is NADPH-inde-

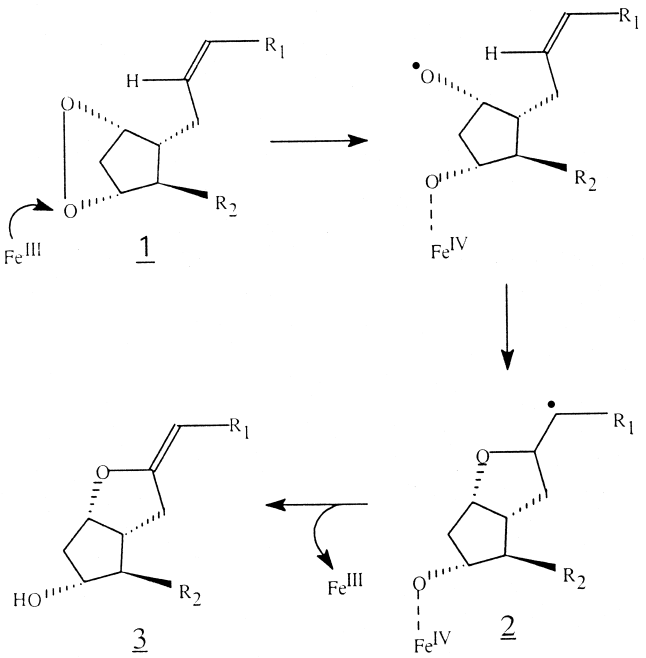

Fig. 6. Reaction mechanism for biosynthesis of PGI_2 from PGH_2 by CYP8A. The scheme is modified from Hecker and Ullrich [15]. It seems likely 5-hydroxy-PGI_1 is formed from PGH_2 by recombinant cytochromes P450 in the presence of NADPH cytochrome P450 reductase and NADPH in a process which also involves the same PGI_1 compound with a radical at C-5. This radical may react with molecular ion and form 5-hydro(per-o)xy-PGI_1 as described in the text. **1**, PGH_2; **2**, PGI_1 with a radical at C-5; **3**, PGI_2. R_1 denotes CH_2-CH_2-CH_2-COOH, and R_2 denotes HC=CH-CH(OH)-CH_2-CH_2-CH_2-CH_2-CH_3.

pendent [15], whereas formation of 5-hydroxy-PGI_1 is NADPH-dependent. NADPH-cytochrome P450 reductase reduces the ferric heme iron of cytochromes P450 to ferrous. Further work is needed to determine the redox states of heme iron in the biosynthesis of 5-hydroxy-PGI_1.

Electrospray ionization LC–MS with MS/MS is a powerful and convenient tool for metabolic and structural studies. It offers many advantages over GC–MS with electron impact ionization, for example, speed, no need for derivatization, sensitivity, and structurally informative MS/MS spectra. However, GC–MS is still the method of choice for conclusive analysis of ω-side chain hydroxy metabolites of prostanoids [39], as the interpretation is facilitated by a large number of published mass spectra of PGs and related compounds [42].

CYP2C19 and CYP4A11 catalyze ω-side chain hydroxylation of three PGH_2 analogues, i.e. U44069, U46619, and U51605. These enzymes did not form detectable amounts of ω1-, ω2-, or ω3-hydroxy metabolites of PGH_2. It seems likely that several biochemical criteria must be fulfilled for cytochromes P450 to metabolize PG endoperoxides. First, cytochromes P450 must have catalytic competence with high turnover numbers and low K_m values to cope with unstable metabolites like PGH_2, which often is formed in low concentration. Turnover numbers for most cytochromes P450 are between 1–50 mol product/min/mol cytochrome P450, whereas PGH synthases can form PGH_2 with turnover numbers up to 3200 [1]. Only very efficient enzymes can be expected to metabolize PGH_2. Second, PGH_2 biosynthesis and PGH_2 metabolism should be closely linked. It seems likely that PGH_2 is more stable in the lipophilic environment of the endoplasmic reticulum than in buffer, and may diffuse in the endoplasmic reticulum to cytochromes P450 and to other microsomal enzymes. In the seminal vesicles, PGH synthase-1 and CYP4F8 appears to be functionally linked, and CYP4F8 is catalytically competent to metabolize PGH_2. It remains to be determined whether cytochromes P450 and PGH synthases are functionally coupled in any other tissues.

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