

## CONVERSION OF PROSTAGLANDIN ENDOPEROXIDES TO C<sub>17</sub>-HYDROXY ACIDS CATALYZED BY HUMAN PLATELET THROMBOXANE SYNTHASE

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### 1. Introduction

Thromboxane A<sub>2</sub> 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<sub>2</sub>, 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) was enzymatically formed from prostaglandin (PG)H<sub>2</sub> by platelet microsomes and purified thromboxane synthase [2]. The present paper provides evidence that TXB<sub>2</sub> and HHT are formed by the same enzyme. It also shows that the formation of IHT does not involve TXA<sub>2</sub> as an intermediate.

### 2. Materials and methods

Human platelet microsomes, partially purified

**Abbreviations:** APA, 9 $\alpha$ ,11 $\alpha$ -azo-15(S)hydroxyprosta-5(*cis*)-13(*trans*)-dienoic acid; EMPA, 9 $\alpha$ ,11 $\alpha$ -epoxymethano-15(S)hydroxyprosta-5(*cis*)-13(*trans*)-dienoic acid; GLC-MS, gas-liquid chromatography – mass spectrometry; HHT, 12L-hydroxy-8,10-heptadecatrienoic 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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thromboxane synthase, [1-<sup>14</sup>C]PGH<sub>1</sub> (spec. act. 0.34 Ci/mol) and [1-<sup>14</sup>C]PGH<sub>2</sub> (spec. act. 1.0 Ci/mol) were prepared as described [2]. The endoperoxides moved slightly ahead of PGB<sub>1</sub> on silica gel G thin-layer chromatography (TLC) and were converted to PGF<sub>1 $\alpha$</sub>  and PGF<sub>2 $\alpha$</sub> , respectively, by SnCl<sub>2</sub> reduction. 9 $\alpha$ ,11 $\alpha$ -Azo-15(S)hydroxyprosta-5(*cis*), 13(*trans*)-dienoic acid (APA) was generously given by Dr E. J. Corey. 9 $\alpha$ ,11 $\alpha$ -Epoxymethano-15(S)hydroxyprosta-5(*cis*), 13(*trans*)-dienoic acid (EMPA) and TXB<sub>2</sub> were kindly provided by the Upjohn Company. 2-Iso-propyl-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<sub>2</sub> 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-<sup>14</sup>C]-PGH<sub>1</sub> (154  $\mu$ M) or [1-<sup>14</sup>C]PGH<sub>2</sub> (120  $\mu$ M). 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).

## 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  $\text{TXB}_2$  and HHT, respectively. Unlabeled  $\text{TXB}_2$  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  $\text{TXB}_2$  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  $37^\circ\text{C}$  with  $[1-^{14}\text{C}]\text{-PGH}_2$  (120  $\mu\text{M}$ ). Reactions were stopped after either 10 s or 2 min by the addition of 80 vol. methanol to convert  $\text{TXA}_2$  to *O*-methyl  $\text{TXB}_2$  [4]. After 1 h at  $24^\circ\text{C}$ , 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.  $\text{SnCl}_2$  in ethanol (5 mg/ml) to reduce  $\text{PGH}_2$  to  $\text{PGF}_{2\alpha}$  [5]. In this case, products were extracted after 2 min at  $24^\circ\text{C}$ . Ether extracts were methylated and analyzed by TLC, using solvents (a–c) (section 2.1). For analyses with solvent (a),  $\text{TXB}_2$  was added prior to methylation as an internal reference.

## 3. Results

### 3.1. Products formed from $\text{PGH}_1$ and $\text{PGH}_2$

Figure 1 shows thin-layer radiochromatograms of products isolated after incubations of  $[1-^{14}\text{C}]\text{PGH}_1$  (upper panel) and  $[1-^{14}\text{C}]\text{PGH}_2$  (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,  $\cdot\text{CH}_3$ ), 337 (M-31,  $\cdot\text{OCH}_3$ ), 321 (M-15-32,  $\cdot\text{CH}_3 + \text{CH}_3\text{OH}$ ), 297 (base peak, M-71,  $\cdot\text{CH}_2(\text{CH}_2)_3\text{CH}_3$ ), 278 (M-90,  $(\text{CH}_3)_3\text{SiOH}$ ) and 225 (M-143,  $\cdot\text{CH}_2(\text{CH}_2)_5\text{COOCH}_3$ ). Small amounts of  $\text{TXB}_1$  (*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  $\times$  90), 368, 325, 301, 297, 258, 243 (base peak), 217 and 173) were also detected.

