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Regioselectivity of Hydroxylation of Prostaglandins by Liver Microsomes Supported by NADPH versus H_2O_2 in Methylcholanthrene-Treated and Control Rats: Formation of Novel Prostaglandin Metabolites

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The effects of methylcholanthrene (MC) treatment of male rats on the regioselectivity of hydroxylation of prostaglandins E_1 and E_2 (PGE_1 and PGE_2) by liver microsomes, supplemented with NADPH or H_2O_2, was examined. In the presence of NADPH, control microsomes catalyzed the hydroxylation at ω-1 (C_{19}) and at ω-(C_{20}) sites with minimal formation of novel monohydroxy metabolites of PGE_1 and PGE_2, referred to as compounds X_1 and X_2, respectively. Similarly, H_2O_2 supported the 19-hydroxylation and the formation of compounds X_1 and X_2, but yielded only minimal amounts of 20-hydroxy products. With NADPH, MC-treated microsomal incubations demonstrated only minor quantitative change in the 19- and 20-hydroxylation as compared with controls, but showed a 7- to 11-fold increase in formation of compound X_1 and a 10-fold increase in formation of X_2. By contrast with H_2O_2, MC-treatment increased by about 3-fold the 19- and 20-hydroxylation of PGE_1 and by 35- to 46-fold the formation of X_1; similarly, there was an approximate 2-fold increase in 19- and 20-hydroxylation of PGE_2 and a 10-fold increase in formation of X_2. These findings suggest that several monooxygenases are involved in catalyzing the hydroxylation at the various sites of the PGE molecule. Inhibitors of monooxygenases (SKF 525A, α-naphthoflavone, and imidazole derivatives) provided further evidence that the hydroxylation at the three sites of PGEs is catalyzed by different *P*-450 monooxygenases. It is striking that the inhibitors had a much lesser effect on the 20-hydroxylation of PGE_1 as compared with other sites of hydroxylation. Structural identification of compounds X_1 and X_2 was elucidated as follows. Resistance of the PGB derivative of X_1 to periodate oxidation and mass fragmentation analysis of the *t*-butyldimethylsilyl ether methyl ester, placed the hydroxylation at C_{17} or C_{18}. Finally, mass fragmentation of trimethylsilyl ether methyl ester PGB derivatives of X_1 and X_2 provided conclusive evidence that X_1 and X_2 are 18-hydroxy-PGE_1 and 18-hydroxy-PGE_2, respectively. The above findings indicate that the high regioselectivity of hydroxylation of PGE_1 and PGE_2, resulting in the formation of 18-hydroxy-PGE_1 and 18-hydroxy-PGE_2, respectively, is catalyzed by *P*-450 isozyme(s) which are induced by MC, possibly by *P*-450_c.

Prostaglandins (PGs)[2] are excreted in the urine of mammals primarily as dicarboxylic acid derivatives (1-5). Also, significant amounts of (ω-1)-hydroxy-PGs in

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[2] Abbreviations used: PGs, prostaglandins; PGE_1 and PGE_2, prostaglandin E_1 and E_2, respectively; PGB_1 and PGB_2, prostaglandins B_1 and B_2, respectively; GC, gas chromatography; MS, mass spectrometry; MC, methylcholanthrene; ANF, α-naphthoflavone; PI, 1-phenylimidazole; IPI, 1-phenyl-(2-isopropyl)-imidazole; SKF 525A, β-diethylaminoethyl-2,2-diphenylpentanoate; BuDMS, *t*-butyldimethylsilyl group; TMS, trimethylsilyl; BSA, bovine serum albumin.

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biological fluids have been observed (3, 4, 6-9). Evidence strongly suggests that the ω-carboxyl of PGs is generated via an initial ω-hydroxylation (10-12), followed by further oxidation (13). The ω- and (ω-1)-hydroxylations appear to be catalyzed by cytochrome *P*-450 monooxygenases, present in the endoplasmic reticulum (microsomes) in a variety of tissues (12, 14-17). Studies in several laboratories demonstrated that liver microsomes from rat, guinea pig, hamster, and rabbit catalyze the hydroxylation of PGs at the ω- and (ω-1)-positions (12, 15, 18-20). Based on studies with various inducers and inhibitors of hepatic monooxygenases, it was concluded that several microsomal monooxygenases catalyze the hydroxylation of PGs (19-21). Additionally, K_m determinations provided evidence that in rabbit liver, different monooxygenases are involved in catalysis of hydroxylation at the different sites of the PG molecule (20). Studies with reconstituted monooxygenases of purified *P*-450 isozymes from rabbit liver, demonstrating high regioselectivity of hydroxylation of PGs (22, 23, 35), provided support for the above conclusion.

It is generally accepted that NADPH is the native cofactor of cytochrome *P*-450 monooxygenases involved in activating molecular oxygen for incorporation into a given substrate. Nevertheless, it is possible that in certain instances, endogenous H_2O_2 or organic peroxides could serve as the source of oxygenating species without the need for participation of NADPH and 0_2. In fact, several *in vitro* studies have shown H_2O_2- and organic hydroperoxide-supported hydroxylation of various substrates by liver microsomes and by reconstituted systems with purified cytochrome *P*-450 isozymes (24-28).

