CONVERSION OF PROSTAGLANDIN ENDOPEROXIDES TO C_{17}-HYDROXY ACIDS CATALYZED BY HUMAN PLATELET THROMBOXANE SYNTHASE

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1. Introduction

Thromboxane A_2 is a new, unstable bioregulator which induces the platelet release reaction, platelet aggregation and smooth muscle contractions [1]. An enzyme catalyzing the conversion of prostaglandin endoperoxides to thromboxanes (thromboxane synthase) has been isolated from platelet microsomes [2] and the inhibition of this enzyme by various compounds has been investigated [3]. In addition to thromboxane (TX)B_2, 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) was enzymatically formed from prostaglandin (PG)H_2 by platelet microsomes and purified thromboxane synthase [2]. The present paper provides evidence that TXB_2 and HHT are formed by the same enzyme. It also shows that the formation of HHT does not involve TXA_2 as an intermediate.

2. Materials and methods

Human platelet microsomes, partially purified thromboxane synthase, [1-^{14}C]PGH_1 (spec. act. 0.34 Ci/mol) and [1-^{14}C]PGH_2 (spec. act. 1.0 Ci/mol) were prepared as described [2]. The endoperoxides moved slightly ahead of PGB_1 on silica gel G thin-layer chromatography (TLC) and were converted to $PGF_{1\alpha}$ and $PGF_{2\alpha}$, respectively, by $SnCl_2$ reduction. 9α,11α-Azo-15(S)hydroxyprosta-5(*cis*), 13(*trans*)-dienoic acid (APA) was generously given by Dr E. J. Corey. 9α,11α-Epoxymethano-15(S)hydroxyprosta-5(*cis*), 13(*trans*)-dienoic acid (EMPA) and TXB_2 were kindly provided by the Upjohn Company. 2-Isopropyl-3-nicotinylindole (L-8027) and sodium *p*-benzyl-4-[1-oxo-2-(4-chlorobenzyl)-3-phenylpropyl] phenyl phosphonate (N-0164) were given by Labaz and Nelson Research, respectively. Imidazole and $SnCl_2$ were purchased from Sigma.

2.1. *Analyses of products formed from prostaglandin endoperoxides*

Partially purified thromboxane synthase in 0.1 M Tris–HCl, pH 7.4, was incubated at 24°C with [1-^{14}C]-PGH_1 (154 μM) or [1-^{14}C]PGH_2 (120 μM). Reactions were interrupted after 1 min by addition of cold 0.2 M citric acid and products were immediately extracted with diethyl ether. After conversion to methyl esters aliquots were analyzed by TLC (solvent systems: (a) diethyl ether/methanol 49:1 v/v; (b) and (c) the organic phases of ethyl acetate/2,2,4-trimethylpentane/water 50:100:100 and 75:75:100 v/v/v, respectively). The remainder of the products was further converted to trimethylsilyl ether derivatives and analyzed by gas–liquid chromatography (1% OV-1) – mass spectrometry (GLC–MS).

Abbreviations: APA, 9α,11α-azo-15(S)hydroxyprosta-5(*cis*)-13(*trans*)-dienoic acid; EMPA, 9α,11α-epoxymethano-15(S) hydroxyprosta-5(*cis*)-13(*trans*)-dienoic acid; GLC–MS, gas–liquid chromatography – mass spectrometry; HHD, 12L-hydroxy-8,10-heptadecadienoic acid; HHT, 12L-hydroxy-5,8,10-heptadecatrienoic acid; L-8027, 2-isopropyl-3-nicotinylindole; N-0164, *p*-benzyl-4[1-oxo-2-(4-chlorobenzyl)-3-phenylpropyl] phenyl phosphonate; PG, prostaglandin; TLC, thin-layer chromatography; TX, thromboxane

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2.2. *Enzyme inhibition experiments*

Incubations were performed at twice the scale described before [3]. Extracts were divided into two parts, for the determination of TXB_2 and HHT, respectively. Unlabeled TXB_2 was added as an internal reference for the former analyses. Methylated samples were analyzed by TLC using solvents (a) and (b) (section 2.1) for TXB_2 and HHT determinations, respectively.

