Production of platelet thromboxane A₂ inactivates purified human platelet thromboxane synthase

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Human platelet thromboxane synthase was partially purified by DEAE-cellulose, Affi-Gel Blue, and Sephacryl S-300 chromatography to a specific activity of 259 nmol of thromboxane B₂/min per mg. Thromboxane synthase retained 75–90% of its enzymic activity when bound to phenyl-Sepharose. The immobilized enzyme was inactivated at pH 3.0 and inhibited by 1-benzylimidazole and U-63,557A. The ability of the enzyme to produce thromboxane A₂ from prostaglandin H₂ was dramatically reduced by multiple additions of prostaglandin H₂. Our data suggest that the production of thromboxane A₂ by the enzyme is self-limiting and that the enzyme is inactivated during the reaction.

INTRODUCTION

TXA₂ is an extremely potent but labile compound that is a key mediator in platelet secretion [1]. In resting platelets the biosynthesis of TXA₂ is insignificant. However, when platelets are activated it is rapidly synthesized and released. The biosynthesis of TXA₂ is a multistep process involving the release of arachidonic acid from membrane phospholipids, the oxygenation and cyclization of arachidonate to the prostaglandin endoperoxides (PGG₂ and PGH₂), and the rearrangement of the endoperoxides to form TXA₂. Despite the efforts of a number of laboratories [2–4], thromboxane synthase (which catalyses the synthesis of TXA₂ from PGH₂), has not been purified to homogeneity and is not well characterized. The present investigation was designed to investigate the effect of immobilizing thromboxane synthase in a lipophilic environment on the production of both TXA₂ and HHT. To date it is still unclear whether TXA₂ and HHT are both products of the reaction catalysed by thromboxane synthase, even though the formation of both TXA₂ and HHT are inhibited to the same degree by a variety of inhibitors. Our results show that the enzymic conversion of PGH₂ to both TXA₂ and HHT is limited and that the enzyme is inactivated during TXA₂ production.

EXPERIMENTAL

Materials

Lubrol PX was purchased from Sigma Chemical Co. Arachidonic acid was obtained from Nu-Chek, Elysian, MN, U.S.A., and 1-benzylimidazole from Aldrich Chemical Co. Prostaglandin standards, TXB₂ and sodium 5-(3'-pyridinylmethyl)benzofuran-2-carboxylate (U-63,557A) were kindly supplied by the Upjohn Company, Kalamazoo, MI, U.S.A. Affi-Gel Blue was purchased from Bio-Rad Laboratories, DE-52 cellulose from Whatman, and Sephacryl S-300 and phenyl-Sepharose CL-4B from Pharmacia. [5,6,8,9,11,12,14,15-³H]TXB₂ was obtained from New

England Nuclear. All other reagents were analytical grade and obtained from commercial sources.

Preparation of human platelet thromboxane synthase

Human platelet concentrates were obtained from the Rush Medical Center blood bank (Chicago, IL, U.S.A.) and were usually used within 2 days of their expiration date. Platelet concentrates were pooled and centrifuged at 200 *g* for 10 min to remove contaminating red blood cells. The platelets were then sedimented by centrifugation at 2000 *g* for 15 min. The platelet pellet was resuspended in 50 mM-potassium phosphate buffer, pH 7.4, and washed twice with the same buffer. The pellets from the last centrifugation were resuspended with a Polytron homogenizer (4 × 30 s, at a setting of 10) and centrifuged at 8000 *g* for 15 min. The supernatant was further centrifuged at 105000 *g* for 1 h in a Beckman ultracentrifuge. The microsomal pellets were suspended by homogenization in the same buffer in a volume equivalent to three times the wet weight of the platelets. Microsomes were divided into portions and stored at –70 °C.