The current study compares, in MC-treated and control rats, the regioselectivity of hydroxylation of PGE_1 and PGE_2 by rat liver microsomes in the presence of NADPH versus H_2O_2. Also, this investigation demonstrates that MC-treatment induces dramatically the formation of a novel monohydroxylated metabolites of PGE_1 and PGE_2, which exhibit lower polarity in HPLC when analyzed as the PGB derivatives than the corresponding (ω-1)- and ω-hydroxylated derivatives. In a previous study, using a different HPLC column ("fatty acid" column), this metabolite of PGE_1 cochromatographed with the corresponding 20-hydroxy derivative and thus it was erroneously assumed that MC and polyhalogenated hydrocarbons increased the formation of 20-hydroxy-PGE_1 (21); in fact, the current study shows that the increase in "20" hydroxylation was largely due to a marked increase in this novel metabolite, formed only in minute amounts in control animals. Also, the current findings provide structural identification of these PGE metabolites and suggest that an MC-inducible cytochrome *P*-450 [possibly cytochrome $P\text{-}450_c$ (38)] is responsible for the high regioselectivity of hydroxylation of PGs at this hitherto undescribed site.

MATERIALS AND METHODS

Compounds. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49), D-glucose 6-phosphate monosodium slat, NADP, NADPH, and lauric acid were purchased from Sigma Chemical Company (St. Louis, Mo.). Hydrogen peroxide (50%) was obtained from Fisher Scientific Company (Fairlawn, N. J.). [5,6-^{3}H]-PGE_1 (50 Ci/mmol) and [5,6,8,11,12,14,15-^{3}H]-PGE_2 (160 Ci/mmol) were purchased from Amersham (Arlington Heights, Ill.). Prostaglandins (unlabeled) were a gift from Upjohn Company (Kalamazoo, Mich.) and from Ono Pharmaceutical Company (Japan). α-Naphthoflavone was from Aldrich (Milwaukee, Wisc.). Methylcholanthrene was purchased from Eastman-Kodak (Rochester, N. Y.) Acetonitrile for HPLC, a Burdick and Jackson product, was obtained from American Laboratory Supply Company (Natick, Mass.). The silylation reagents *t*-butyldimethylsilyl chloride and trimethylsilyl imidazole (Tri-Sil Z) were obtained from Applied Science Laboratories (State College, Pa.) and Pierce Chemical Company (Rockford, Ill.). SKF 525A was a gift from Smith Kline and French Company (Philadelphia, Pa.). Phenylimidazole and 1-phenyl-(2-isopropyl)imidazole were gifts from Dr. C. F. Wilkinson (Cornell University, Ithaca, N. Y.).

Authentic prostaglandin derivatives. 19-OH-PGB_1 and 19-OH-PGB_2 were isolated from pooled human semen, as previously described by us (29), with the following modifications: The frozen semen is thawed and mixed with 10:1 ratio of acetone to semen, and the resulting precipitate is discarded. The supernate is evaporated to dryness, the residue is dissolved in

methanol followed by addition of 0.5 M KOH, and the mixture is left standing for 2 h (to convert the 19-OH-PGEs into corresponding PGBs). The solution is neutralized with HCl and finally acidified with citrate buffer to pH 3-4. The resulting mixture is extracted with ethyl acetate and the extract is evaporated to dryness under N_2. The residue is passed through a Sep-Pak C_{18} column (Waters Assoc.) and the eluted material is subjected to purification by HPLC, using a "fatty acid" column (Waters Assoc.) (29). The isolated 19-OH-PGB_1 and 19-OH-PGB_2 were used as such or derivatized as methyl esters and as *t*-butyldimethylsilyl products, followed by purification on HPLC as previously described (20).

Animals and hepatic microsomes. Sprague-Dawley albino male rats weighing 110-120 g were obtained from Charles River Breeding Labs (Wilmington, Mass.). Rats were treated daily for 3 days with MC (25 mg/kg) in corn oil; controls received an equivalent dose of corn oil. Animals were decapitated 48 h after the last dosing and liver microsomes were prepared as previously described (30). The microsomal pellet was resuspended in 1.15% aqueous KCl solution and centrifuged again for 1 h at 105,000*g*. The microsomal pellet was covered with 1.15% KCl solution and stored frozen at −70°C. Prior to use, the microsomes were thawed and resuspended in fresh KCl solution. Protein determinations were made by modification (31) of the Lowry procedure (32), using BSA as standard.

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 and the ethanol was evaporated to dryness at room temperature under a stream of nitrogen. The incubation contained potassium phosphate (50 mM, pH 7.4), $MgCl_2$ (10 mM), and EDTA·4 Na (1 mM). The microsomal suspension was added (for protein concentration see tables) and the vials were preincubated for 2 min at 37°C. Reactions were initiated with NADPH (1.22 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 (19, 20); linearity of product formation for at least 30 min was previously established. NADPH and the regenerating system were omitted when the reaction was initiated with H_2O_2 (usually 50 mM),[3] and incubations were carried out for 10 min at 37°C; although a preliminary study with H_2O_2 demonstrated completion of reaction within 5 min, for convenience we utilized 10-min incubation. The reactions were terminated by addition of 10 ml ethanol and the subsequent workup was as previously described (20). It should be noted that, in the course of the above workup, PGEs and their respective metabolites were converted by base to the corresponding PGB derivatives to facilitate their detection by uv.

