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Short communication

Oxygenation of 5,8,11-eicosatrienoic acid by prostaglandin H synthase-2 of ovine placental cotyledons: isolation of 13-hydroxy-5,8,11-eicosatrienoic and 11-hydroxy-5,8,12-eicosatrienoic acids

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Abstract

Prostaglandin H synthase-1 of ram vesicular glands metabolises 5,8,11-eicosatrienoic (Mead) acid to 13*R*-hydroxy-5,8,11-eicosatrienoic and to 11*R*-hydroxy-5,8,12-eicosatrienoic in a 5:1 ratio. We wanted to determine the metabolism of this fatty acid by prostaglandin H synthase-2. Western blot showed that microsomes of sheep and rabbit placental cotyledons contained prostaglandin H synthase-2, while prostaglandin H synthase-1 could not be detected. Microsomes of sheep cotyledons metabolised [1-^{14}C]5,8,11-eicosatrienoic acid to many polar metabolites and diclofenac (0.05 m*M*) inhibited the biosynthesis. The two major metabolites were identified as 13-hydroxy-5,8,11-eicosatrienoic and 11-hydroxy-5,8,12-eicosatrienoic acids. They were formed in a ratio of 3:2, which was not changed by aspirin (2 m*M*). 5,8,11-Eicosatrienoic acid is likely oxygenated by removal of the pro-*S* hydrogen at C-13 and insertion of molecular oxygen at either C-13 or C-11, which is followed by reduction of the peroxy derivatives to 13-hydroxy-5,8,11-eicosatrienoic and 11-hydroxy-5,8,12-eicosatrienoic acids, respectively. Prostaglandin H synthase-1 and -2 oxygenate 5,8,11-eicosatrienoic acid only slowly compared with arachidonic acid.

Keywords: 5,8,11-Eicosatrienoic acid; Prostaglandin H synthase; Hydroxyeicosatrienoic acid

1. Introduction

Prostaglandin H synthase-1 (PGHS-1) is abundant in ram vesicular glands, which is the classical source of this enzyme [1]. A second form of the enzyme, PGHS-2, was discovered more recently [2–6]. PGHS-2 is often referred to as the "inducible" form of PGHS, since it was detected as an immediate-early gene product in stimulated cultured cells [2–4]. Gonadotropins and pregnancy may induce PGHS-2 in ovary and placenta [5,7,8]. PGHS-2 levels in sheep placental cotyledons increased up to 25 times near term and this tissue is a useful source of the enzyme [7,8]. Targeted gene disruption of PGHS-2 in mice causes female infertility [9]. PGHS-2 may also be important in human reproduction. The mRNA of PGHS-2 and PGHS-1 are present in a 100:1 ratio in the human amnion [10].

The main difference between PGHS-1 and -2 is that PGHS-2 contains a unique protein sequence near the C terminus [2–4]. The catalytic properties of the two enzymes are similar but with subtle differences.

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In short, the active site of PGHS-2 appears to be larger and PGHS-2 can metabolise many polyunsaturated fatty acids more efficiently than PGHS-1 [2,11,12].

5Z,8Z,11Z-Eicosatrienoic acid (20:3*n*-9) is of biological interest for two reasons. It is the major eicosanoic fatty acid in normal cartilage [13]. Second, essential fatty acid deficiency leads to pathophysiological changes in animals as arachidonic acid (20:4*n*-6) decreases and 20:3*n*-9 accumulates in phospholipids [14,15]. The pathological changes are largely due to a reduced formation of prostanoids but 20:3*n*-9 and its metabolites may also contribute. 20:3*n*-9 can be metabolised by 5-lipoxygenase to leukotrienes, by arachidonate 12-lipoxygenase, by cytochrome P450 and by PGHS-1 [16–20].

