A STRUCTURAL REQUIREMENT FOR THE CONVERSION OF PROSTAGLANDIN ENDOPEROXIDES TO THROMBOXANES

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1. Introduction

Human platelets contain an enzyme, thromboxane synthase, which converts the prostaglandin (PG) endoperoxide PGH_2 to thromboxane A_2 [1,2]. The same enzyme also forms 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) from PGH_2 [3]. When PGH_1 was incubated with partially purified platelet enzyme, only small amounts of thromboxane B_1 were formed. The major product was 12-hydroxy-8,10-heptadecadienoic acid (HHD) [3].

Enzyme inhibitors and substrate competition experiments have now shown that the conversion of PGH_1 to HHD is catalyzed by thromboxane synthase. The lack of a Δ^5-double bond in PGH_1, seems to preclude the formation of thromboxane A_1. To further investigate this point, a positional isomer of PGH_2 with the *cis* double bond at Δ^4 was prepared. This substrate was converted to Δ^4-HHD but not appreciably to a thromboxane. Therefore, the enzyme appears to specifically require a Δ^5-double bond in order to isomerize prostaglandin endoperoxides to thromboxanes.

Abbreviations: BSTFA, bis(trimethylsilyl) trifluoroacetamide; DEAE, diethylaminoethyl; HHD, 12-hydroxy-8,10-heptadecadienoic acid; HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid; HPHT, 12-hydroperoxy-5,8,10-heptadecatrienoic acid; $HPTXA_2$, 15-hydroperoxythromboxane A_2; L-8027, 2-isopropyl-3-nicotinylindole; PG, prostaglandin; SD, standard deviation; TLC, thin-layer chromatography; TX, thromboxane

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2. Materials and methods

Human platelet microsomes, [1-^{14}C]PGH_1 (spec. act. 0.34 Ci/mol) and [1-^{14}C]PGH_2 (spec. act. 1 Ci/mol) were prepared as in [1]. 4,8,11,14-Eicosatetraynoic acid was a generous gift from Drs Nugteren and van Dorp, Unilever Research, Vlaardingen, The Netherlands. Lindlar catalyst was purchased from Fluka AG, Buchs SG, Switzerland.

2.1. *4,8,11,14-(all cis)-[$^3H_8$]Eicosatetraenoic acid*

To a solution of 15 mg 4,8,11,14-eicosatetraynoic acid in 0.75 ml methanol, 3 mg Lindlar catalyst and 3 mg quinoline was added. The acid was reduced with H_2 at room temperature for 80 min with vigorous stirring.

Tritium-labeled 4,8,11,14-eicosatetraenoic acid was prepared in a similar way using $^3H_2$ instead of $^1H_2$.

Part of the crude products from these two reductions were pooled, water was added and the mixture acidified to pH 3. After extraction with diethyl ether the solvent was evaporated under reduced pressure and the residue was dissolved in methanol/water/acetic acid (85:15:0.01, v/v/v). This material was purified by reversed phase high pressure liquid chromatography (HPLC), first with methanol/water/acetic acid (85:15:0.01, v/v/v) as solvent system, and in a second run with methanol/water/acetic acid (80:20:0.01, v/v/v).

2.2. cis-Δ^4-*[4,5,8,9,11,12,14,15-$^3H_8$]PGH_1*

The labeled eicosatetraenoic acid (5 mg, 3.58 Ci/mol) was incubated with sheep vesicular gland micro-

somes as in [4]. The products were chromatographed on silicic acid. A major radioactive compound eluted in the fraction reported to elute PGH_2 [4].

Confirmation of the structure was made by the following experiments. The product cochromatographed with authentic PGH_2 on silica gel G (TLC) with ethyl acetate/trimethyl pentane/acetic acid (50:50:0.5, v/v/v) as solvent system. Incubation of the product with 0.1 M phosphate buffer (pH 7.4) for 30 min at room temperature yielded a compound which cochromatographed with authentic PGE_2 on TLC (solvent diethyl ether/methanol (49:1, v/v)). Reduction of the material with stannous chloride produced a substance which cochromatographed with $PGF_{2\alpha}$ on TLC in the same solvent system.

