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BIOSYNTHESIS OF THROMBOXANE B_2

Introduction

The transformation of arachidonic acid in human platelets into a non-prostanoate product, thromboxane (Tx) B_2, was first described by M. Hamberg and B. Samuelsson [1]. A further study on the mechanism of this bioconversion led to the discovery of a highly unstable intermediate designated TxA_2 which induced irreversible platelet aggregation and contracted arterial smooth muscle [2]. The formation of thromboxanes from arachidonic acid (AA) takes place by two-step biosynthesis. The first step is the conversion of arachidonic acid to an unstable prostaglandin endoperoxide (PGH_2) catalyzed by prostaglandin endoperoxide synthetase (EC 1.14.99.1). The second step consists in the formation of TxA_2 and consequently TxB_2 from PGH_2 affected by thromboxane synthetase. In addition to affording TxA_2, this enzyme catalyzes the fragmentation of PGH_2 to a C_{17}-hydroxy acid (HHT) and malondialdehyde [3].

Tx-synthetase is a microsomal enzyme which has been isolated from platelets and lung [4, 5, 6], but mainly due to its lability has not yet been purified to homogeneity.

Experiments with partially purified enzyme have shown that the enzymatic formation of TxA_2 is limited and the enzyme itself is inactivated during catalysis [7].

Up to date some thromboxane biosynthesis methods have been described by several investigators [8, 9], but no convenient preparative method has been published yet.

The present paper reports on the results of our studies aimed at investigating different routes of TxB_2 biosynthesis and determining optimal conditions for TxB_2 preparative biosynthesis.

Materials and methods

Materials. Arachidonic acid and PG-standards were the gifts from the Pilot-Production Plant of the Institute of Chemistry, Tallinn. [1-^{14}C]arachidonic acid was purchased from Amersham; hydroquinone, glutathione, bovine serum albumin, diethyldithiocarbamic acid Na salt (DEDTC) and 2-(N-morpholino)ethanesulfonic acid (MES) from Sigma; Tris, EDTA, hemin, N-ethylmaleimide and Tween 20 from Serva; Fractogel DEAE 650 and precoated silica gel 60 TLC sheets from Merck; Ultrogel ACA 34 from LKB; all other chemicals and solvents were analytical grade, Reakhim.

Purification of cyclooxygenase from ram seminal vesicles. Ram seminal vesicles (RSV) were obtained from a local slaughter house where they were immediately frozen with liquid nitrogen and stored at $-70\,°C$. The frozen RSV were powdered with an MPW-302 homogenizer (Mechanica Precyzyna, Poland) in liquid nitrogen and added to Tris-HCl buffer (20 mM, pH 8.0) containing 10 mM EDTA and 1 mM DEDTC. This mixture was then homogenized and centrifuged at 2000 g for 20 min. The supernatant was filtered through cheese cloth and recentrifuged at

150 000 g for 1 h. The pelleted microsomes were suspended in the same buffer containing 5 mM EDTA and 0.5 mM DEDTC and centrifuged at 150 000 g for 1 h. The final microsomal pellet was solubilized in the same buffer, containing 1% (v/v) Tween-20, 1 mM EDTA, 0.1 mM DEDTC, for 0.5 h and centrifuged (150 000 g, 1 h). The supernatant was divided into portions and stored at −70 °C.

An aliquot of supernatant was applied to a Fractogel DEAE 650 (s) 12×160 mm column equilibrated with the same buffer used for solubilization, except for the Tween-20 concentration of 0.1%. The elution was started with the same buffer ($v_1 = v_o$ ml) and continued gradiently up to 100% of MES buffer (20 mM, pH 4.4) containing all the above additives.

The cyclooxygenase fraction eluted in a sharp narrow peak was concentrated on an Amicon XM-50 filter and brought into an Ultrogel ACA 34 16×700 mm column equilibrated with 20 mM Tris-HCl buffer, pH 8.0, containing 0.1% Tween-20, 1 mM EDTA and 0.1 mM DEDTC [10]. Cyclooxygenase was eluted from 91 to 100 ml. After concentration on the Amicon XM-50 filter the enzyme preparation was stored at −70 °C. The enzyme cyclooxygenase activity was estimated by measuring oxygen consumption with a Clark oxygen electrode [11]. The specific activity of the enzyme preparations used in this work was 15—30 μmoles O_2/mg min. The protein content was measured by the method of Lowry. All the procedures were carried out between 0 and 4 °C.