The first step in biosynthesis of prostaglandins from 20:4*n*-6 is abstraction of the pro-*S* hydrogen from C-13 and insertion of molecular oxygen at C-11 [1,2]. Prostaglandins are not formed from 20:3*n*-9, possibly due to the lack of an *n*-6 double bond [21], but this fatty acid is oxygenated by an "abortive" cyclooxygenase reaction. PGHS-1 transforms 20:3*n*-9 to 13*R*-hydroxy-5Z,8Z,11Z-eicosatrienoic acid (13-HETrE) as the main product and to small amounts of 11*R*-hydroxy-5Z,8Z,12E-eicosatrienoic acid (11-HETrE) [19,20]. This is consistent with the first step in prostaglandin biosynthesis. The two metabolites are apparently formed by attack of molecular oxygen at C-11 or C-13 of the carbon-centered radical formed by abstraction of the pro-*S* hydrogen at C-13 [19,20]. In addition, the 11-peroxy radical can form a series of polar metabolites non-enzymically [20].

In the present report we evaluated two sources of PGHS-2, sheep and rabbit placental cotyledons. Our goal was to determine whether PGHS-2 can oxygenate 20:3*n*-9 and to determine the major products. This seemed of interest for several reasons. First, PGHS-2 has been shown to metabolise some fatty acids quantitatively and qualitatively different from PGHS-1 [11,12]. Many fatty acids are better substrates for PGHS-2 than PGHS-1 and the products differ. Linolenic acid is, for example, subject to hydrogen abstraction at the *n*-5 carbon and not the *n*-8 carbon by PGHS-2 [11,12]. 20:3*n*-9 was found to be a poor substrate of recombinant human PGHS-2, but the metabolites were not characterised [12]. Second, high concentrations of aspirin block biosynthesis of prostaglandins from 20:4*n*-6 by PGHS-1 and PGHS-2, but PGHS-2 may still oxygenate 20:4*n*-6 to 15*R*-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid [6,22]. It therefore seemed possible that PGHS-2 may oxygenate 20:3*n*-9 differently than PGHS-1. Finally, 20:3*n*-9 is a major fatty acid in hyaline cartilage and its metabolism by PGHS-2 of inflammatory cells could be of importance in joint disorders.

2. Experimental

2.1. Materials

[1-^{14}C]20:3*n*-9 (56 Ci/mol) was from American Radiolabeled Chemicals (St. Louis, MO, USA) and 20:3*n*-9 (>98%) from Cascade (Reading, UK). [1-^{14}C]20:4*n*-6 (56 Ci/mol) was from Amersham (Amersham, UK) and 20:4*n*-6 (99%) was from Sigma (St. Louis, MO, USA). SepPak C_{18} were from Waters (Milford, MA, USA). Diclofenac sodium was from Ciba-Geigy AG (Basel, Switzerland) and aspirin was obtained locally. 13-HODE (13*S*-hydroxy-9Z,11E-octadecadienoic acid) was prepared with soybean lipoxygenase [20]. Bis(trimethylsilyl)trifluoroacetamide was from Supelco (Bellefonte, PA, USA). 5% Pd/$CaCO_3$ and most chemicals and solvents were from E. Merck (Darmstadt, Germany). Monoclonal mouse antibodies against PGHS-1 (cyo-5) were purchased from Oxford Biomedical Research (Oxford, MI, USA). Polyclonal rabbit antibodies against PGHS-2 were a kind gift of Dr. J. Otto (Michigan State University, MI, USA), and alkaline phosphatase conjugated antirabbit IgG and antimouse IgG were obtained from Sigma. The antibodies were diluted 1:10, 1:200 and 1:30 000, respectively. Equipment for gel electrophoresis and blotting (MiniProtean II, Mini Trans-Blot), high-range molecular size markers and nitrocellulose membranes were from BioRad (Hercules, CA, USA).