High performance liquid chromatography and quantitation of metabolites. A small volume of methanol is added to the dried extracts containing the PGB derivatives. To determine recoveries, an aliquot is taken for radioactive determination in a scintillation spectrometer and another aliquot, typically 5-10% of the extract, is subjected to HPLC (for conditions and typical chromatogram, see Fig. 1). Quantitation of hydroxylated metabolites is achieved from areas under the corresponding HPLC peaks (using standard curves) and corrected for recoveries (20). Loss of radioactivity in conversion of PGE_2 to PGB_2 was corrected for in calculating recoveries. The remainder of the extract is kept dry under argon at −20°C to be utilized for eventual characterization of products.

Characterization of metabolites. The extracts from several incubations are combined and subjected to HPLC. The relevant uv-absorbing (280 nm) compounds corresponding in retention time to PGB derivatives of authentic 19-OH-PGE_1, 20-OH-PGE_1, and the less polar compound X_1 (subscript 1 indicates originating from PGE_1; subscript 2 is from PGE_2) are collected. The isolated fractions are evaporated at room temperature under a stream of N_2 and stored under argon. For eventual characterization, the individual compounds are subjected again to HPLC and care is taken to collect only the major areas under the relevant peaks. The fractions are again evaporated to dryness. The three proucts are converted to methyl esters, using diazomethane, and are chromatographed on HPLC, and the collected fractions are evaporated to dryness under a stream of N_2. A portion of the resulting methyl esters are derivatized into either trimethylsilyl or *t*-butyldimethylsilyl ethers, as previously described (20). The isolation and determination of purity of the *t*-butyldimethylsilyl ether-derivatized products was by HPLC, using similarly derivatized, authentic 19-OH-PGB_1 and 20-OH-PGB_1 as standards.

GC/MS. Mass fragmentation of the various *t*-butyldimethylsilyl(BuDMS)- and trimethylsilyl (TMS)-PGB derivatives was carried out essentially as previously described (20).

RESULTS AND DISCUSSION

Extracts from incubations of PGE_1 with liver microsomes from MC-treated rats, in the presence of NADPH versus H_2O_2, were analyzed as PGB derivatives by HPLC. Using a C_8 reverse-phase column, the formation of a novel metabolite (re-

[3] The concentration of the stock solution of H_2O_2 was routinely determined from its extinction coefficient of 43.6 M^{-1} cm^{-1} at 240 nm (33).

ferred to as compound X_1), less polar than the other products (19-OH-PGE_1 and 20-OH-PGE_1), was observed (Figs. 1A and B). However, chromatography of the above incubation extract on a "fatty acid" column, used by us in previous studies (21), did not permit the separation of 20-OH-PGB_1 from base-treated compound X_1 (Figs. 1C and D).

To characterize compound X_1, the BuDMS ether methyl ester of the corresponding PGB derivative was prepared. As expected from the behavior of X_1, the BuDMS-X_1 methyl ester also exhibited a longer retention time on HPLC than the corresponding BuDMS derivative of 19-hydroxy-PGB_1 methyl ester (not shown).

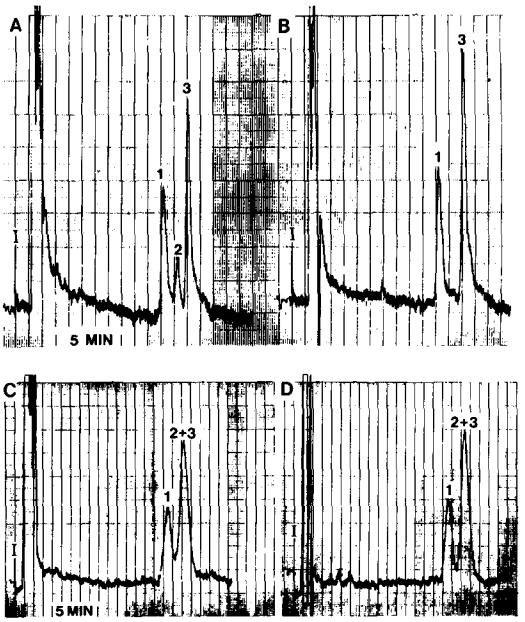


FIG. 1. HPLC profile of an extract from an incubation of PGE_1 with liver microsomes from MC-treated rats. (A) Incubation in the presence of NADPH; used a C_8 (5 μm) Whatman column. Conditions: Mobile phase, 25% CH_3CN:75% H_2O [0.01% acetic acid (HAc)]; run at 2 ml/min; monitored at 280 nm (0.005 AUFS). (B) Incubation in the presence of H_2O_2 (50 mM); used a C_8 (5 μm) column; conditions as in (A). (C) Same as (A), but used a "fatty acid" Waters Associates column and the mobile phase was 20% CH_3CN/80% H_2O containing 0.01% HAc. (D) Same as (B), but used a "fatty acid" column and conditions of (C). Notations: I, injection point; peaks 1, 19-OH-B_1; peak 2, 20-OH-B_1, peak 3, PGB derivative of compound X_1.