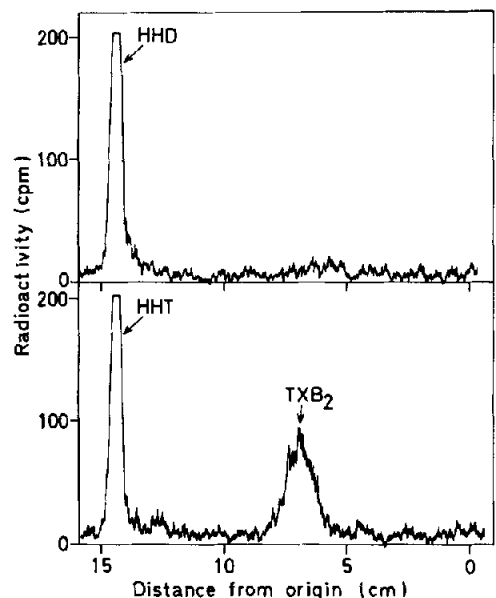


Fig.1. Thin-layer radiochromatograms of products formed from  $[1-^{14}\text{C}]\text{PGH}_1$  (upper) and  $[1-^{14}\text{C}]\text{PGH}_2$  (lower) during incubations ( $24^\circ\text{C}$ , 1 min) with partially purified thromboxane synthase from human platelets. HHD, 12-hydroxy-8,10-heptadecadienoic acid; HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid and  $\text{TXB}_2$ , thromboxane  $B_2$  (solvent (a), section 2.1.).

The two major products formed from  $\text{PGH}_2$  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,  $\text{CH}_2\text{CH}=\text{CH}(\text{CH}_2)_3\text{COOCH}_3$ )) and  $\text{TXB}_2$  (*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  $\times$  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  $[1-^{14}\text{C}]\text{PGH}_2$  in the presence of the inhibitors. Two dose–inhibition curves were deter-

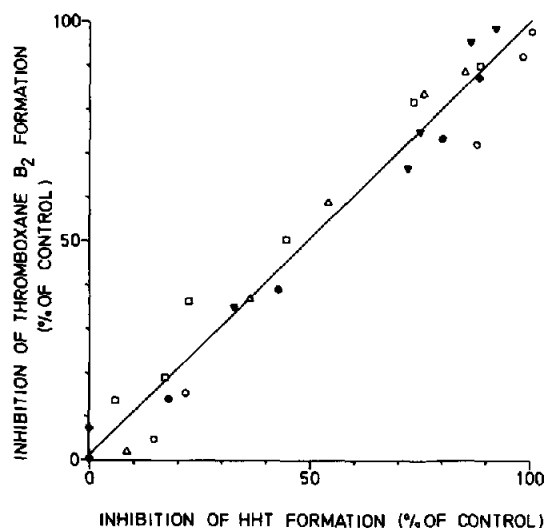


Fig. 2. Linear regression of the inhibition of thromboxane B<sub>2</sub> and HHT formation by L-8027 ( $\Delta$ ), N-0164 ( $\circ$ ), imidazole ( $\square$ ), 9 $\alpha$ ,11 $\alpha$ -epoxymethano-15(S)-hydroxyprosta-5(*cis*), 13(*trans*)-dienoic acid ( $\bullet$ ) and 9 $\alpha$ ,11 $\alpha$ -azo-15(S)-hydroxyprosta-5(*cis*), 13(*trans*)-dienoic acid ( $\blacktriangledown$ ).

mined for each substance, one for TXB<sub>2</sub> and one for HHT formation. For a given inhibitor these curves were always nearly identical. Analogous experiments with [ $1^{14}\text{C}$ ]PGH<sub>1</sub> as substrate also gave similar dose-inhibition curves for HHT formation. In fig. 2 the inhibition of TXB<sub>2</sub> 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<sub>2</sub> to TXB<sub>2</sub>

PGH<sub>2</sub> 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<sub>2</sub> to *O*-methyl TXB<sub>2</sub>. Parallel incubations, stopped by addition of SnCl<sub>2</sub> in ethanol, showed that no endoperoxide remained after 10 s incubation. At this time the reaction mixture contained 21% TXA<sub>2</sub> (measured as *O*-methyl TXB<sub>2</sub>) 22% TXB<sub>2</sub> and 55% HHT (fig. 3, upper). After 2 min incubation the products consisted of < 3% TXA<sub>2</sub>, 42% TXB<sub>2</sub> and 55% HHT (fig. 3, lower).