2.2. Incubation and extraction

Microsomes of ram vesicular glands were prepared by differential centrifugation (10 000 *g*, 20 min; 100 000 *g*, 60 min; 4°C), and stored at −80°C. Microsomes were incubated with [^{14}C]20:3*n*-9 as

described [20]. Sheep placental cotyledons were obtained immediately after delivery and frozen on dry ice or in liquid nitrogen. Rabbit cotyledons were obtained by section on gestation days 27 and 29 and immediately frozen at −20°C or −80°C. Microsomes of the cotyledons were prepared from a 20% homogenate as above and stored at −80°C. The placental microsomes were usually suspended in buffer (0.05 *M* Tris–HCl, pH 7.4, 1 m*M* EDTA, 1 m*M* EGTA) to a concentration of 1.5–3 mg of microsomal protein/ml and incubated with [^{14}C]20:3*n*-9 (18 μ*M*, 55 Ci/mol) or 20:3*n*-9 (0.1–0.3 m*M*) for 30 min at 37°C. Microsomes were suspended in 0.1 *M* Tris–HCl (pH 8.0) with 5 m*M* EDTA and 2 m*M* phenol in experiments with the oxygen electrode [8] and in some experiments with radiolabelled substrate. The effect of a freshly prepared solution of aspirin on the metabolism of [^{14}C]20:3*n*-9 was investigated using 13-HODE (7 nmol) as an internal standard. Aspirin was preincubated with the microsomes on ice for 20 or 90 min and diclofenac sodium for 5 min. The incubations were terminated by 4 volumes of ethanol and processed as described [20].

After extractive isolation on a SepPak C_{18} [20], the metabolites were analysed first by reversed-phase high-performance liquid chromatography (RP–HPLC) and then, after methylation, by capillary gas chromatography–mass spectrometry (GC–MS). The equipment for HPLC and the UV detector was as described previously [20]. The columns contained octadecasilane silica (5 μm; 150×4.6 mm I.D. or 200×8.0 mm I.D.) for RP–HPLC. The RP–HPLC columns were eluted with methanol–water–acetic acid (75:25:0.01 or 80:20:0.01, v/v) at 1–2 ml/min.

2.3. Western blot

Microsomal proteins of sheep and rabbit placental cotyledons and ram vesicular glands were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using 10% polyacrylamide gels for separation. Proteins were transferred to nitrocellulose membranes by electrophoresis. The membranes were incubated with primary antibodies (30 min), washed and incubated with secondary antibodies (30 min). After final washings, bound secondary antibodies were detected with *p*-nitrophenyl phosphate (Sigma).

2.4. GC–MS analysis

Methyl esters were prepared with ethereal diazomethane and Me_3Si ether derivatives by treatment with bis(trimethylsilyl)trifluoroacetamide and pyridine [20]. Hydrogenation was performed with $Pd/CaCO_3$ and C-values were determined as described [20]. GC–MS analyses were performed with a capillary GC (Varian 3400), a non-polar column (DB-5, J&W Scientific; film, 0.25 μm; 30 m×0.25 mm I.D.; carrier He, 15 p.s.i.) and an ion trap mass spectrometer (ITS40, Finnigan MAT) as described [20].

2.5. Other analyses

Radioactivity was determined by liquid scintillation (Beckman LS2800). Protein was determined with the Bradford method [23] using bovine gamma globulin (fraction II) as a standard. Oxygen consumption was measured with an oxygen monitor (Model 5300, YSI., Yellow Springs, OH, USA), an oxygen electrode (YSI 5331), and a jacketed Gilson cell (1.5 ml), operated at 25°C [8].

3. Results

3.1. Western blot of PGHS and cyclooxygenase activity of microsomes of placental cotyledons

Immunoblotting with antibodies specific for PGHS-2 indicated the presence of this protein in both sheep and rabbit placental cotyledons. Immunoblotting of proteins from sheep cotyledons showed a strong band at 70 000 amu and a weak band at 68 000 amu. Proteins of rabbit cotyledons were immunostained at 68 000–70 000 amu. Some immunostaining was also noted at 47 000 amu, possibly due to partial proteolysis of PGHS-2 [8]. Immunoblotting with the PGHS-1 antibodies failed to detect PGHS-1 in the placental microsomes, but PGHS-1 was readily detected as a single band at about 68 000 amu in microsomes of ram vesicular glands.