Each of the three BuDMS ether methyl ester PGB_1 derivatives yielded on GC/MS a molecular ion at $m/z = 594$ and a fragment at M^+ minus (t-butyl) = 537. This finding indicated that, like the 19-OH-PGB_1 and 20-OH-PGB_1 derivatives, compound X_1 is also a monohydroxy-PGB_1 derivative (Figs. 2A, B, and C, and Table IA). Based on a mass fragmentation ($m/z = 393$), the hydroxylation of PGE_1 was placed at C_{16}, C_{17}, or C_{18}. Hydroxylation at C_{16} (vicinal hydroxylation) was excluded because exposure of base-treated X_1 to sodium periodate in aqueous solution did not alter absorbance at 278 nm or chromatographic behavior on HPLC. Thus it was concluded that X_1 represented hydroxylation at C_{17} or C_{18} (Fig. 3).[4] Based on the finding that MC treatment of rats stimulates the (ω-2)-hydroxylation of hexane by liver microsomes (36), we speculated that X_1 was derived from (ω-2)-hydroxylation of PGE_1 and hence represents hydroxylation at C_{18}. It was previously observed that mass fragmentation of the TMS ether derivative of 19-OH-PGB_1-methyl ester allowed assignment of hydroxyl at C_{19} (10, 20). Hence, to attempt to assign the site of hydroxylation in compound X_1, the methyl ester of the base-treated X_1 was derivatized with trimethylsilylchloride and subjected to GC/MS analysis. The GC/MS fragmentation of this derivative identified compound X_1 as 18-hydroxy-PGE_1 (Table IB).

Similar observations were made with base-treated extracts from incubations of PGE_2 except that the novel product, referred to as compound X_2, is more polar on HPLC (shorter retention) than similarly prepared X_1 but, like X_1, is also less polar than the corresponding 19- and 20-hydroxylated PGB_2 derivatives (Fig. 4). Compound X_2 was identified by GC/MS to be 18-hydroxy-PGE_2 (Table IC).

The inclusion of sodium azide (1 mM),

[4] The possibility that compounds X_1 and X_2 are the 19(S) enantiomers of 19(R)-19-OH-PGE_1 and 19(R)-19-OH-PGE_2, respectively, was excluded. We observed that 19(S)-19-OH-PGB_2 and 19(R)-19-OH-PGB_2 do not separate on HPLC under the conditions separating 19(R)-19-OH-PGB_2 and base-treated X_2.

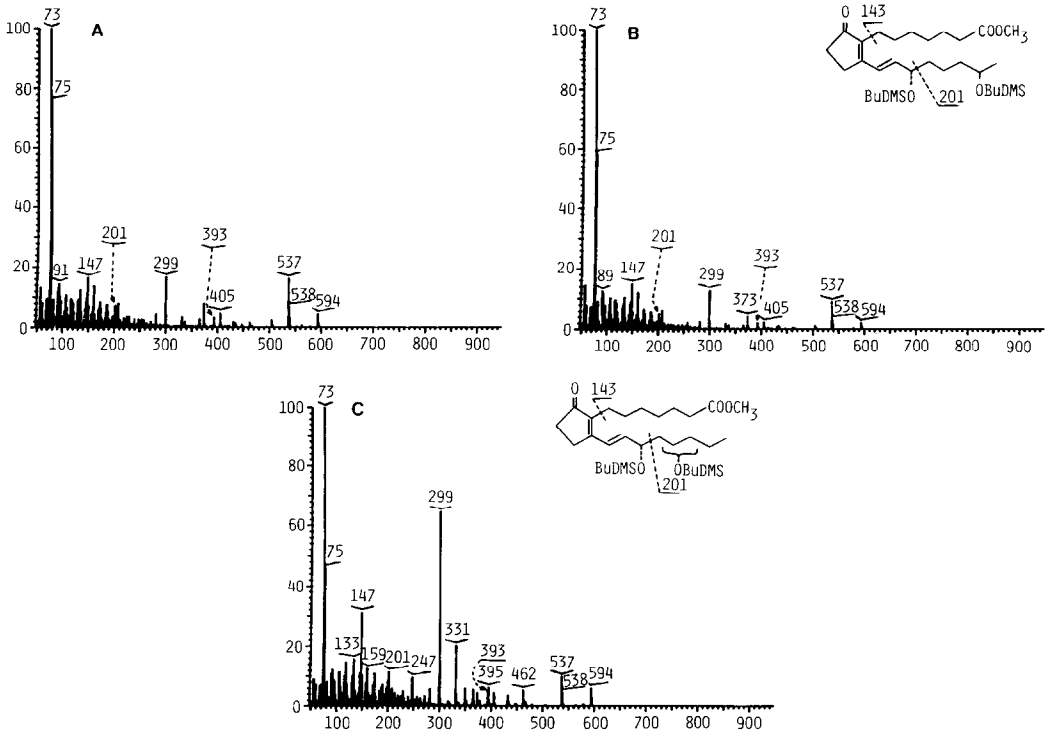


FIG. 2. GC/MS fragmentation of BuDMS ether methyl esters of metabolites of PGE_1 from incubation with MC-treated liver microsomes. The products in the form of PGB_1 derivatives were purified by HPLC and derivatized as described under Experimental Procedures. (A) 19-OH-PGB_1 isolated from semen and derivatized as BuDMS ether and methyl ester; used as representative authentic sample. (B) HPLC-metabolite 1 (Figs. 1A and B), corresponding in retention time to authentic 19-OH-PGB_1, was further purified by HPLC and derivatized as BuDMS ether methyl ester. (C) Metabolite 3 (Figs. 1A and B), PGB form of compound X_1, was isolated by HPLC and derivatized as BuDMS ether methyl ester.

to inhibit microsomal catalase activity in incubations supported by H_2O_2 (50 mM), had no effect on PGE_1 hydroxylation. In contrast, the addition of catalase (50 units) completely inhibited product formation in H_2O_2-supported, but had no effect in NADPH-supported, reactions (not shown). These findings demonstrate that endogenous catalase has little or no effect on the H_2O_2-supported prostaglandin hydroxylations and that the NADPH-supported hydroxylation does not involve endogenously generated H_2O_2. Since azide slightly inhibited 19-hydroxylation by the NADPH-supported system, azide was omitted from all subsequent incubations to maintain uniformity between H_2O_2- and NADPH-supported reactions. Additionally, it was observed that there was no product formation by H_2O_2 when microsomes were either omitted or exposed to ca. 90°C for 10 min prior to addition to the incubations.