Human platelet microsomes (10 ml) were then mixed with an equal volume of 50 mM-potassium phosphate, pH 7.4, containing 2 mM-EDTA, 20% (v/v) glycerol and 2% (v/v) Lubrol PX. After 1 h of stirring at 4 °C the mixture was centrifuged at 105000 *g* for 1 h. The supernatant was added to 10 ml of DEAE-cellulose that had been equilibrated in 50 mM-potassium phosphate buffer, pH 7.4, containing 0.1% Lubrol PX, 1 mM-EDTA and 10% (v/v) glycerol. After stirring the solubilized enzyme in the presence of DEAE-cellulose for 30 min at 4 °C the mixture was centrifuged and the supernatant removed. The DEAE-cellulose was washed twice with 10 ml of equilibrating buffer. The supernatant fractions were combined and adsorbed batchwise to 15 ml of Affi-Gel Blue that had been equilibrated in the buffer used for the DEAE-cellulose. The mixture was stirred for 30 min at 4 °C and then centrifuged. The Affi-Gel was then washed several times in the equilibrating buffer. Thromboxane synthase activity was eluted from the Affi-Gel Blue by washing twice with 15 ml of 50 mM-

Abbreviations used: PG, prostaglandin; TX, thromboxane; HHT, 12-L-hydroxy-5,8,10-heptadecatrienoic acid.

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potassium phosphate buffer, pH 7.4, containing 0.1% Lubrol PX, 1 mM-EDTA, 10% (v/v) glycerol and 2.5 M-KCl. The eluted enzyme activity was then dialysed against the elution buffer minus the KCl. The dialysed enzyme preparation was either stored at -70°C or further purified by gel filtration using a Sephacryl S-300 column. Protein concentrations of enzyme samples were estimated by measuring their A_{280} and/or by the method of Bradford [5].

Thromboxane synthase assay

Since TXA_2 is extremely short-lived and is converted into TXB_2 in aqueous medium, enzyme activities were assayed by measuring the formation of TXB_2 immuno-reactivity from PGH_2 . The reaction mixture contained 5 nmol of PGH_2 , enzyme fraction and 50 mM-potassium phosphate, pH 7.4, in a final volume of 1 ml. The reaction was initiated with PGH_2 and allowed to proceed at 23°C for 3 min. The reaction was terminated by acidification with 0.025 ml of 2 M-HCl. The mixture was then neutralized and diluted for TXB_2 measurement by radioimmunoassay.

Radioimmunoassay for TXB_2

TXB_2 immunoreactivity was by a sensitive and specific radioimmunoassay as described previously [6]. Each sample was run in duplicate at three dilutions and values were obtained within the linear portion of the standard curve. Antiserum against TXB_2 was obtained by the repeated immunization of rabbits with TXB_2 coupled to bovine serum albumin. The radioimmunoassay was performed by incubating 0.2 ml of TXB_2 standards or samples with 0.1 ml of diluted antiserum (final resultant dilution of 1:5000) and 0.1 ml of ^3H -labelled TXB_2 (10000 c.p.m.). The incubation was carried out at room temperature for 1 h. Separation of the bound and free antigen was achieved using γ -globulin-coated charcoal. The supernatant obtained after centrifugation was counted in a Packard liquid-scintillation counter. The bound:total ratio was calculated for each sample and the concentration of thromboxane B_2 determined from a standard curve after logit transformation [7]. The antiserum showed little cross-reactivity with $\text{PGF}_{2\alpha}$ (0.02%), PGE_2 (0.02%), PGD_2 (0.02%) or 6-oxo $\text{PGF}_{1\alpha}$ (0.01%). The sensitivity of the assay was 15 pg/200 μl (i.e. $B/B_0 = 80\%$).