A comparison of oxygen consumption by dilutions of microsomes of ram vesicular glands and microsomes of ewe cotyledons with 20:4*n*-6 (0.1 m*M*) as a substrate indicated that the ewe cotyledons con-

tained much less catalytically active enzyme (about 5%). Nevertheless, [^{14}C]20:4*n*-6 was extensively metabolised to prostaglandins by ewe placental microsomes. Microsomes of rabbit placental cotyledons were also catalytically active when prepared from cotyledons, which had been frozen and stored at −80°C.

3.2. HPLC analysis of metabolites of [^{14}C]20:3n-9

Microsomes of ovine placental cotyledons converted [^{14}C]20:3*n*-9 to many radiolabelled products as shown in Fig. 1. Rabbit placental microsomes yielded similar results. The most polar products, which eluted with 10–40 ml, were not further characterised. The two major peaks of radioactivity (peak I and II in Fig. 1), which eluted with 56 and 62 ml, were identified as 13-HETrE and 11-HETrE, respectively, by GC–MS analysis (see Section 3.3) and by comparison with standards. Both metabolites had the same elution volume on RP–HPLC as 11-HETrE and 13-HETrE obtained by biosynthesis with PGHS-1. 13-HETrE and 11-HETrE were formed in a ratio of about 3:2, but with wide variations between experiments. The mean ratios of six experiments were 1.61 to 1. Corresponding figures from several incubations with ram seminal microsomes yielded a ratio of 5-6 to 1. Finally, [^{14}C] 20:3*n*-9 was not metabolised to polar products by the high speed supernatant of sheep placental cotyledons.

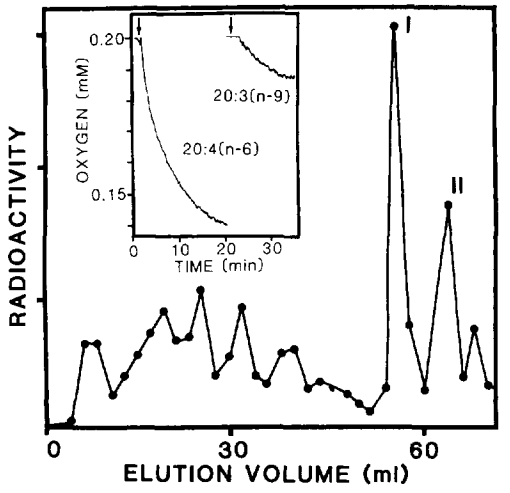

Fig. 1. RP–HPLC of metabolites formed from [^{14}C]20:3*n*-9 by PGHS-2 of microsomes of ovine placental cotyledons. Peaks I and II were found to contain 13-HETrE and 11-HETrE, respectively. The reversed-phase column (5 μm octadecasilane silica, 200×8 mm I.D) was eluted with methanol–water–acetic acid, (80:20:0.01, v/v) at 2.0 ml/min. The insert shows oxygen consumption by microsomes of ovine cotyledons (1.6 mg protein/ml, 25°C) after addition of 0.3 m*M* 20:4*n*-6 (left) or 0.3 m*M* 20:3*n*-9 (right) as indicated by arrows.

20:4*n*-6 will react with two molecules of oxygen during prostaglandin biosynthesis, while 20:3*n*-9 will react with only one. In comparison with 20:4*n*-6, 20:3*n*-9 was nevertheless a very poor substrate of PGHS-2 as judged from the oxygen consumption after addition of 20:4*n*-6 and 20:3*n*-9 (insert in Fig. 1). With 0.3 m*M* 20:4*n*-6 as a substrate the initial oxygen consumption was approximately 20 nmol O_2/min/mg and with 0.3 m*M* 20:3*n*-9 about 1 nmol O_2/min/mg or even less in some experiments.