The optimal concentration of H_2O_2 for PGE_1 metabolism was determined in control and MC-treated rats (Fig. 5). It appears that 50–100 mM H_2O_2 was optimal. Table II demonstrates that incubations of PGE_1 (0.1 mM) with hepatic microsomes, from control rats, and NADPH yielded primarily 19-OH-PGE_1, negligible amounts of 18-OH-PGE_1, and small amounts of the 20-OH-product; however, with nearly saturating concentrations of PGE_1 (0.5 mM), there was an approximate threefold increase in formation of all the metabolites. These results (0.1 vs 0.5 mM PGE_1) and previous observations (21) indicate

TABLE IA

ION ASSIGNMENT AND INTENSITIES IN THE MASS SPECTRA OF BuDMS METHYL ESTER PGB_1 DERIVATIVES GENERATED FROM PGE_1 METABOLITES AND AUTHENTIC 19-OH-PGE_1

Ion assignment (authentic 19-OH-B_1-BuDMS methyl ester) (m/z)	
594	M^+
537	M^+-[*t*-butyl]
393	M^+-[201]
299	M^+-[2(BuDMSOH) + $CH_3O^\cdot$]
201	$[CH_3CHOBuDMSCH_2CH_2CH_2]$
143	$[(CH_2)_6COOCH_3]$

	m/z (intensities in %)[b]						
Intensities[a]	594	537	393	299	201	143	73
19-OH-PGB_1 (authentic)	5	16	3	17	7	6	100
19-OH-PGB_1 (metabolite)	2	9	2	13	5	5	100
X_1 (base-treated)	6	10	5	65	12	5	100

[a] PGs as PGB_1-*t*-butyldimethylsilyl ether methyl ester derivatives.
[b] Obtained from a computer printout of the expanded scale of Figs. 2A-C.

that in the rat, the hepatic monooxygenases exhibit a relatively low affinity for PGE_1, as compared with the rabbit where a striking difference between the high affinity of the ω- vs the low affinity of (ω-1)-hydroxylases was evident (20). With H_2O_2 as the oxidant, a similar pattern of effect on the rate of hydroxylation with 0.5 vs 0.1 mM concentration of PGE_1 was observed (Table II), suggesting that H_2O_2 is most probably utilized by the same *P*-450 monooxygenases which also use

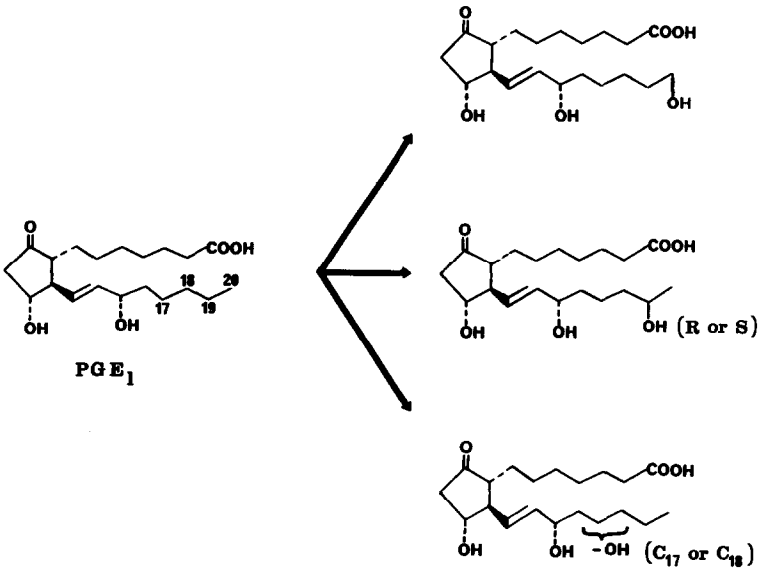
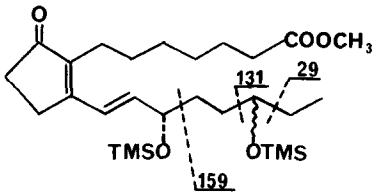


FIG. 3. Structures of hydroxylated metabolites of PGE_1.

TABLE IB

ION ASSIGNMENT AND INTENSITIES IN THE MASS SPECTRUM OF THE TMS ETHER METHYL ESTER PGB DERIVATIVE OF COMPOUND X_1 (18-OH-PGE_1)



Ion assignment (m/z)			Intensities (as %)[a]
510		M^+	80
420	M^+-90 =	M^+-[TMSOH]	28
391	M^+-[90 + 29] =	M^+-[TMSOH + CH_3CH_2]	25
364	M^+-[131 + 15] =	M^+-[$CH_3CH_2CHOTMS$ + CH_3]	48
351	M^+-159 =	M^+-[$CH_3CH_2CHOTMSCH_2CH_2$]	50
323	M^+-187	Unidentified	100
131		[$CH_3CH_2CHOTMS$]	45

[a] Estimated from the computer-generated mass fragmentation pattern.

