Isolation and chemical conversion of two novel prostaglandin endoperoxides: 5(6)-epoxy-PGG_1 and 5(6)-epoxy-PGH_1

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5(6)-Epoxy-PGG_1 and 5(6)-epoxy-PGH_1 were isolated after incubation of microsomes of RSV with ^{3}H-labelled 5(6)-epoxy-C20:3 for 45 s at 37°C. The endoperoxides were methylated and characterized by conversion to prostaglandins. In buffer, the endoperoxides were converted to methyl-5(6)-epoxy-PGE_1 and methyl-5(6)-epoxy-15-hydroperoxy-PGE_1, while treatment with $SnCl_2$ reduced the endoperoxides to methyl-5-hydroxy-$PGI_{1\alpha}$ and methyl-5-hydroxy-$PGI_{1\beta}$. Significant amounts of methyl-5(6)-epoxy-HHD were also formed. The endoperoxides could be separated by silicic acid chromatography and when 1 mM phenol was present in the incubation, 5(6)-epoxy-PGH_1 was obtained as the main product.

Arachidonic acid *Prostaglandin* *Cytochrome P-450* *Metabolism* *HPLC* *GC–MS*

1. INTRODUCTION

The isolation of the prostaglandin endoperoxides PGG_2 and PGH_2 was reported in [1–3]. The prostaglandin endoperoxides were soon realized to be of considerable biological interest [4]. They are key intermediates in the biosynthesis of prostaglandins, thromboxanes and prostacyclin and exert biological effects, which are different from many of their metabolites [1–4]. Consequently, many stable analogs of prostaglandin endoperoxides have been developed [5].

5(6)-Epoxy-C20:3 is one of the 4 epoxides formed from arachidonic acid by cytochrome *P*-450 in the liver and renal cortex [6–8]. This epoxide was recently found to be metabolized to prostaglandins by fatty acid cyclooxygenase [9]. The main products were identified as 5(6)-epoxy-PGE_1, 5-hydroxy-$PGI_{1\alpha}$ and 5-hydroxy-$PGI_{1\beta}$ [9,10], which all were assumed to be derived from the unstable 5(6)-epoxyprostaglandin endoperoxides, 5(6)-epoxy-PGG_1 and 5(6)-epoxy-PGH_1. The isolation of these endoperoxides as methyl esters is described here.

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Abbreviations: PG, prostaglandin; RSV, ram seminal vesicles; Me_3Si, trimethylsilyl; 5(6)-epoxy-C20:3, *cis*-5(6)-epoxy-8,11,14-eicosatrienoic acid; 5(6)-epoxy-HHD, *cis*-5(6)-epoxy-*trans*-8,11-heptadecadienoic acid; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; UV, ultraviolet

2. MATERIALS AND METHODS

2.1. *Materials*

Arachidonic acid (99%) and sodium *p*-hydroxymercuribenzoate were purchased from Sigma. [^{3}H]Arachidonic acid (100 Ci/mmol) and [^{14}C]arachidonic acid (56 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, England. Methoxyamine HCl and *N,O*-bis(trimethylsilyl)trifluoroacetamide were obtained from Pierce. Liquefied phenol was from Fisher. Silicic acid (Silicar CC-4) was from Mallinckrodt. Other chemicals were from Merck. HPLC was performed as in [9,10]. Radioactivity

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was determined with liquid scintillation [9]. RSV were obtained from the Department of Physiological Chemistry, Karolinska Institutet. ^{3}H-labelled 5(6)-epoxy-C20:3 (21 and 0.1 mCi/mmol) and ^{14}C-labelled 5(6)-epoxy-C20:3 (56 mCi/mmol) were synthesized as in [9,11]. Methyl-5-hydroxy-$PGI_{1\alpha}$, methyl-5-hydroxy-$PGI_{1\beta}$, methyl-5(6)-epoxy-PGB_1 and methyl-5,6-dihydroxy-PGB_1 were synthesized as in [9,12].

2.2. *Experimental*

Microsomes of RSV were resuspended in 0.1 M KH_2PO_4/K_2HPO_4 buffer (pH 8.0) with 1 mM sodium *p*-hydroxymercuribenzoate and 1 mM EDTA. In some experiments 1 mM phenol was added. Incubations were performed for 45 s in a water bath at 37°C by addition of the microsomal suspension to an equal volume of buffer, which was kept at 37°C and contained the substrate. ^{3}H-labelled 5(6)-epoxy-C20:3 ($15–20 \times 10^6$ dpm) was incubated with microsomes from 0.5 g RSV in a total volume of 4 ml, while 1.5–2 mg epoxide (added in 30 μl ethanol) were incubated with microsomes from 6 g RSV in 44 ml. The two incubations were combined, acidified to pH 4 (0.05 M HCl) and immediately extracted twice with cold diethyl ether. The ether extracts were kept on ice and dried over $MgSO_4$. After evaporation, the residue was dissolved in methanol (0.5 ml) and an ether solution of diazomethane (−20°C) was immediately added in excess. After 1 min the solvents were evaporated to dryness and the residue dissolved in dry acetone.