Radioactivity on thin-layer chromatograms was determined as described [3].

2.3. *Trapping of thromboxane A_2*

Partially purified thromboxane synthase in 0.1 M Tris–HCl, pH 7.4, was incubated at 37°C with [1-^{14}C]-PGH_2 (120 μM). Reactions were stopped after either 10 s or 2 min by the addition of 80 vol. methanol to convert TXA_2 to *O*-methyl TXB_2 [4]. After 1 h at 24°C, the methanol was removed in vacuo, water was added and the products were extracted with diethyl ether. Parallel incubations were stopped by adding 5 vol. $SnCl_2$ in ethanol (5 mg/ml) to reduce PGH_2 to $PGF_{2\alpha}$ [5]. In this case, products were extracted after 2 min at 24°C. Ether extracts were methylated and analyzed by TLC, using solvents (a–c) (section 2.1). For analyses with solvent (a), TXB_2 was added prior to methylation as an internal reference.

3. Results

3.1. *Products formed from PGH_1 and PGH_2*

Figure 1 shows thin-layer radiochromatograms of products isolated after incubations of [1-^{14}C]PGH_1 (upper panel) and [1-^{14}C]PGH_2 (lower panel) with purified thromboxane synthase from human platelets. Based on GLC–MS analyses the major product from the first incubation was identified as 2-hydroxy 8,10-heptadecadienoic acid (HHD; *C*-value 19.3; ions at *m/e* 368 (M), 353 (M-15, · CH_3), 337 (M-31, · OCH_3), 321 (M-15-32, · CH_3 + CH_3OH), 297 (base peak, M-71, · $CH_2(CH_2)_3CH_3$), 278 (M-90, $(CH_3)_3SiOH$) and 225 (M-143, · CH_2 $(CH_2)_5COOCH_3$)). Small amounts of TXB_1 (*C*-value 24.9; ions at *m/e* 602 (M), 587 (M-15), 531 (M-71), 512 (M-90), 441 (M-71-90), 422 (M-2 × 90), 368, 325, 301, 297, 258, 243 (base peak), 217 and 173) were also detected.

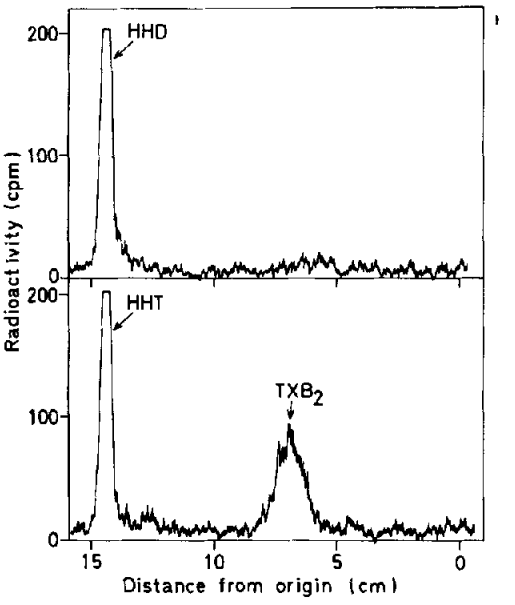

Fig.1. Thin-layer radiochromatograms of products formed from [1-^{14}C]PGH_1 (upper) and [1-^{14}C]PGH_2 (lower) during incubations (24°C, 1 min) with partially purified thromboxane synthase from human platelets. HHD, 12-hydroxy-8,10-heptadecadienoic acid; HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid and TXB_2, thromboxane B_2 (solvent (a), section 2.1.).