Diclofenac sodium (0.05 m*M*), a potent PGHS inhibitor, was found to decrease the formation of 13-HETrE and 11-HETrE by over 90%. Treatment of the microsomes with 2 m*M* aspirin for 20 or 90 min on ice did not change the ratio of 13-HETrE and 11-HETrE although the oxygenation of 20:4*n*-6 was reduced by about 70% after preincubation for 60 min in parallel experiments.

3.3. Identification of the two major metabolites

3.3.1. 13-HETrE

This material had an elution volume of 56 ml on RP–HPLC (peak I in Fig. 1) and it lacked significant UV absorbency at 235 nm. The mass spectrum of the Me_3Si ether methyl ester derivative of the compound before and after hydrogenation is shown in Fig. 2. The C values were 21.1 and 21.9, respectively. The parent compound showed signals at *m/z* 393 (M^+−15), 377 (M^+−31), 318 (M^+−90), 309 (M^+−99, loss of C-14–C-20), 281, 253, 187, 169 and 73 (base peak). After hydrogenation, strong signals were noted at *m/z* 383 (M^+−31), 367 (M^+−47), 315 (M^+−99) and 201 [Me_3SiO^+=CH–(CH_2)6–CH_3]. The results were consistent with previous reports [19,20].

3.3.2. 11R-HETrE

This metabolite was identified in peak II of Fig. 1 (elution volume 62 ml) and it also lacked significant UV absorbency at 235 nm. The mass spectrum of

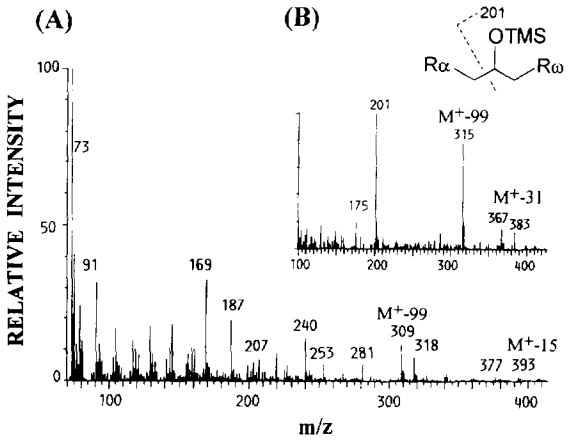

Fig. 2. Mass spectra of methyl 13-hydroxy-5,8,11-eicosatrienoate before and after hydrogenation. Me_3Si ether derivatives. (A) Mass spectrum before hydrogenation. (B) Mass spectrum after hydrogenation. R_α and R_ω of the insert designate the carboxyl side chain and the omega sida chain, respectively. Ion trap mass spectrometer (electron impact ionisation).

this compound was as reported [19,20] with a strong signal at m/z 227 [Me_3SiO^+=CH–CH=CH–$(CH_2)_6$–CH_3]. After hydrogenation, informative and strong signals were present, inter alia, at m/z 287 (M^+-117, loss of C-12–C-20) and 229 [Me_3SiO^+=CH–$(CH_2)_8$–CH_3]. The C values were 21.2 and 21.9, respectively, as reported [20].

4. Discussion

Microsomes of ovine placental cotyledons at term were found to contain PGHS, although the enzyme activity was much lower than in microsomes of ram vesicular glands. Rabbit placental cotyledons were also enzymically active. Western blot indicated that PGHS-2 was present in microsomes of ewe cotyledons at term, which is in agreement with previous reports [7,8], and in microsomes of rabbit cotyledons near term. We could not detect PGHS-1 in the placental cotyledons by Western blot, but small amounts of this ubiquitous enzyme cannot be excluded [7].