2.3. *Chromatography*

The endoperoxides were dissolved in 30% diethyl ether in hexane and applied to an open column of silicic acid (1 g), which was eluted with 80 ml. The eluent was then changed to 50% (60 ml) and to 80% diethyl ether (60 ml) in hexane. Fractions of 5 ml were collected. The column was finally eluted with ethyl acetate. HPLC was performed as described in table 1.

2.4. *Chemical conversion of endoperoxides*

Reduction with $SnCl_2$ in methanol (5 mg/ml) was performed as in [3]. The endoperoxides were also allowed to decay in 0.1 M K_2HPO_4 buffer (pH 7.4) with 1 mM EDTA for 30 min at 37°C [2,3].

2.5. *Analyses*

The GC–MS analyses were performed on a quadrupole mass spectrometer (Finnigan 4000) equipped with a data acquisition system (Incos). An open capillary column of fused silica (20 m SE-54 CB) was operated isothermally at 270 or 280°C. Splitless injections were performed with a 'falling needle' device [13]. The MS conditions were: electron energy, 70 eV; emission current, 0.2 A; temperature of ion source, 300°C. *C* values were estimated from the retention times of saturated fatty acid methyl esters (C20–C26). The UV analysis was performed with Zeiss Spektralphotometer PM 6 using ethanol as solvent.

Table 1

Elution volumes of prostaglandins on reversed-phase HPLC

Compound	Elution volume (ml) (55:45:0.2)	Compound	Elution volume (ml) (65:35:0.2)
5-HO-$PGI_{1\alpha}$	126	5-HO-$PGI_{1\alpha}$	35
5-HO-$PGI_{1\beta}$	98	5-HO-$PGI_{1\beta}$	30
5(6)-Epoxy-15-hydroperoxy-PGE_1	94	5(6)-Epoxy-HHD	72
5(6)-Epoxy-PGE_1	78	5(6)-Epoxy-PGB_1	44
5,6-$(HO)_2$-PGB_1	81		

All compounds were chromatographed as methyl ester derivatives on 10 μm octadecasilane silica (7.8 × 300 mm, Nucleosil). The eluent was methanol–water–acetic acid as indicated (v/v). Flow rate was 1.5–2 ml/min and fractions were collected every minute

2.6. *Derivatization*

Synthesis of Me_3Si and Me_3Si-*O*-methoxime derivatives was performed as in [6,9,10]. Chlorohydrin adducts of epoxides were obtained as in [10].

3. RESULTS

3.1. *Conversion of 5(6)-epoxyprostaglandin endoperoxides to known prostaglandins*

Following incubation of ^{3}H-labelled 5(6)-epoxy-C20:3 with microsomes of RSV and extractive isolation, 35–50% of radioactivity was recovered. The methylated crude extract from one experiment was divided into two parts. One part was reduced with $SnCl_2$ and the polar products were separated by reversed-phase HPLC as shown in fig.1A. Two major peaks of radioactivity appeared, which had the same elution volume as authentic methyl-5-hydroxy-$PGI_{1\alpha}$ and methyl-5-hydroxy-$PGI_{1\beta}$ (table 1). The products could be conclusively identified as these compounds by GC–MS and by differences in *C* values (Me_3Si derivative [9]). The other part was allowed to decompose in buffer and the polar products were separated by reversed-phase HPLC (fig.1B). The most polar peak of radioactivity (peak I) contained methyl-5(6)-epoxy-PGE_1, which was identified by GC–MS of the chlorohydrin adduct (Me_3Si-*O*-methoxime derivative; [10]) and by conversion to methyl-5,6-dihydroxy-PGB_1 by treatment with 0.1 M KOH (UV absorbance maximum at 278 nm; mass spectrum of the Me_3Si derivative [10]). Treatment of the material in peakII (fig.1B) with base also resulted in formation of a compound with maximal UV absorbance at 278 nm. Following reduction with GSH [2] and methylation, a mass spectrum of methyl-5,6-dihydroxy-PGB_1 was obtained. Finally, reduction of the material in peak II with $SnCl_2$ converted it to a major product, which had the same elution volume as methyl-5(6)-epoxy-PGE_1 on reversed-phase HPLC (fig.1C) and the product was identified as this compound by GC–MS as described above. The material in peak II was thus identified as methyl-5(6)-epoxy-15-hydroperoxy-PGE_1 (cf. [3]).