NADPH and O_2. However, one contrasting observation is the relatively low ω-hydroxylation in incubations of PGE_1 containing H_2O_2 (vs NADPH). A similar finding was made when lauric acid hydroxylation by untreated rat liver microsomes was supported with cumene hydroperoxide vs NADPH (25); whereas NADPH supported both ω- and (ω-1)hydroxylation, cumene hydroperoxide yielded solely (ω-1)-hydroxylation. Whether the low ω-hydroxylating activity in H_2O_2-supported reactions is due to inactivation of the ω-hydroxylating enzyme by H_2O_2 or whether that enzyme does not utilize effectively H_2O_2 is not known. Tables IIIA and B describe the effect of MC on the regioselectivity of hydroxylation of PGE_1 and PGE_2 (0.5 mM). In the presence of NADPH, there was little or no effect by MC treatment on the (ω-1)- and ω-hydroxylation; however, the formation of the (ω-2)-hydroxy products was markedly elevated by MC. By constrast, when H_2O_2 was used instead of NADPH, MC treated exhibited a moderate increase in (ω-1)- and ω-hydroxylation (see 19-OH and 20-OH), but there was a dramatic increase in formation of the (ω-2)-hydroxy products (see 18-OH).

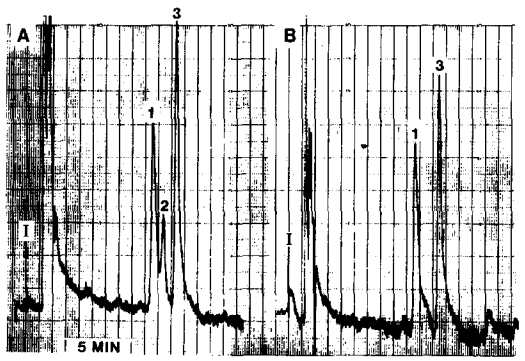
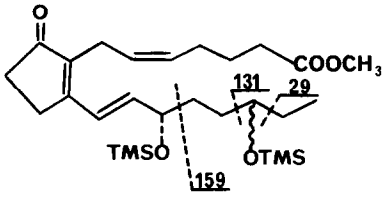


FIG. 4. HPLC profile of an extract from incubation of PGE_2 with liver microsomes from MC-treated rats. Analysis with a C_8 (5 μm) column. Conditions as in Fig. 1A. (A) Incubation in the presence of NADPH. (B) Incubation in the presence of H_2O_2 (50 mM). Notations: I, injection point; peaks 1, 19-OH-B_2; peak 2, 20-OH-B_2; peak 3, PGB derivative of compound X_2.

Incidentally, a preliminary study on the effects of treatment with PCBs (Aroclor 1254), in female rats, on PGE_1 hydroxylation by liver microsomes also demonstrated an increase in the formation of compound X_1, presumably 18-OH-PGE_1,

TABLE IC

ION ASSIGNMENT AND INTENSITIES IN THE MASS SPECTRUM OF THE TMS ETHER METHYL ESTER PGB DERIVATIVE OF COMPOUND X_2 (18-OH-PGE_2)



Ion assignment (m/z)			Intensities (as %)[a]
508		M^+	45
418	M^+-90 =	M^+-[TMSOH]	14
389	M^+-[90 + 29] =	M^+-[TMSOH + CH_3CH_2]	20
362	M^+-[131 + 15] =	M^+-[$CH_3CH_2CHOTMS$ + CH_3]	18
349	M^+-159 =	M^+-[$CH_3CH_2CHOTMSCH_2CH_2$]	18
321	M^+-187	Unidentified	100
131		[$CH_3CH_2CHOTMS$]	62

[a] Estimated from the computer-generated mass fragmentation pattern.

with little or no effect on the ω-hydroxylation (A. S. Perry and D. Kupfer, unpublished). These findings suggest that, in the previous study (21), PCBs elevated the formation of the novel metabolite, rather than that of the ω-hydroxy product. More recently we observed that the *P*-450 isozyme 6, from rabbit liver, catalyzes the formation of PGE_1 and PGE_2 metabolites with chromatographic characteristics suggesting 18-hydroxylation (35). To establish whether these metabolites are, in fact, 18-hydroxy derivatives will require rigorous structure characterization.

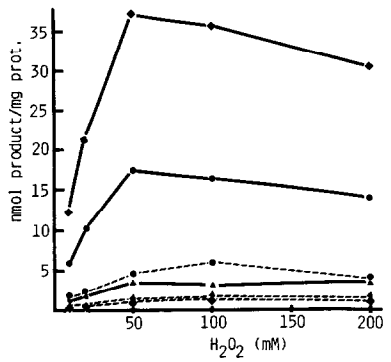


FIG. 5. The effect of varying H_2O_2 concentrations on PGE_1 hydroxylation by MC and control rat liver microsomes. —, MC treated; ---, Control; ♦, 18-OH; ●, 19-OH; ▲, 20-OH.