The first step in prostaglandin biosynthesis consists of abstraction of the pro-*S* hydrogen from C-13 of the eicosanoic prostaglandin precursor fatty acids, i.e., 20:3*n*-6, 20:4*n*-6, 20:4*n*-3 and 20:5*n*-3 [1–3]. The steric course of these oxygenations involves elimination of hydrogen and attack by molecular oxygen in an antarafacial way. The fatty acid we have studied, 20:3*n*-9, lacks an *n*-6 double bond and cannot be converted to prostaglandins [21].

Previous studies have shown that 20:3*n*-9 undergoes an "abortive" cyclooxygenase reaction with PGHS-1 [19,20]. The present work now extends these results to PGHS-2. The major oxygenated metabolites of 20:3*n*-9 of ovine placental microsomes were identified as 13-HETrE and 11-HETrE (Fig. 3), which were formed in a 3:2 ratio. In comparison with arachidonic acid, 20:3*n*-9 is a poor substrate of both PGHS-1 [19,20] and PGHS-2 (Fig. 1). This is in agreement with Percival et al. [12], who found that recombinant human PGHS-2 oxy-

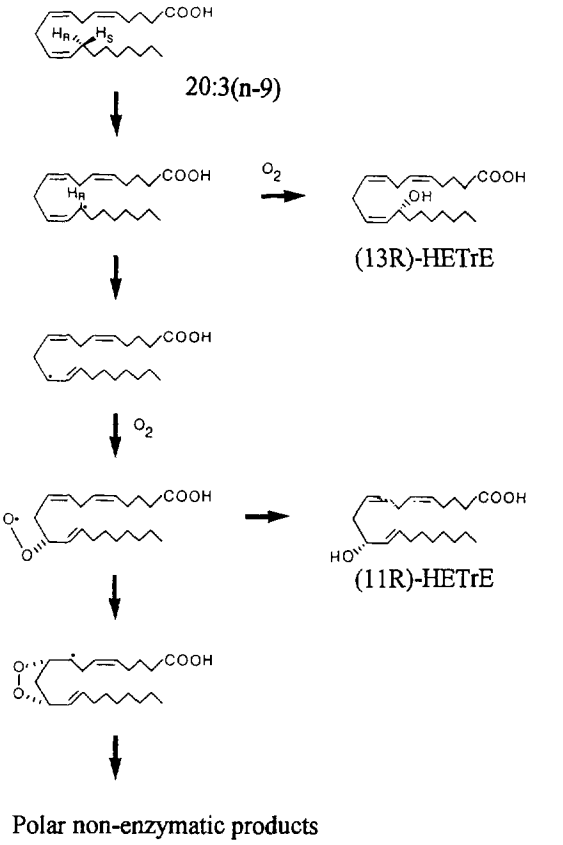

Fig. 3. Summary of the metabolism of 20:3*n*-9 by PGHS-1 and PGHS-2. The unstable intermediates in the left column have not been isolated. The 11*R*-peroxy radical can yield a series of products non-enzymically [19,20].

genated 20:3*n*-9 slowly in comparison with a series of other fatty acids. It is therefore likely that the oxygenation of 20:3*n*-9 might be significant only in essential fatty acid deficiency, in inflammatory conditions involving hyaline cartilage or in other situations with a high concentration of 20:3*n*-9.

The mechanism of oxygenation of 20:3*n*-9 by PGHS is of scientific interest. PGHS-1 and -2 can apparently abstract hydrogen from the monoallylic C-13 of 20:3*n*-9 (Fig. 3). This requires 15% more energy (about 10 kcal/mol) than abstracting the bis-allylic hydrogen from C-13 of 20:4*n*-6 [24]. In addition to PGHS-1 and PGHS-2, there is only one enzyme that catalyses a similar dioxygenation. Linoleic acid 8R-dioxygenase is a fungal enzyme, which catalyses hydrogen abstraction from C-8 of linoleic acid and antarafacial insertion of molecular oxygen at C-8 [25]. PGHS and linoleic acid 8R-dioxygenase are hemoproteins [1–4,26]. It will be of interest to determine whether their oxygenation mechanisms are related.

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