Preparation of human platelet microsomes. Human platelet (HP) rich plasma was obtained from the Blood Service Center of Estonia, Tallinn, and was used on the day of expiration. The plasma concentrates were pooled and centrifuged at 200 g for 10 min to remove contaminant red blood cells. The platelets were then sedimented by centrifugation at 2000 g for 20 min. The washed pellet, resuspended in 50 mM Tris-HCl buffer, pH 8.0, was sonicated with a UD-11 Techpan Ultrasonic desintegrator (4×30 s, set 4) and centrifuged at 2000 g for 10 min. The clear supernatant was further centrifuged at 100 000 g for 1 h. The microsomal pellet was suspended in the same buffer, divided into portions and stored at −70 °C.

Thromboxane synthesis. Since TxA_2 is extremely short-lived in aqueous medium, the yield of thromboxane synthesis was estimated by measuring the formation of [1-^{14}C]-TxB_2. Using [1-^{14}C]-PGH_2 as a substrate, the incubation mixture contained 50 mM Tris-HCl, pH 8.0, and an enzyme preparation in the final volume of 0.2 ml. The reaction was initiated with the addition of 7.5 nmoles of substrate in 2μl of acetone and allowed to proceed at 30 °C for 5 min. In the case of arachidonic acid, the reaction mixture contained an enzyme preparation, 50 mM Tris-HCl buffer, (pH 8.0), 0.7 mM hydroquinone and 2 μM hemin in the final volume of 0.2 ml. The reaction was initiated with the addition of 20 nmoles of [1-^{14}C]AA (100 000 cpm) and stirred at 30 °C for 15 min. In both cases the reaction was terminated by acidification with 2M citric acid up to pH 3.0, extracted thrice with 2 ml of diethyl ether, washed with 2 ml of water and dried over $MgSO_4$. The solvent was evaporated in an Ar stream and the residue was separated after adding unlabelled standards by TLC using chloroform-methanol-acetic acid (90:10:2) as an eluting mixture. The spots were visualized with an anisaldehyde solution [12]. The plates were sectioned, the sections were scraped into vials and counted on an LKB Betarack Scintillation Counter.

Synthesis and purification of [1-^{14}C]PGH_2. [1-^{14}C]PGH_2 was synthesized from [1-^{14}C]labeled arachidonic acid using the partially acetone-pentane delipidized powder from ram seminal vesicles in the presence of 0.7 mM hydroquinone and 3 mM p-mercury benzoate as described previously [13, 14, 15].

Results and discussion

The incubation of human platelets microsomes with [1-^{14}C]PGH_2 afforded 4 main radioactive products: PGE_2, PGD_2, TxB_2 and HHT. The content of the latter was not determined.

Fig. 1 shows the results of an experiment in which increasing enzyme amounts were tested. It is clear that the response linearity occurs only at low enzyme concentrations and the maximum yield of TxB_2 is achieved at a protein content of 1—2 mg/ml. Above this concentration the increasing, enzyme increments lead to a decrease of TxB_2 formation. The TxB_2 synthesis was inhibited to 50% with 1 mM imidazole, N-ethylmaleimide, a potent PGH—PGE isomerase inhibitor, was ineffective (the Table, *A*).

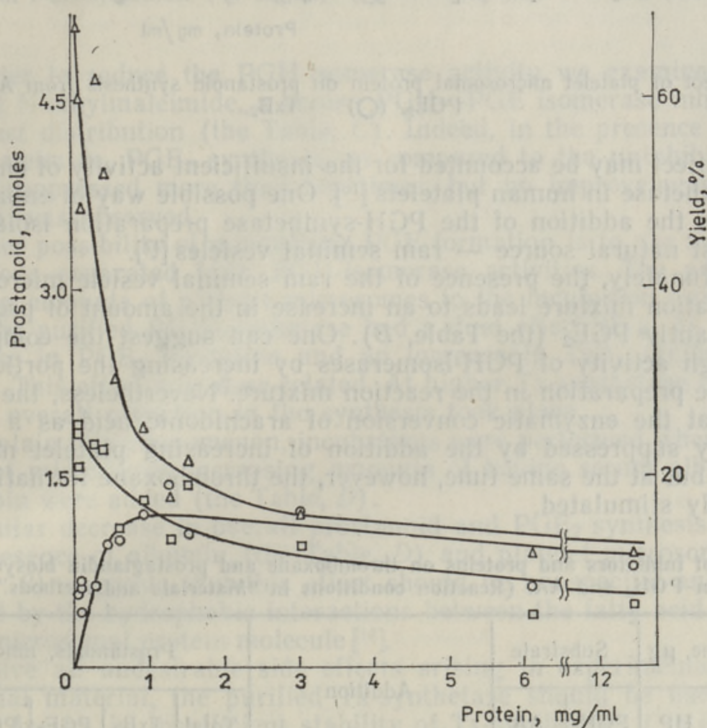

Fig. 1. Effect of platelet microsomal protein on prostanoid synthesis from PGH_2. (□) — PGD_2, (△) — PGE_2, (○) — TxB_2.