The two major products formed from PGH_2 were identified as 12-hydroxy-5,8,10-heptadecatrienoic acid (*C*-value 19.2; ions at *m/e* 366 (M), 351 (M-15), 335 (M-31), 319 (M-15-32), 295 (base peak, M-71), 276 (M-90) and 225 (M-141, $CH_2CH{=}CH(CH_2)_3COOCH_3$)) and TXB_2 (*C*-value 24.6; ions at *m/e* 600 (M), 585 (M-15), 529 (M-71), 510 (M-90), 439 (M-71-90), 420 (M-2 × 90), 366, 323, 301, 295, 256 (base peak), 217 and 173), respectively.

3.2. *Inhibition of thromboxane B_2, HHT and HHD formation*

In a previous study data were presented on the inhibition of thromboxane synthase by several compounds [3]. The most potent inhibitors, APA, EMPA, L-8027, N-0164 and imidazole, were selected for the present investigation. Human platelet microsomes were incubated with [1-^{14}C]PGH_2 in the presence of the inhibitors. Two dose–inhibition curves were deter-

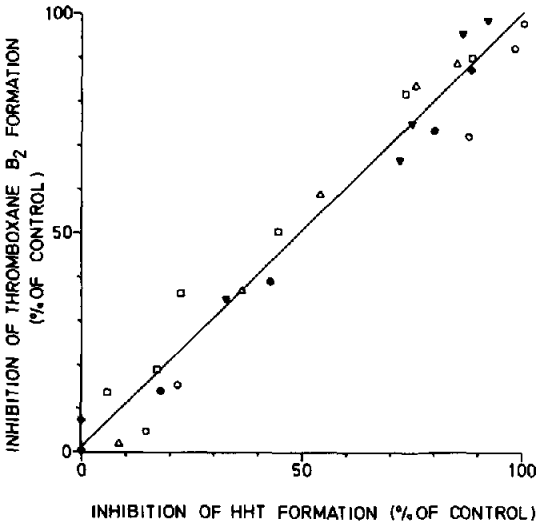

Fig.2. Linear regression of the inhibition of thromboxane B_2 and HHT formation by L-8027 (△), N-0164 (○), imidazole (□), 9α,11α-epoxymethano-15(S)-hydroxyprosta-5(*cis*), 13(*trans*)-dienoic acid (●) and 9α,11α-azo-15(S)hydroxy-prosta-5(*cis*), 13(*trans*)-dienoic acid (▼).

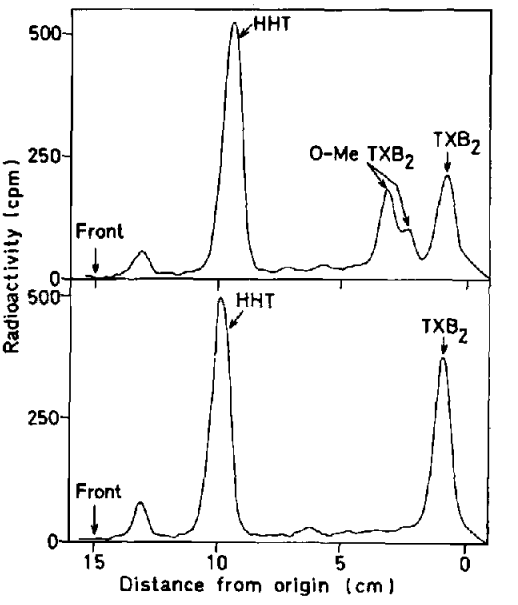

Fig.3. Thin-layer radiochromatograms of products formed from [1-^{14}C]PGH_2 during incubations with partially purified thromboxane synthase at 37°C for 10 s (upper) and 2 min (lower). Incubations were stopped by the addition of 80 vol. methanol, to convert thromboxane A_2 to *O*-methyl thromboxane B_2 (O-Me TXB_2) (Solvent (c), section 2.1.).

mined for each substance, one for TXB_2 and one for HHT formation. For a given inhibitor these curves were always nearly identical. Analogous experiments with [1-^{14}C]PGH_1 as substrate also gave similar dose–inhibition curves for HHD formation. In fig.2 the inhibition of TXB_2 formation has been plotted against the inhibition of HHT formation for all five inhibitors. Linear regression gave a slope of 0.99, an intercept of 1.38 and a coefficient of determination of 0.96.

