INHIBITORS OF THROMBOXANE SYNTHASE IN HUMAN PLATELETS

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1. Introduction

Thromboxanes A are a new group of compounds with extreme potency to induce platelet aggregation and smooth muscle contractions. They are derived from prostaglandin endoperoxides and have a half-life of only 30–40 s in aqueous solution (pH 7.4, 37°C). Thromboxanes B, the products of the spontaneous degradation, are biologically inactive [1]. Two enzymes are required to convert polyunsaturated fatty acids to thromboxanes: prostaglandin endoperoxide synthase (cyclooxygenase) and thromboxane synthase (fig.1). These enzymes from human platelets were recently solubilized and chromatographically resolved [2]. In the present report the ability of various compounds to inhibit thromboxane synthase from human platelets is described.

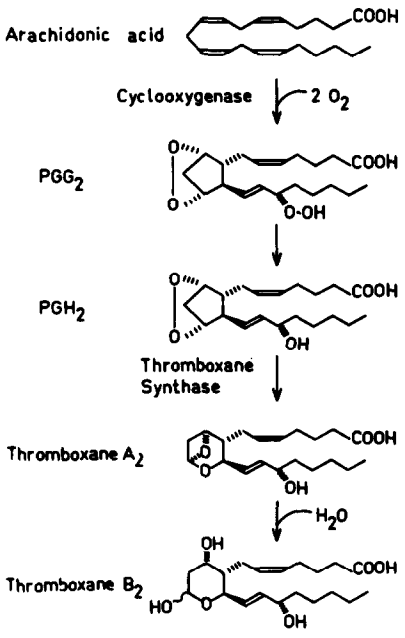

Fig.1. Enzymes required to convert arachidonic acid to thromboxane B_2.

2. Materials and methods

[1-^{14}C]Prostaglandin H_2 (1 Ci/mol) was prepared from [1-^{14}C]arachidonic acid (Radiochemical Centre, 55 Ci/mol) [3]. The incubation mixture contained 5 mM L-tryptophan, a cofactor for the conversion of prostaglandin G_2 to prostaglandin H_2 [4]. Phtalazinol was kindly provided by Dr T. Shimamoto. 9α,11α-Azo-15(S)-hydroxy-prosta-5(*cis*), 13(*trans*)-dienoic acid was a generous gift from Dr E. J. Corey. 9α,11α-Epoxymethano-15(S)-hydroxy-prosta-5(*cis*), 13(*trans*)-dienoic acid, 9α,11α-methanoepoxy-15(S)-hydroxy-prosta-5(*cis*), 13(*trans*)-dienoic acid, prostaglandins and thromboxane B_2 were kindly provided by the Upjohn Company. 5,8,11,14-Eicosatetrayonic acid, 2-isopropyl-3-nicotinyl-indole and sodium *p*-benzyl-4-[1-oxo-2-(4-chlorobenzyl)-3-phenyl propyl] phenyl phosphonate were generously provided by Hoffman La Roche, Labaz and Nelson Research, respectively. Imidazole was purchased from Sigma.

Abbreviation: ETA, 5,8,11,14-eicosatetraynoic acid

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2.1. *Platelet microsomes*

Washed platelets were prepared from human blood collected with 7.5% (v/v) of 77 mM sodium EDTA

[3]. The platelets from 400 ml blood were resuspended in 10 mM Tris–HCl, pH 7.4 to vol. 10 ml and sonicated at 0°C using a Branson sonifier model S-125 (setting no 4); six 5 s treatments separated by 1 min intervals for cooling. The fraction sedimenting between 1500 X *g* X 15 min and 100 000 X *g* X 60 min was resuspended in 50 mM Tris buffer, pH 7.4, to vol. 20 ml. Platelet disruption and subsequent manipulations were performed at 0–4°C.