The main advantage of synthesis of TxB_2 from PGH_2 is the relative simplicity of product purification. But due to rather a complicated isolation and purification process of PGH_2 itself, the method described above is inefficient for TxB_2 synthesis on a preparative scale.

On the other hand, in addition to the Tx-synthetase activity, a considerable amount of PGH-synthetase activity is detected in the platelet microsomal fraction [11]. Consequently, the direct synthesis of TxB_2 from arachidonic acid may seem rather promising. The results (Fig. 2) obtained by incubation of arachidonic acid with the platelet microsomal fraction indicate that TxB_2 and prostaglandins were generated in somewhat lower yields and the protein concentration necessary for maximum TxB_2 formation is slightly higher than in the case of PGH_2.

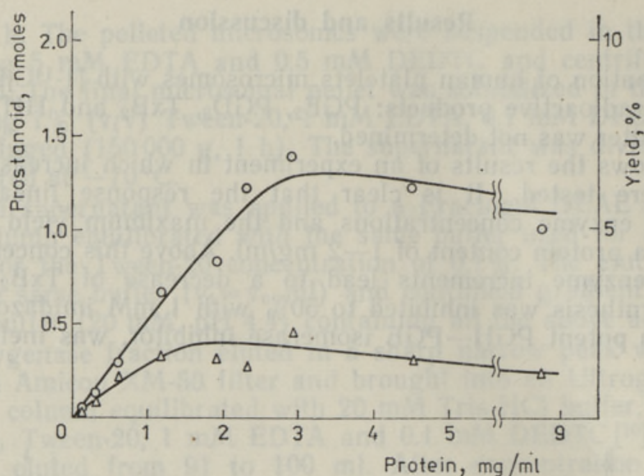

Fig. 2. Effect of platelet microsomal protein on prostanoid synthesis from AA. (△) — PGE_2, (○) — TxB_2.

This effect may be accounted for the insufficient activity of endogenous PGH-synthetase in human platelets [16]. One possible way of enhancing its activity is the addition of the PGH-synthetase preparation isolated from the richest natural source — ram seminal vesicles [17].

Unfortunately, the presence of the ram seminal vesicle microsomes in the incubation mixture leads to an increase in the amount of prostanoids, predominantly PGE_2 (the Table, *B*). One can suggest the compensation of the high activity of PGH-isomerases by increasing the portion of Tx-synthetase preparation in the reaction mixture. Nevertheless, the Table, *B* shows that the enzymatic conversion of arachidonic acid as a whole is drastically suppressed by the addition of increasing platelet microsome amounts, but at the same time, however, the thromboxane formation is not remarkably stimulated.

Effect of inhibitors and proteins on thromboxane and prostaglandin biosynthesis from PGH_2 and AA. (Reaction conditions in "Materials and methods".)

No	Enzyme, μg		Substrate		Addition	Prostanoids, nmoles				
	RSV	HP	PGH_2	AA		Total	TxB_2	PGF_2	PGE_2	PGD_2
	—	580	+	—	—	3.94	2.00	0.28	1.33	0.33
A	—	580	+	—	N-ethylmaleimide, 3 mM	3.64	2.08	0.39	0.92	0.25
	—	580	+	—	Imidazole, 1 mM	4.38	1.07	0.82	2.16	0.33
	260	—	—	+	—	15.47	—	0.46	14.23	0.78
	260	100	—	+	—	14.52	0.65	0.39	12.82	0.66
B	260	300	—	+	—	12.10	1.10	0.27	10.15	0.58
	260	600	—	+	—	9.00	1.82	0.20	6.46	0.52
	260	900	—	+	—	5.86	2.01	0.46	2.81	0.58
	520	300	—	+	—	12.04	1.06	0.32	10.0	0.66
C	520	300	—	+	N-ethylmaleimide, 3 mM	3.30	0.94	0.74	0.90	0.72
	20*	—	—	+	—	6.23	—	0.73	3.88	1.62
D	20*	—	—	+	Albumin, 1 mg/ml	6.31	—	0.55	3.79	1.97
	20*	—	—	+	Albumin, 10 mg/ml	2.15	—	0.04	1.52	0.59
	20*	—	—	+	Hemoglobin, 10 mg/ml	2.28	—	0.43	1.12	0.73

* purified

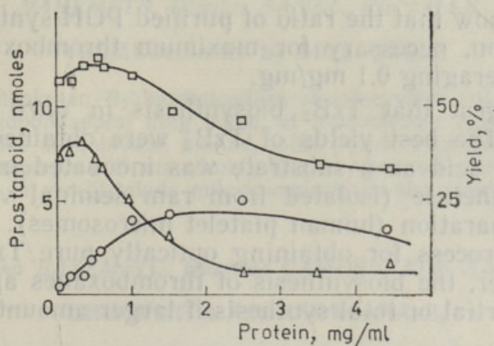

Fig. 3. Effect of platelet microsomal protein on prostanoid synthesis from AA in the presence of PGH-syntethase (0.1 mg/ml). (○) — TxB_2, (△) — PGE_2, (□) — ΣPG.