2.3. *Inhibition experiments*

Human platelet microsomes were preincubated for 15 s with various concentrations of either L-8027 (2-isopropyl-3-nicotinyl-indole) or imidazole, two thromboxane synthase inhibitors [5–7]. [1-^{14}C]PGH_1 or [1-^{14}C]PGH_2 was added and the incubation was continued for 60 s at 24°C. The substrate concentrations were 124 μM and 143 μM, respectively. The reaction was stopped by addition of cold (−78°C) citric acid in ether/methanol [7]. The reaction mixtures were analyzed for 12-hydroxy-8,10-heptadecadienoic acid (HHD) or 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) [3].

2.4. *Variations of pH in incubation medium*

PGH_2 was incubated with platelet microsomes, solubilized microsomes and solubilized microsomes which had been further purified by DEAE-cellulose chromatography [1] in buffers of varying pH. The following buffers were used: pH < 6, Na-acetate; 6 < pH < 8, K-phosphate; pH > 8, Tris–HCl. Final buffer concentrations were 80 mM. After 60 s at 24°C the incubation mixtures were analyzed for HHT and TXB_2.

2.5. *Competition experiments*

A constant amount (5.65 μg) of [1-^{14}C]PGH_1 was incubated with 200 μl human platelet microsomes and varying amounts of unlabeled PGH_2 for 1 min at 24°C. Percent conversion of PGH_1 to HHD was measured.

2.6. *Incubations with PGH_1, cis-Δ^4-PGH_1, PGG_2 and PGH_2*

Human platelet microsomes (200 μl) were incubated for 1 min at 24°C with [1-^{14}C]PGH_1, *cis* Δ^4-[$^3H_8$]PGH_1 or [1-^{14}C]PGH_2 (124 μM, 100 μM and 143 μM, respectively). [1-^{14}C]PGH_2 was incubated under the same conditions with DEAE-cellulose fractionated solubilized enzyme [1]. After acidification and extraction with ether, thromboxanes and C-17 hydroxy acids were analyzed by thin-layer chromatography as in [3]. A series of incubations of [1-^{14}C]-PGG_2 and [1-^{14}C]PGH_2 were performed with DEAE-cellulose purified thromboxane synthase to generate thromboxane A_2 and 15-hydroperoxy thromboxane A_2 [4] and to determine the ratios of C-17 acids to thromboxanes formed.

3. Results

3.1. *Inhibition of HHD formation*

Dose–inhibition curves for the conversion of PGH_1 and PGH_2 to HHD and HHT by human platelet microsomes are shown in fig.1. Both endoperoxides were converted to the corresponding C-17 acids when inhibitor was absent. When the microsomes were boiled prior to incubation, 5% of the endoperoxides were converted to the C-17 acids. The non-enzymatic decomposition of endoperoxides to C-17 acids has been corrected for in fig.1.

Figure 2 shows the effect of unlabeled PGH_2 on the conversion of [1-^{14}C]PGH_1 to HHD. As the ratio of PGH_2 to PGH_1 increases the production of HHD decreases linearly.

3.2. *Ratios of C-17 acid to thromboxane formation: Effects of pH and ionic strength*

When PGH_1 was incubated with platelet microsomes, the ratio of HHD to TXB_1 in the ether extract was about 40. PGH_2 was incubated with DEAE-purified thromboxane synthase in a series of experiments. The mean ratio of HHT to TXB_2 was 2.3 ± 0.05 (± SD, n=17). Variations in pH from 5–8 did not affect enzyme activity markedly. At pH > 8 the activity usually decreased. The ratio of HHT to TXB_2 formed in these experiments was relatively constant.

Solubilized platelet microsomes were incubated with PGH_2 in the presence of varying concentrations

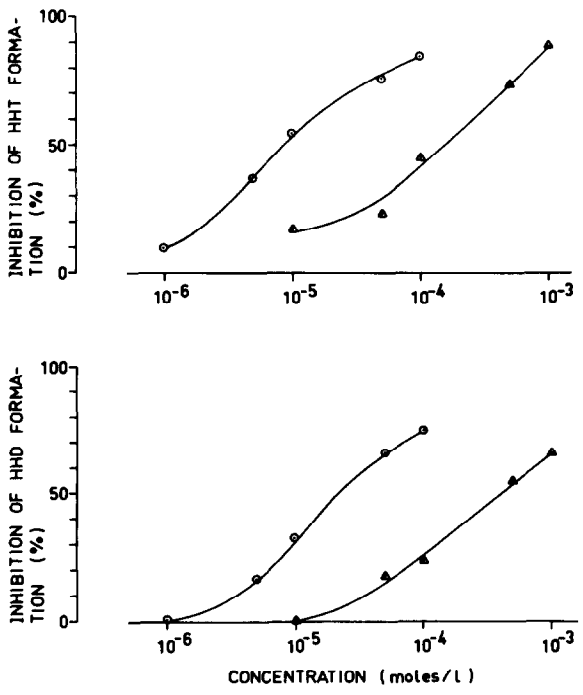

Fig.1. Dose–inhibition curves for the conversion of PGH_1 and PGH_2 to HHD and HHT by human platelet microsomes: (⊙) L-8027; (△) imidazole.