To study the characteristics of the hepatic monooxygenase(s) from MC-treated rats and to further examine the evidence that several enzymes are involved in the hydroxylation of PGEs, a variety of inhibitors were utilized (Table IV). Both the NADPH- and the H_2O_2-supported 18- and 19-hydroxylations were inhibited by monooxygenase inhibitors, albeit the 20-hydroxylation was much less inhibited. There were significant differences in potency of inhibition and in preferential sites of inhibition among the various compounds examined. Whereas SKF 525A inhibited to similar extent the 18- and 19-hydroxylation, ANF suppressed 18-hydroxylation much more effectively than that of the 19-hydroxy product. Similarly, phenylimidazole (PI) and isopropylphenylimidazole (IPI) inhibited 18-hydroxylation most effectively; IPI being the more potent inhibitor, at 5 μM inhibiting the 18-hydroxylation by 90%. Lauric acid, at a twofold higher concentration than PGE_1, inhibited PGE_1 hydroxylation at the three sites, suggesting that laurate is an alter-

TABLE II

REGIOSELECTIVITY OF HYDROXYLATION OF PGE_1 BY HEPATIC MICROSOMES FROM CONTROL AND MC-TREATED RATS IN THE PRESENCE OF NADPH OR H_2O_2

PGE_1 (mM)	Microsomes	Cofactor	PGE_1 hydroxylation (nmol/mg protein)[a] 19-OH	20-OH	18-OH
0.1	Control	NADPH	1.3	0.6	0.3
0.5	Control	NADPH	3.9 (3.0)[b]	1.9 (3.2)	0.9 (3.0)
0.1	Control	H_2O_2	0.6	0.1	0.2
0.5	Control	H_2O_2	2.2 (3.7)	0.4 (4.0)	0.5 (2.5)
0.1	MC	NADPH	1.5	0.5	3.0
0.5	MC	NADPH	3.4 (2.3)	1.4 (2.8)	6.6 (2.2)
0.1	MC	H_2O_2	2.0	0.3	4.5
0.5	MC	H_2O_2	7.4 (3.7)	1.0 (3.3)	17.6 (3.9)

Note. Each incubation contained microsomes from control (2.3 mg protein; 2.0 nmol *P*-450/mg protein) or from MC-treated (2.0 mg protein; 3.2 nmol [*P*-450 + 448]/mg protein) rats. The reaction was started with NADPH + NADPH-regenerating system or with H_2O_2 (50 mM), and was conducted for 30 and 10 min, respectively, as described under Materials and Methods.

[a] Each value represents a mean of duplicate incubations, using liver microsomes pooled from five control or five MC-treated rats.

[b] Numbers in parentheses represent ratio of values of PGE_1 hydroxylation at 0.5 to 0.1 mM PGE_1.

TABLE IIIA

THE EFFECT OF METHYLCHOLANTHRENE TREATMENT OF RATS ON THE REGIOSELECTIVITY OF HYDROXYLATION OF PGE_1 BY HEPATIC MICROSOMES IN THE PRESENCE OF NADPH VS H_2O_2

Treatment	Cofactor	PGE_1 hydroxylation (nmol/mg protein)[a] 19-OH (T/C)[b]	20-OH (T/C)	18-OH (T/C)
Experiment 1[c]				
—	NADPH	3.9	1.9	0.9
MC	NADPH	3.4 (0.9)	1.4 (0.7)	6.6 (7.3)
—	H_2O_2	2.2	0.4	0.5
MC	H_2O_2	7.4 (3.4)	1.0 (2.5)	17.5 (35.0)
Experiment 2[c]				
—	NADPH	4.1	2.0	0.7
MC	NADPH	4.5 (1.1)	1.8 (0.9)	8.1 (11.6)
—	H_2O_2	4.5	1.1	0.8
MC	H_2O_2	17.3 (3.8)	3.3 (3.0)	37.0 (46.3)

[a] Each value represents a mean of duplicate incubations, using microsomes pooled from five rats.

[b] T/C: In parentheses are ratios of values of MC-treated to controls.

[c] Incubations of control and MC-treated microsomes contained 2.3 and 2.0 mg protein, respectively (Experiment 1); Control and MC, 0.8 mg (Experiment 2). Incubations were conducted with 0.5 mM PGE_1. Reactions were started with NADPH + NADPH-regenerating system or with H_2O_2 (50 mM). Conditions were as in Table II and as under Materials and Methods.

TABLE IIIB

THE EFFECT OF METHYLCHOLANTHRENE TREATMENT OF RATS ON THE REGIOSELECTIVITY OF HYDROXYLATION OF PGE_2 BY HEPATIC MICROSOMES IN THE PRESENCE OF NADPH VS H_2O_2

		PGE_2 hydroxylation (nmol/mg protein)[a]		
Treatment	Cofactor	19-OH (T/C)[b]	20-OH (T/C)	18-OH (T/C)
Experiment 1[c]				
—	NADPH	5.9	2.1	1.6
MC	NADPH	6.8 (1.1)	2.5 (1.2)	16.6 (10.4)
—	H_2O_2	8.0	2.1	1.8
MC	H_2O_2	13.3 (1.7)	4.5 (2.1)	21.2 (11.8)
Experiment 2[c]				
— (4)[d]	NADPH	2.6 ± 0.1	0.9 ± 0.0	0.8 ± 0.1
MC (4)	NADPH	4.0 ± 0.2 (1.5)*	1.2 ± 0.1 (1.3)**	9.8 ± 0.4 (12.3)*
— (4)	H_2O_2	4.7 ± 0.4	1.0 ± 0.1	1.1 ± 0.1
MC (4)	H_2O_2	6.7 ± 0.6 (1.4)**	1.0 ± 0.3 (1.0)	11.4 ± 0.6 (10.4)*

[a] Experiment 1, each value represents a mean of duplicate incubations, using microsomes pooled from five rats; Experiment 2, values represent means ± SE of duplicate incubations of microsomes from individual rats.

[b] T/C: In parentheses are ratios of values of MC-treated to controls.