3.3. *Exclusive transformation of TXA_2 to TXB_2*

PGH_2 was incubated with partially purified thromboxane synthase for either 10 s or 2 min at 37°C. The reactions were stopped by addition of excess methanol to convert TXA_2 to *O*-methyl TXB_2. Parallel incubations, stopped by addition of $SnCl_2$ in ethanol, showed that no endoperoxide remained after 10 s incubation. At this time the reaction mixture contained 21% TXA_2 (measured as *O*-methyl TXB_2) 22% TXB_2 and 55% HHT (fig.3, upper). After 2 min incubation the products consisted of < 3% TXA_2, 42% TXB_2 and 55% HHT (fig.3, lower).

4. Discussion

HHD was earlier detected as a by-product of prostaglandin biosynthesis in sheep vesicular gland [6]. It was suggested to be formed from a prostaglandin endoperoxide. The other product of the reaction was identified as malondialdehyde [6]. It was later found that HHT and TXB_2 were formed by aggregating human platelets in approximately equal amounts [7]. During previous studies on the solubilization and purification of platelet thromboxane synthetase we observed that HHT was enzymically formed from PGH_2 by the same fractions that formed TXB_2 [2]. Furthermore, the purified enzyme converted PGH_1 predominantly to HHD (fig.1). Five structurally unrelated inhibitors of thromboxane formation [3] inhibited TXB_2 and HHT formation from PGH_2 identically (fig.2) and in addition inhibited HHD formation from PGH_1. These results strongly suggest that platelet thromboxane synthase catalyzes the conversion of prostaglandin endoperoxides to C_{17} hydroxy acids plus malondial-

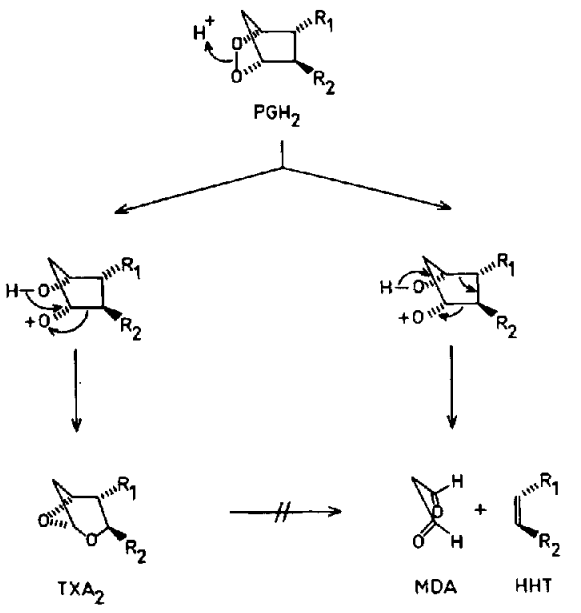

Fig.4. Possible mechanism of thromboxane A_2 (TXA_2), HHT and malondialdehyde (MDA) formation from PGH_2, catalyzed by thromboxane synthase.

dehyde. This reaction might or might not involve thromboxane A as an intermediate. TXA_2 was generated during a short incubation of PGH_2 with purified thromboxane synthase and the products formed during its spontaneous decay were determined (fig.3). The results showed that TXA_2 was exclusively transformed to thromboxane B_2 and that HHT was formed independently. This is consistent with the predominant formation of HHD from PGH_1 and the approx. 1:1 formation of TXB_2 and HHT from PGH_2 (fig.1). A hypothetical reaction mechanism is outlined in fig.4. It is postulated that the isomerization of PGH to thromboxane A is initiated by protonation of the oxygen at C-9 of the substrate. The cation formed after cleavage of the O–O bond can rearrange in two ways to yield either thromboxane A or C_{17} hydroxy acid plus malondialdehyde. The results showing that TXA_2 is exclusively converted to TXB_2 has important implications for the quantitative determination of thromboxane synthesis in various biological systems.

Acknowledgements

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