In order to reduce the PGH-isomerase activity we examined the influence of N-ethylmaleimide, a known PGH—PGE isomerase inhibitor, on the product distribution (the Table, *C*). Indeed, in the presence of 3 m*M* N-ethylmaleimide, PGE_2 synthesis, as compared to the uninhibited control, was suppressed more than ten times, but no improvement in TxB_2 formation was observed.

Another possibility of minimizing PGE formation is to use the enzyme preparations separated from PGH-isomerase activities. The addition of increasing amounts of platelet microsomes to the incubation mixture containing the purified PGH-synthetase had a dual effect (Fig. 3). Initially, a decrease in PGE_2 formation and an increase in TxB_2 synthesis were observed. This effect was dose-related. At higher Tx-synthetase concentrations the overall reduction in the synthesis took place.

To explain this phenomenon, incubations were performed where instead of platelet microsomes increasing amounts of bovine serum albumin and hemoglobin were added (the Table, *D*).

A similar decrease in overall prostanoid and PGE_2 synthesis observed in the presence of albumin (the Table, *D*) and platelet microsomal material (Fig. 3) suggests that this effect should be nonspecific and may be explained by the hydrophobic interactions between the fatty acid substrate and the microsomal protein molecule [18].

To solve all undesirable side effects arising in experiments with the microsomal material, the purified Tx-synthetase should be used. Unfortunately, due to the insufficient stability of Tx-synthetase it is impossible to obtain active preparations for preparative work.

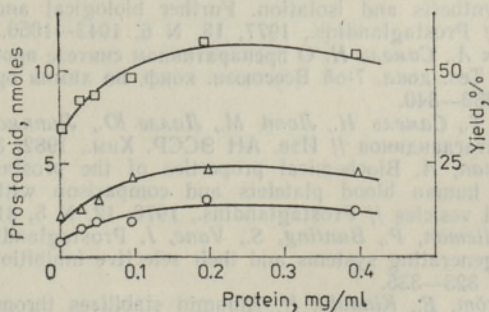

Fig. 4. Effect of PGH-synthetase on prostanoid synthesis from AA in the presence of platelet microsomal protein (0.64 mg/ml). (○) — TxB_2, (△) — PGE_2, (□) — ΣPG.

Figs 3 and 4 show that the ratio of purified PGH-synthetase to platelet microsomal fraction, necessary for maximum thromboxane formation is fairly constant, averaging 0.1 mg/mg.

Our data suggest that TxB_2 biosynthesis *in vitro* is not a highly efficient process. The best yields of TxB_2 were obtained in experiments where arachidonic acid as a substrate was incubated in the presence of purified PGH-synthetase (isolated from ram seminal vesicles) and the Tx-synthetase preparation (human platelet microsomes). This method can be considered a process for obtaining optically pure TxB_2 in milligram quantities. However, the biosynthesis of thromboxanes appears to be less promising than partial or total synthesis if larger amounts are desired.

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TROMBOKSAAN B_2 BIOSÜNTEES

On uuritud tromboksaan B_2 biosünteetilise saamise võimalusi, lähtudes prostaglandiin H_2-st või arahhidoonhappest ning leitud, et tromboksaan B_2 preparatiivseks sünteesiks milligrammskaalas on sobivaim meetod, mille puhul arahhidoonhapet inkubeeritakse kombineeritud ensüümpreparaadi manulusel. Viimane koosneb puhastatud tsüklooksügenaasist EC 1.14.99.1 ja vereliistakute mikrosoomfraktsioonist vahekorras 1:10.

Ивар ЯРВИНГ, Айно ВАХЕМЕТС, Нигулас САМЕЛЬ, Юло ЛИЛЛЕ

БИОСИНТЕЗ ТРОМБОКСАНА B_2

Изучен процесс биосинтеза тромбоксана B_2 исходя из простагландина H_2 или арахидоновой кислоты. Установлено, что для препаративных целей целесообразно инкубировать арахидоновую кислоту в присутствии комбинированного ферментного препарата, в состав которого входят очищенная циклооксигеназа (ЕС 1.14.99.1) из везикулярных желез барана и микросомные фракции из тромбоцитов человека в соотношении 1 : 10.