PGH_2 synthesis and purification

PGH_2 was prepared using arachidonic acid and ram seminal vesicle microsomes as previously described [8,9]. The biosynthetically produced PGH_2 was purified over both a gravity flow silicic acid column and a cyanobonded phase h.p.l.c. self-packed column using a hexane/propan-2-ol gradient [10]. The identity and purity of the PGH_2 was confirmed by NH_3 direct chemical ionization mass spectrometry [11,12]. The biological activity of purified PGH_2 was assayed by its ability to aggregate human platelets. [^{14}C] PGH_2 (4.5 Ci/mol) was prepared as described above. During the synthesis and purification of PGH_2 some HHT was also produced. The HHT was isolated by h.p.l.c. and identified by NH_3 direct chemical ionization mass spectrometry also [12]. This HHT was used as a standard on the octadecyl (C_{18}) reversed-phase h.p.l.c. column.

Characterization of products formed from PGH_2

Thromboxane synthase reaction supernatants were collected and acidified to pH 3.0. The acidified samples were loaded onto Sep-Pak C_{18} cartridges that had been prewashed with 5 ml of methanol and 5 ml of 0.5% aqueous acetic acid. After the sample had been added, the column was washed with 5 ml of 25% (v/v) methanol containing 0.5% acetic acid. Finally, the eicosanoid products were eluted with two 2 ml portions of 90% (v/v) methanol containing 0.5% acetic acid. The eluted samples were dried under N_2 , taken up in 50 μl of methanol and injected (five injections, 10 μl each) onto a reversed-phase h.p.l.c. column (u-PAK C_{18} , 5 μm ; Varian Associates, Palo Alto, CA, U.S.A.). The eicosanoids were eluted with a gradient of 0.1% aqueous acetic acid (pH 3.2)/acetonitrile from 72:28 to 0:100 (v/v) at 1.5 ml/min.

RESULTS AND DISCUSSION

Table 1 represents a typical purification of thromboxane synthase from human platelet microsomes. A 259-fold purification was obtained with a 17% recovery of enzyme activity. The M_r of the enzyme was determined by measuring the elution volume of enzymic activity from a Sephacryl S-300 column equilibrated with 50 mM-potassium phosphate, pH 7.4, containing 0.1% Lubrol PX, 1 mM-EDTA and 10% (v/v) glycerol. The M_r of the platelet thromboxane synthase was calculated to be

Table 1. Purification of thromboxane synthase from human platelet microsomes

Microsomes were prepared from 600 ml of human platelet concentrates by differential centrifugation. Catalytic activity is expressed in nmol of TXB_2 formed/min under the conditions described in the Experimental section. All values represent the mean of four preparations, $\pm$ S.E.M.

Procedural step	Protein (mg)	Activity (nmol/min)	Specific activity (nmol/min per mg)	Yield (%)	Purification (-fold)
Microsomes	249 $\pm$ 42	234 $\pm$ 88	0.94 $\pm$ 0.23	100	1.0
Solubilized	50 $\pm$ 13	152 $\pm$ 44	3.01 $\pm$ 0.97	65 $\pm$ 4	3.2 $\pm$ 0.5
DEAE-cellulose	29 $\pm$ 7	108 $\pm$ 27	3.67 $\pm$ 0.99	46 $\pm$ 9	3.9 $\pm$ 0.5
Affi-Gel Blue	3.6 $\pm$ 1.1	68 $\pm$ 17	18.6 $\pm$ 3.4	29 $\pm$ 8	19.8 $\pm$ 2.6
Sephacryl S-300	0.20 $\pm$ 0.15	39 $\pm$ 16	195.0 $\pm$ 63.4	17 $\pm$ 6	259.0 $\pm$ 53.1

Fig. 1. Effect of increased temperatures on the catalytic activity of human platelet thromboxane synthase

The relative amounts (%) of TXB₂ (○) and HHT (△) formation by thromboxane synthase after 5 min incubations at various temperatures are plotted. Each point represents the mean of two experiments done in duplicate.

165000 by extrapolation from a plot of the log of the M_r of standard proteins versus their relative elution volumes from the columns.