[c] Incubations of control and MC-treated microsomes contained 0.8 mg protein (Experiment 1); and (0.8-1.3 mg) in control and (1.1-1.3 mg) in MC (Experiment 2). Experiment 1 of this table was conducted simultaneously with Experiment 2 of Table IIIa.

[d] In parentheses, number of animals. Conditions were as in Table IIIa, except that PGE_2 (0.5 mM) was used.

* $P \leq 0.001$.

** $P \leq 0.05$.

TABLE IV

EFFECT OF INHIBITORS OF MONOOXYGENASES ON THE REGIOSELECTIVITY OF PGE_1 HYDROXYLATION SUPPORTED BY NADPH VS H_2O_2 IN LIVER MICROSOMES FROM MC-TREATED RATS

		Hydroxylation of PGE_1 (nmol/mg protein)[a]		
Inhibitor (μM)		19-OH	20-OH	18-OH
Experiment 1[a]				
—	NADPH	1.6	0.6	2.9
SKF 525A (200)	NADPH	0.7 (44)[d]	0.5 (83)	1.0 (34)
PI (10)	NADPH	0.6 (38)	0.3 (50)	0.7 (24)
Experiment 1				
—	NADPH	1.9	0.8	3.6
IPI (10)	NADPH	0.2 (11)	0.6 (75)	0.2 (6)
Experiment 2[b]				
—	NADPH	1.5	0.7	2.7
ANF (5)	NADPH	0.6 (40)	0.5 (71)	0.2 (7)
Experiment 3[b]				
—	NADPH	1.2	0.5	2.0
Laurate (200)	NADPH	0.6 (50)	0.3 (60)	1.2 (60)

TABLE IV—*Continued*

Inhibitor (μM)		Hydroxylation of PGE_1 (nmol/mg protein)[a]		
		19-OH	20-OH	18-OH
Experiment 4				
—	H_2O_2	4.1	0.7	8.5
SKF 525A (100)	H_2O_2	1.4 (34)	ND[c]	3.0 (35)
PI (5)	H_2O_2	1.9 (46)	ND	3.4 (40)
PI (10)	H_2O_2	1.3 (32)	ND	1.5 (18)
IPI (5)	H_2O_2	0.7 (17)	ND	0.9 (11)
IPI (10)	H_2O_2	0.6 (15)	ND	0.6 (7)
Experiment 5[b]				
—	H_2O_2	3.5	0.6	7.2
ANF (5)	H_2O_2	0.8 (23)	ND	0.2 (3)
Experiment 6[b]				
—	H_2O_2	2.9	0.5	6.7
Laurate (200)	H_2O_2	1.7 (59)	ND	4.0 (60)

[a] Incubations contained 0.8–1.0 mg microsomal protein/ml and PGE_1 (0.1 mM). When NADPH was used, incubations were for 30 min; with H_2O_2 (50 mM) they were for 10 min. Values among duplicates varied between 7 and 15%.

[b] Experiments 2, 3, 5, and 6: The inhibitors were added in 5 μl ethanol, the control contained 5 μl ethanol. In other experiments, inhibitors were added in H_2O.

[c] Not determined.

[d] In parentheses, percentage of corresponding control.

nate substrate for the same monooxygenases. These findings raise a question whether, in previous studies of hydroxylation of straight-chain fatty acids, such as laurate, products other than those hydroxylated at the ω and (ω-1) carbons could have been formed, but were merely not detected by the methods utilized.

Cytochromes *P*-450_c and *P*-450_d appear to be the prime MC-inducible isozymes (37, 38); however, *P*-450_c is induced to a much larger extent than *P*-450_d (38). This tempts a speculation that in MC-treated microsomes, *P*-450_c is responsible for catalyzing the 18-hydroxylation of prostaglandins. Also, from findings with NADPH-supported reactions, it appears that the 19- and 20-hydroxylations are not significantly mediated by the MC-induced *P*-450 isozymes. However, with H_2O_2 as the oxidant, both the 19- and 20-hydroxylations were elevated about threefold by MC treatment (Table IIIA). Since *P*-450_d is induced to a lesser extent than *P*-450_c (38), it is likely that 19- and 20-hydroxylations are catalyzed by *P*-450_d. It is possible that in NADPH-supported reactions, the limiting amounts of NADPH-cytochrome *P*-450 reductase did not permit the full expression of catalytic activity of the various *P*-450 isozymes. Additionally, the difference in regioselectivity of hydroxylations of PGEs with NADPH versus H_2O_2 was not entirely unexpected, since different oxidants were previously found to exhibit wide variations in regioselectivity of hydroxylation of steroids and warfarin (26, 34). Studies with isolated *P*-450 isozymes from MC-treated rats or with monospecific antibodies to *P*-450_c and *P*-450_d will help resolve these questions.

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Note added in proof. Since submission of the manuscript, we became aware that *P*-450_e from *Long-Evans* rats exhibits only 70% homology in the first 10 amino acids to the major *P*-450 from MC-treated *Sprague-Dawley* rats (Cheng *et al.* (1984) *Biochem. Biophys. Res. Comm.* **123:** 1201; Kuwara *et al.* (1984) *J. Biochem.* **95:** 703; Sakaki *et al.* (1984) *J. Biochem.* **96:** 117). Thus, we would like to revise our speculation to propose that the (ω-2)-hydroxylation of PGs is catalyzed by the major MC-inducible *P*-450 in *Sprague-Dawley* rats. However, since this *P*-450 exhibits similar characteristics to *P*-450_e, it is likely that the (ω-2)-hydroxylation will occur also in *Long-Evans* rats.

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