2.2. *Enzyme assay*

Resuspended microsomes, 100 μl, (0.65 mg protein/ml) were preincubated with various presumed inhibitors at 24°C. After 15 s, 2.5 μg [1-^{14}C]prostaglandin H_2 was added and the incubation was continued for another 60 s at 24°C. The reaction was stopped by adding 0.3 ml diethyl ether/methanol/0.2 M citric acid (30:4:1, v/v/v) precooled to −70°C. After rapid mixing and phase separation the lower phase was frozen. The ether phase was aspirated, mixed with 10 μg unlabeled thromboxane B_2, methylated with etheral diazomethane and chromatographed on silica gel G thin-layer chromatography with 2% methanol in diethyl ether as solvent system. The thin-layer chromatograms were scanned for radioactivity on a Berthold Dünnschichtsscanner II and sprayed with phosphomolybdic acid to detect reference compounds. Appropriate zones were then scraped off into scintillation vials and counted in a Packard Tri Carb 3385 liquid scintillation counter, after the addition of 10 ml Permablend® (4.4 g/l toluene/ethanol, 4:1 v/v) to each vial. Percent conversion to thromboxane B_2 was obtained by relating the radioactivity which co-chromatographed with the internal reference thromboxane B_2 to the total radioactivity recovered from the plate.

3. Results and discussion

Radiochromatograms of products from prostaglandin H_2 incubations with native and boiled platelet microsomes are shown in fig.2. The positions of unlabeled thromboxane B_2 (A), prostaglandin E_2 (B) prostaglandin $F_{2\alpha}$ (C) used as internal (A) and external (B and C) reference compounds are also shown. The radioactive material co-chromatographing with unlabeled thromboxane B_2 was analyzed by gas–liquid chromatography–mass spectrometry and identified as thromboxane B_2 [2].

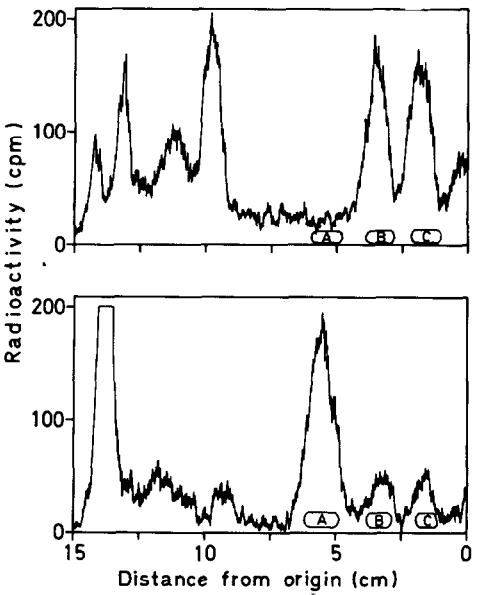

Fig.2. Thin-layer radiochromatogram of products isolated after incubations of [1-^{14}C]prostaglandin H_2 with native (lower panel) or boiled human platelet microsomes (upper panel). Positions of internal (A) and external (B, C) reference compounds are shown with striped areas. (A, thromboxane B_2; B, prostaglandin E_2; C, prostaglandin $F_{2\alpha}$).

Recently a number of stable analogues of the prostaglandin endoperoxides have been synthesized [5–7]. Three of these analogues were tested in the present investigation. One, 9α,11α-azo-15(S)-hydroxy-prosta-5(*cis*), 13(*trans*)-dienoic acid (analogue I), was a potent inhibitor of thromboxane synthase with an ID_{50} of approximately 2 μM. Another relatively potent inhibitor was 9α,11α-epoxymethano-15(S)-hydroxy-prosta 5(*cis*), 13(*trans*)-dienoic acid (analogue II) which gave an ID_{50} 2×10^{-5} M. The third analogue, 9α,11α-methanoepoxy-15(*S*)-hydroxy-prosta-5(*cis*), 13(*trans*)-dienoic acid (analogue III) was not effective in inhibiting thromboxane synthase (figs. 3–4). The relative potency of these compounds as inhibitors of thromboxane synthase parallelled their binding to platelet microsomes*, suggesting that the analogues inhibit