3.2. *Separation of methyl-5(6)-epoxy-PGG_1 and methyl-5(6)-epoxy-PGH_1 on silicic acid*

Addition of ^{14}C-labelled 5(6)-epoxy-C20:3 at the time of the extractive isolation showed that most of the substrate was eluted from the silicic acid column with the first 1–2 fractions of 30% diethyl ether in hexane. On changing the eluent to 50% ether, one major peak of radioactivity eluted and another peak eluted with 80% ether in hexane. The material in both peaks was converted to methyl-5-hydroxy-$PGI_{1\alpha}$ and methyl-5-hydroxy-$PGI_{1\beta}$ by reduction with $SnCl_2$. In buffer the first eluting compound decomposed mainly to methyl-5(6)-epoxy-15-hydroperoxy-PGE_1 as judged from reversed-phase HPLC, while the more polar compound was converted to methyl-5(6)-epoxy-PGE_1 (cf. fig.3).

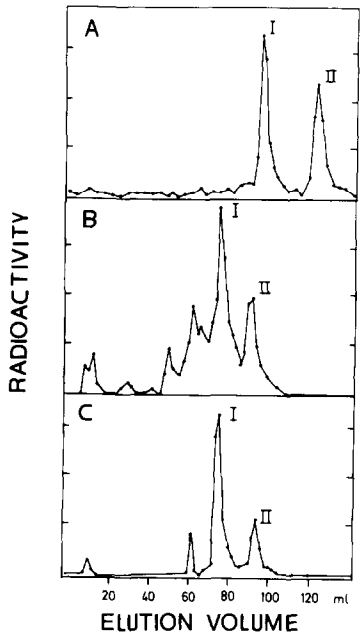

Fig.1. Reversed-phase HPLC of polar products derived from methyl-5(6)-epoxy-PGG_1 and methyl-5(6)-epoxy-PGH_1. (A) Endoperoxides were reduced by treatment with $SnCl_2$ in methanol. Peak I, methyl-5-hydroxy-$PGI_{1\beta}$; peak II, methyl-5-hydroxy-$PGI_{1\alpha}$. (B) Endoperoxides were allowed to decompose in buffer. Peak I, methyl-5(6)-epoxy-PGE_1; peak II, methyl-5(6)-epoxy-15-hydroperoxy-PGE_1. (C) Part of the material in peak II of the chromatogram in B was reduced with $SnCl_2$ and rechromatographed as shown. The main product (peak I) was identified as methyl-5(6)-epoxy-PGE_1. Column, 10 μm octadecasilane silica (7.8 × 300 mm); eluent, methanol–water–acetic acid (55:45:0.2). Flow rate 2 ml/min.

3.3. *Identification of methyl-5(6)-epoxy-HHD*

In buffer or by reduction with $SnCl_2$, the endoperoxides were also converted to a product, which was less polar than the prostaglandins on reversed-phase HPLC (table 1). This compound was identified as methyl-5(6)-epoxy-HHD as follows: the UV analysis showed an absorbance maximum at 231 nm indicating the presence of two conjugated *trans* double bonds [13]. A mass spectrum is shown in fig.2A (Me_3Si derivative). Strong signals were noted at *m/z* 382 (M^+), 364 ($M^+ - 18$, loss of water), 311 ($M^+ - 71$), 293 ($M^+ - 99$, possibly loss of water from *m/z* 311), 292 ($M^+ - 90$), 281 (cf. inset, fig.2A), 263 (possibly 381 – 18), 251, 225, 199, 181, 167, 143 (inset), 129 and 111. The base peak was *m/z* 73 and the *C* value was 21.1. The GC–MS analysis of the chlorohydrin adducts (Me_3Si derivative) showed that two major products were formed. The mass spectrum of the 5-chloro-6-hydroxy adduct (*C* value 22.7) is shown in fig.2B. Signals were noted at *m/z* 490 (M^+), 475 ($M^+ - 15$), 459 ($M^+ - 31$), 419 ($M^+ - 71$), 383, 365 ($M^+ - 125$, loss of Me_3SiOH and Cl), 341 (cf. inset), 292, 251 (cf. inset), 219 (251 – 32, loss of methanol), 191, 161, 143, 119, 105 and 73 (base peak). The 5-hydroxy-6-chloro adduct (*C* value 22.9) also showed signals at *m/z* 490, 475, 459, 419, 383 and 365. Other strong signals were noted at *m/z* 353, 242, 225, 203 (cleavage between C5 and C6), 191, 173, 171, 143, 129 and 73 (base peak). The UV and GC–MS analyses were consistent with the proposed structure.