of NaCl. At < 0.5 M no effect on enzyme activity or on the ratio of HHT to TXB_2 in ether extracts was observed.

When PGG_2 was incubated with DEAE-purified thromboxane synthase a mean ratio of 12-hydroperoxy-5,8,10-heptadecatrienoic acid to 15-hydroperoxy thromboxane B_2 (cf. [4]) was 1.6 ± 0.04 (± SD, n=15).

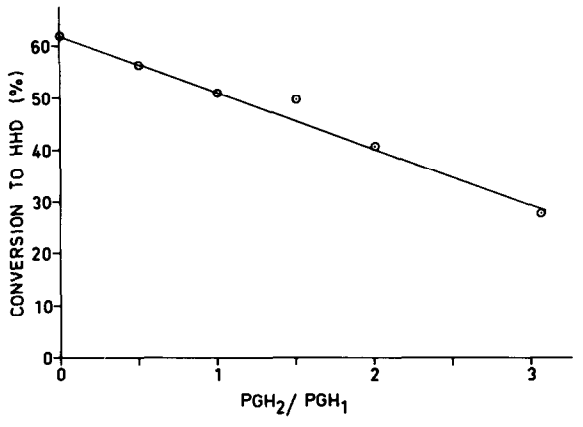

Fig.2. Competition between [1-^{14}C]PGH_1 and unlabeled PGH_2 for human platelet thromboxane synthase.

3.3. *Product formed from* cis-Δ^4-*PGH*$_1$

cis-Δ^4-PGH_1 was synthesized from 4,8,11,14-eicosatetraynoic acid as outlined in fig.3. Incubations of this endoperoxide with human platelet microsomes yielded a product which cochromatographed with authentic HHT on TLC. This material was derivatized with diazomethane followed by BSTFA. Analyses by gas-liquid chromatography–mass spectrometry showed a *C*-value of 19.35 on 1% OV-1 and major ions at *m/e* 366 (M), 351 (M-15, loss of $\cdot CH_3$), 335 (M-31, loss of $\cdot OCH_3$), 298, 295 (M-71, loss of $\cdot(CH_2)_4CH_3$), 276 (M-90, loss of $(CH_3)_3SiOH$), 239, 225 (M-141, loss of $\cdot CH_2-CH_2-CH{=}CH-(CH_2)_2-COOCH_3$), 205 (M-71-90), and 173.

These data suggest that the product is a 12-hydroxy-heptadecatrienoic acid (cf. [8]). The ion at *m/e* 239 is probably formed by cleavage between C-6 and C-7 and charge retention on the C-7 to C-17 fragment. The ion, which is absent from the mass spectrum of

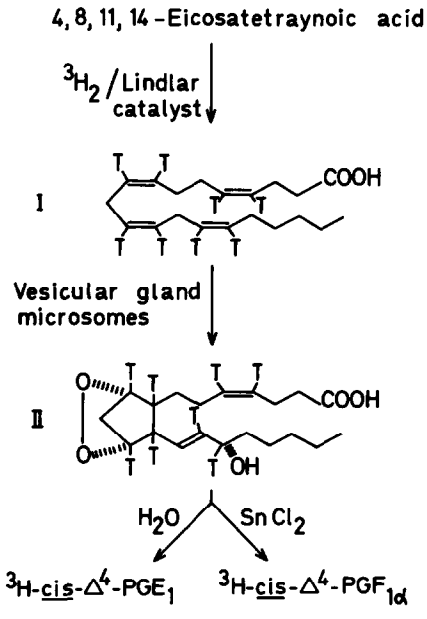

Fig.3. Synthesis of *cis*-Δ^4-[$^3H_8$]PGH_1. I: 4,8,11,14-(all *cis*)-[$^3H_8$]eicosatetraenoic acid; II: *cis*-Δ^4-[$^3H_8$]PGH_1.