*Diczfalusy, U., Powell, W. S., Hammarström, S. and Samuelsson, B., unpublished observations

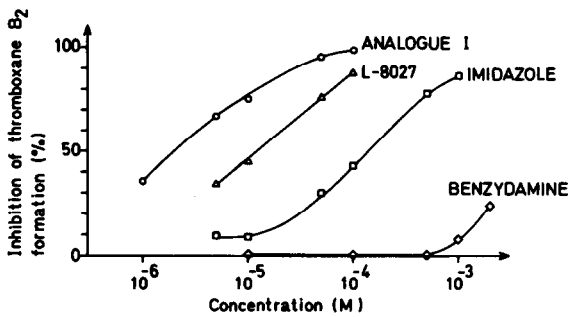

Fig.3. Inhibition of thromboxane B_2 formation from prostaglandin H_2 as a function of inhibitor concentration.

thromboxane synthase by binding to the substrate site of this enzyme.

It was recently reported that an anti-inflammatory compound, 2-isopropyl-3-nicotinyl-indole (L-8027) selectively inhibits thromboxane A_2 synthesis [8]. This compound was tested in our system and was found to be a potent inhibitor (ID_{50} approx. 10 μM, fig.3). Benzydamine, which was reported to inhibit thromboxane formation by horse platelet microsomes [9], was not effective in our system at concentrations below 1 mM. It is thus doubtful if this compound should be considered a specific inhibitor of thromboxane synthase. It has been reported that imidazole is a selective inhibitor of thromboxane formation [10]. Our results (fig.3) confirm that imidazole inhibits the conversion of prostaglandin H_2 to thromboxane B_2. The potency of imidazole was however 10–100-times less than that of the compounds described above (ID_{50} 1.5×10^{-4} M). The acetylenic analogue of arachidonic acid 5,8,11,14-eicosatetraynoic acid, was not effective in inhibiting thromboxane synthesis (fig.4). Sodium *p*-benzyl-4-[1-oxo-2-(4-chlorobenzyl)-3-phenyl propyl] phenyl phosphonate (N-0164) has been reported to inhibit the formation of thromboxane A_2-like activity from prostaglandin endoperoxides by human platelet microsomes [11] as assayed on rabbit aorta. The reported ID_{50} (2.4×10^{-5} M) is identical to that obtained in our system (fig.4). Phtalazinol (EG-626) did not inhibit the conversion of prostaglandin H_2 to thromboxane B_2 catalyzed by human platelet microsomes (fig.4). This agrees with a proposal that EG-626 is an antagonist of thromboxane action [12] rather than an inhibitor of thromboxane synthase.

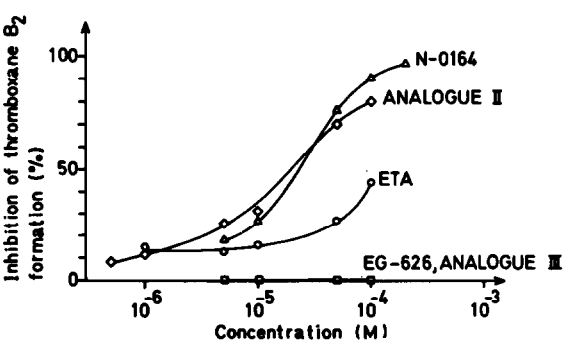

Fig.4. Inhibition of thromboxane B_2 formation from prostaglandin H_2 as a function of inhibitor concentration.

In conclusion, four compounds were found to be good inhibitors of microsomal thromboxane synthase from human platelets. These inhibitors (analogue I, L-8027, analogue II and N-0164, decreasing order of potency) had ID_{50} values of 2–24 μM. The endoperoxide analogues I and II have biological activities similar to those of prostaglandin G_2 and prostaglandin H_2 [13]. L-8027 and N-0164, however, appear to be promising compounds for studies on the relative biological importance of prostaglandin endoperoxides and thromboxanes in platelets and other tissues.

Acknowledgements

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