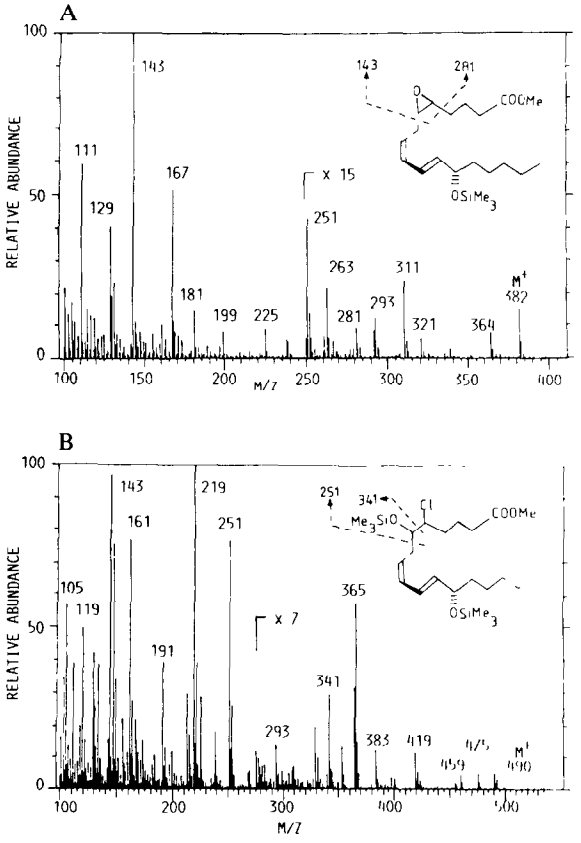

Fig.2. (A) Mass spectrum of methyl-5(6)-epoxy-HHD (Me_3Si derivative). (B) Mass spectrum of one of the chlorohydrin adducts of methyl-5(6)-epoxy-HHD, methyl-5-chloro-6-hydroxy-*trans*-8,11-heptadecadienoate (Me_3Si derivative). The natural abundance of ^{35}Cl (77%) and ^{37}Cl (23%) gives a characteristic appearance of fragments containing chlorine. The insets show important fragments of the mass spectra, which were normalized to the most abundant signal above *m/z* 100.

4. DISCUSSION

Recent reports on the potent effects of 5(6)-epoxy-C20:3 on the release of hormones from isolated pancreatic islets, the median eminence and rat pituitary cells [14–16] stimulated interest in the

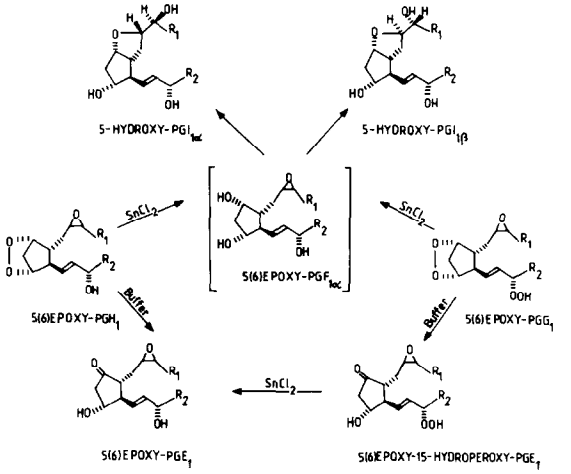

Fig.3. Summary of the chemical conversion of 5(6)-epoxy-PGG_1 and 5(6)-epoxy-PGH_1 to prostaglandins. The compound within brackets, 5(6)-epoxy-$PGF_{1\alpha}$, has not been isolated, but 5-hydroxy-$PGI_{1\alpha}$ and 5-hydroxy-$PGI_{1\beta}$ are likely to be formed from this intermediate by intramolecular hydrolysis of the 5(6)-epoxide by the hydroxyl at C9 (9). $R_1 = (CH_2)_3COOCH_3$; $R_2 = (CH_2)_4CH_3$.

possible metabolism of this epoxide to biologically active products. This report shows that 5(6)-epoxy-C20:3 is metabolized by fatty acid cyclooxygenase to two prostaglandin endoperoxides, 5(6)-epoxy-PGG_1 and 5(6)-epoxy-PGH_1. The endoperoxides were characterized by their chemical conversion into known products as summarized in fig.3. The endoperoxides were also partly transformed into 5(6)-epoxy-HHD.

Phenol stimulates the peroxidase activity of the prostaglandin synthesizing enzymes [17]. In the presence of this cofactor 5(6)-epoxy-PGH_1 was obtained as the main product. This is in agreement with [18] and addition of phenol may be advantageous since PGG compounds are less stable than PGH compounds. PGG compounds can thus be non-enzymatically transformed into the corresponding 15-keto derivatives [18].

It will be of interest to determine whether 5(6)-epoxyprostaglandin endoperoxides are formed in vivo and to determine their biological effect.

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