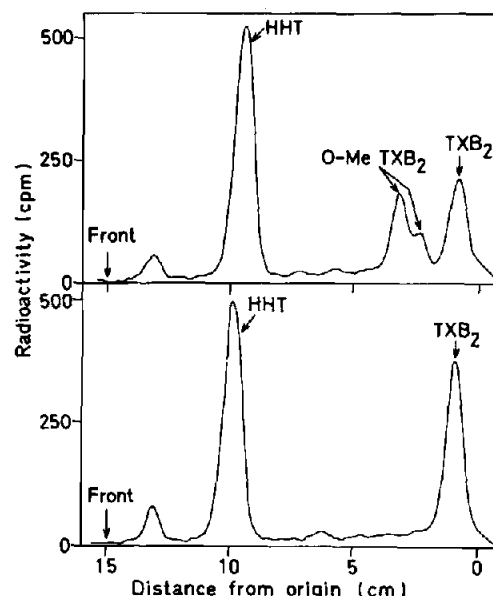


Fig. 3. Thin-layer radiochromatograms of products formed from [ $1^{14}\text{C}$ ]PGH<sub>2</sub> during incubations with partially purified thromboxane synthase at 37°C for 10 s (upper) and 2 min (lower). Incubations were stopped by the addition of 80 vol. methanol, to convert thromboxane A<sub>2</sub> to *O*-methyl thromboxane B<sub>2</sub> (O-Me TXB<sub>2</sub>) (Solvent (c), section 2.1.).

## 4. Discussion

HHT 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<sub>2</sub> 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<sub>2</sub> by the same fractions that formed TXB<sub>2</sub> [2]. Furthermore, the purified enzyme converted PGH<sub>1</sub> predominantly to HHT (fig. 1). Five structurally unrelated inhibitors of thromboxane formation [3] inhibited TXB<sub>2</sub> and HHT formation from PGH<sub>2</sub> identically (fig. 2) and in addition inhibited HHT formation from PGH<sub>1</sub>. These results strongly suggest that platelet thromboxane synthase catalyzes the conversion of prostaglandin endoperoxides to C<sub>17</sub> hydroxy acids plus malondial-

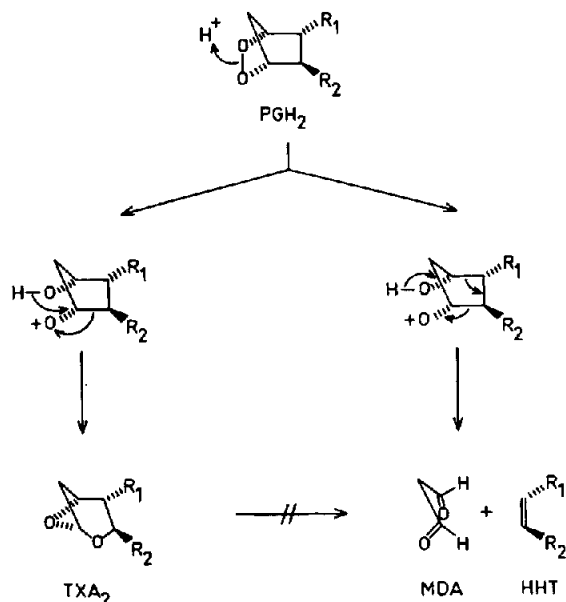


Fig.4. Possible mechanism of thromboxane  $\text{A}_2$  ( $\text{TXA}_2$ ), HHT and malondialdehyde (MDA) formation from  $\text{PGH}_2$ , catalyzed by thromboxane synthase.

dehyde. This reaction might or might not involve thromboxane A as an intermediate.  $\text{TXA}_2$  was generated during a short incubation of  $\text{PGH}_2$  with purified thromboxane synthase and the products formed during its spontaneous decay were determined (fig.3). The results showed that  $\text{TXA}_2$  was exclusively transformed to thromboxane  $\text{B}_2$  and that HHT was formed independently. This is consistent with the predominant formation of HHT from  $\text{PGH}_1$  and the approx. 1:1 formation of  $\text{TXB}_2$  and HHT from  $\text{PGH}_2$

(fig.1). A hypothetical reaction mechanism is outlined in fig.4. It is postulated that the isomerization of  $\text{PGH}$  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  $\text{C}_{17}$  hydroxy acid plus malondialdehyde. The results showing that  $\text{TXA}_2$  is exclusively converted to  $\text{TXB}_2$  has important implications for the quantitative determination of thromboxane synthesis in various biological systems.

#### Acknowledgements

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