The production of both TXB₂ and HHT by the highly purified platelet enzyme was verified. In addition, the stability of these enzymic activities to increased temperatures was investigated. The temperature at which 50% of the enzymic activity remained (T_{50}) after a 5 min incubation period was 51 °C for TXB₂ formation and 53 °C for HHT formation (Fig. 1). The T_{50} values for the platelet enzyme are very similar to those reported for the lung enzyme [13].

Thromboxane synthase binds avidly to phenyl-Sepharose CL-4B and cannot be washed off with 50 mM-potassium phosphate, pH 7.4, containing 0.2% Lubrol PX. Experiments conducted with the enzyme bound to phenyl-Sepharose have shown that the enzyme retains most (i.e. 75–90%) of its activity when bound. The immobilized enzyme can be inactivated at pH 3.0 and inhibited with 1-benzylimidazole and sodium 5-(3'-pyridinylmethyl)benzofuran-2-carboxylate (U-63,557A), demonstrating that the measured biological activity was in fact enzymically mediated (Fig. 2). The thromboxane synthase inhibitors inhibited both TXB₂ and HHT production.

The ability of the phenyl-Sepharose-bound thromboxane synthase to produce TXB₂ was non-linear with increasing concentrations of PGH₂ [i.e., the higher the concentration of PGH₂ incubated with the enzyme the lower the amount (ng) of TXB₂ produced per nmol of PGH₂ added]. In fact, the ability of the immobilized thromboxane synthase to synthesize TXB₂ was dramatically reduced by multiple additions of PGH₂. Briefly, 10 nmol of PGH₂ were added to the immobilized enzyme for 5 min at 23 °C. The reaction was terminated by removing the enzyme by centrifugation. The supernatant

Fig. 2. Inhibition of immobilized thromboxane synthase

Human platelet thromboxane synthase was immobilized on phenyl-Sepharose. The immobilized enzyme was treated with 15 nmol of [¹⁴C]PGH₂ for 5 min (a). The amounts of TXB₂ and HHT produced were determined by the percentage of total radioactivity eluted from the C₁₈ reverse-phase h.p.l.c. column in the fractions where the TXB₂ and HHT standards were eluted. The immobilized enzyme was preincubated for 5 min with either 1 mM-1-benzylimidazole (b) or 0.1 mM-U63,557A (c). Both of these thromboxane synthase inhibitors inhibited TXB₂ and HHT production.

was collected and assayed for TXB₂. The immobilized enzyme was washed with phosphate buffer and incubated with an additional 10 nmol of PGH₂. Only 19 ng of TXB₂ was synthesized from the second addition of PGH₂, whereas 58 ng of TXB₂ had been produced during the first incubation. When a sample of the immobilized enzyme was incubated and washed in the absence of PGH₂ 78% of the enzyme activity was retained (Table 2). Thus the enzyme appears to be inactivated during TXB₂ production.

The inactivation of thromboxane synthase was dependent on the concentration of PGH₂ used in relationship to the amount of enzyme present. For example when 6 µg of immobilized enzyme was incubated with two additions of 5 nmol of PGH₂, the second incubation of PGH₂ produced 61% less TXB₂ than did

Table 2. Decreased TXB₂ production with multiple additions of PGH₂

Thromboxane synthase was immobilized on heads of phenyl-Sepharose. The immobilized enzyme was incubated with 10 nmol of PGH₂ for 5 min, then washed with phosphate buffer and reincubated with another 10 nmol of PGH₂. This process was repeated once more. The TXB₂ in the supernatants of the enzyme reactions was quantified by radioimmunoassay. ○ represents the washing and centrifugation of the thromboxane synthase bound to phenyl-Sepharose beads; ↓ represents the addition of 10 nmol of PGH₂.