Table 1
Conversion of endoperoxides by thromboxane synthase

	Conversion to C-17 acid (%)	Conversion to thromboxane (%)	C-17 acid/ thromboxane
[1-^{14}C]PGH_1	66.5	1.6	41.6
Δ^4-[4,5,8,9,11,12,14,15-$^3H_8$]PGH_1	89.5[a]	2.2[b]	40.7
[1-^{14}C]PGH_2	49.0	23.4	2.1

[a] Corrected for loss of 2 tritium atoms in malondialdehyde
[b] Radioactive material cochromatographing with unlabeled TXB_2

HHT methyl ester, trimethylsilyl ether, suggests the occurrence of a Δ^4-double bond in the present product (Δ^4-HHD).

The ratios of C-17 acids to thromboxanes formed from PGH_1, *cis*-Δ^4-PGH_1 and PGH_2 are summarized in table 1.

4. Discussion

HHT and thromboxane B_2 are the principal metabolites of arachidonic acid, formed via the cyclooxygenase pathway in human platelets [8]. Both compounds arise enzymatically from PGH_2 in reactions catalyzed by thromboxane synthase [3]. A common intermediate in the transformations has been proposed [3].

PGH_1 is not converted efficiently to thromboxane B_1 by partially purified thromboxane synthase. However, it is transformed to the C-17 acid HHD [3]. This reaction was inhibited by L-8027 and imidazole (fig.1). The dose–inhibition curves were similar to those obtained when the conversion of PGH_2 to HHT was determined (fig.1). Furthermore, unlabeled PGH_2 suppressed the formation of HHD from PGH_1 (fig.2). These data suggest that thromboxane synthase converts PGH_1 to HHD and PGH_2 to thromboxane A_2 and HHT. The apparent dual catalysis may be explained if the enzyme can only perform an initial step of the isomerase reaction with PGH_1 as substrate. This initial step may involve protonation of the oxygen atom at C-9 as proposed earlier for PGH_2 [3]. The charged intermediate formed from PGH_2 is converted by further action of the enzyme to thromboxane A_2. Alternatively, the intermediates formed from either endoperoxide may

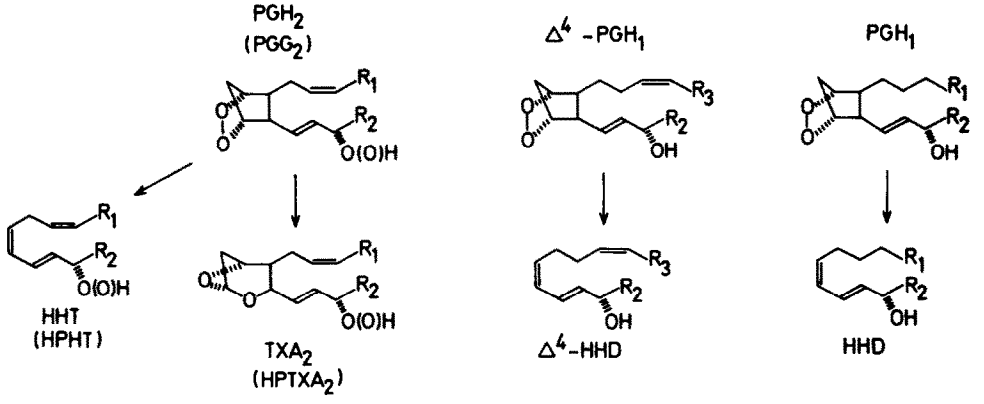

Fig.4. Transformations of endoperoxides by thromboxane synthase: HPHT, 12-hydroperoxy-5,8,10-heptadecatrienoic acid (↑); $HPTXA_2$, 15-hydroperoxy thromboxane A_2 (↑); R_1, $-(CH_2)_3COOH$; R_2, $-(CH_2)_4CH_3$; R_3, $-(CH_2)_2COOH$.

rearrange to the C-17 acids HHD and HHT plus malondialdehyde.

Experiments with *cis*-Δ^4-PGH_1 showed that this endoperoxide is transformed predominantly to Δ^4-HHD. The ratio of C-17 acid to thromboxane was quite similar to that obtained using PGH_1 as substrate (table 1). This suggests a specific requirement of a Δ^5-double bond in the endoperoxide substrate to allow isomerization to thromboxanes.

Figure 4 summarizes the conversions of four prostaglandin endoperoxides catalyzed by human platelet thromboxane synthase.

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