PGH ₂ added	TXB ₂ production	
	(ng/5 min per ml)	(%)
↓ ○	58	100
↓ ↓ ○ ○	19	33
↓ ↓ ↓ ○ ○ ○	8	14
○ ○ ↓	45	100 (78%)
○ ○ ↓ ↓	15	33

the first incubation. When 12 µg of immobilized enzyme was incubated with two additions of 10 nmol of PGH₂ the second incubation with PGH₂ produced 67% less TXB₂ than did the first incubation. Thus, when the ratio of enzyme to substrate is constant the decrease in TXB₂ production observed is similar. On the other hand, when 6 µg aliquots of immobilized enzyme were incubated with two additions of 2 nmol, 5 nmol or 10 nmol of PGH₂ the decrease in TXB₂ production was 45%, 61% and 86% respectively.

The decreased production of both TXB₂ and HHT with subsequent additions of PGH₂ was further investigated. Highly purified [1-¹⁴C]PGH₂ was incubated with phenyl-Sepharose-immobilized thromboxane synthase for 5 min at 23 °C. The reaction mixtures were centrifuged and the eicosanoids in the supernatant were extracted on a C₁₈-Sep Pak. The immobilized enzyme was washed with phosphate buffer and the reaction repeated. The production of TXB₂ and HHT in each incubation was quantified by the percentage of radioactivity eluting from the reversed-phase h.p.l.c. in the same fraction as TXB₂ and HHT standards. The synthesis of both TXB₂ and HHT decreased substantially upon the second and third incubation of the enzyme and PGH₂. The percentage of radioactivity in the TXB₂ peak went from 17.3% with the first incubation to 10.6% with the second incubation to 6.3% with the third incubation. The percentage of radioactivity in the HHT peak decreased from 22.2% in the first incubation, to 15.5% in the second incubation, to 10.2% in the third incubation (Fig. 3). Apparently the enzyme is being inactivated during the conversion of PGH₂ to TXB₂ and HHT.

The inactivation of thromboxane synthase must occur by a different mechanism than that shown for the inactivation of prostaglandin endoperoxide synthase (cyclo-oxygenase). Cyclo-oxygenase is inactivated by free

Fig. 3. Substrate inactivation of thromboxane synthase

Thromboxane synthase immobilized on phenyl-Sepharose was incubated with 15 nmol of [1-¹⁴C]PGH₂ for 5 min (a). The eicosanoids in the reaction supernatant were extracted on C₁₈-Sep Paks and separated by h.p.l.c. The amounts of TXB₂ and HHT produced were determined by the percentage of total radioactivity eluted from the h.p.l.c. with the TXB₂ and HHT standards. The immobilized enzyme was washed in phosphate buffer and incubated with a second 15 nmol of [1-¹⁴C]PGH₂ (b). Finally, the enzyme was incubated with a third sample of [1-¹⁴C]PGH₂ (c). The production of TXB₂ and HHT decreased with each addition of PGH₂.

radicals generated during the peroxidative reduction of PGG₂ to PGH₂, which would be a most unlikely process for the thromboxane synthase since it was inactivated when purified PGH₂, containing no PGG₂, was used as substrate [14]. Ullrich and coworkers [15] have suggested that thromboxane synthase is a haemoprotein of the P-450 type; if true, the observed substrate inactivation of the enzyme may involve a modification of the haem group similar to that described for prostacyclin synthase [16]. If the enzyme's inactivation involves a modification of a haem at the active site, then our data would suggest that the haem participates in the production of both TXB₂ and HHT. Another explanation of thromboxane synthase inactivation might be the depletion of a required cofactor during the reaction. It is interesting to note that thromboxane synthase binds to an Affi-Gel Blue column,

which is generally thought to have a biospecific affinity for nucleotide-requiring enzymes. However, we have been unable to elute thromboxane synthase from Affi-Gel Blue using up to 1 mM concentrations of NAD⁺, NADH, NADP⁺, NADPH, ATP or ADP. In addition, none of the compounds were capable of reactivating thromboxane synthase activity lost during the incubation of the enzyme with PGH₂. Although additional work is necessary to identify the mechanism of enzyme inactivation, this inactivation may well represent an important regulation *in vivo* of TXA₂ production.

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