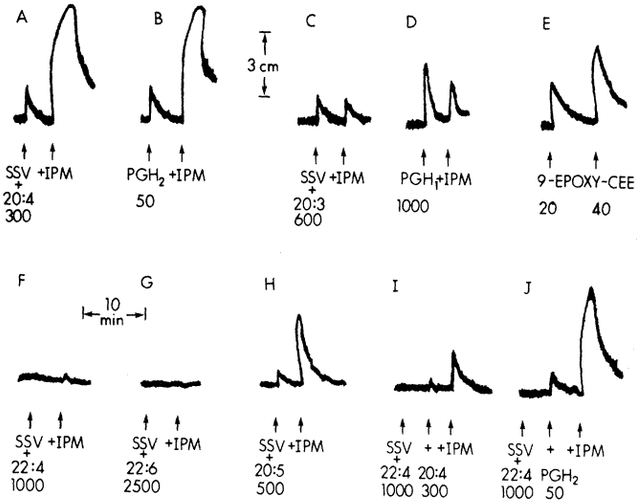
Thromboxanes: Selective Biosynthesis and Distinct Biological Properties

Abstract. *The prostaglandin endoperoxide ring structure alone does not establish suitability as a substrate for thromboxane synthetase, but the degree of unsaturation and carbon chain length are also essential features. Thus, human platelet microsomes can synthesize thromboxane A_2, thromboxane A_3, but not thromboxane A_1 from their respective endoperoxides. The potent vasoconstrictor property of thromboxanes can be dissociated from its capacity to produce platelet aggregation. Furthermore, thromboxane formation is not an essential process in platelet aggregation. The observations indicate the remarkable structural specificity of both the synthetic enzymes, cyclooxygenase and thromboxane synthetase, as well as the vascular and platelet receptor sites.*

Exogenous arachidonic acid [the prostaglandin (PG) E_2 precursor] and the prostaglandin endoperoxides (the biosynthetic intermediates) induce platelet aggregation (*1–3*). Endogenous biosynthesis of the endoperoxides, PGG_2 and PGH_2, has been demonstrated during platelet aggregation (*4*). However, the amount of endoperoxide released either from shocked lungs or from platelets could not account for most of the rabbit aorta contractile activity that was generated (*5*). Both PGG_2 and PGH_2 are rapidly converted by a platelet microsomal enzyme (*6*), thromboxane synthetase, into the highly unstable thromboxane A_2 (TA_2), which is degraded to the stable but biologically inactive thromboxane B_2 (TB_2). Thromboxane A_2 causes platelet aggregation (*7*) and apparently is the potent vasoconstrictor originally described as rabbit aorta contracting substance (*8*). The objectives of our investigation include (i) evaluation of various fatty acids and endoperoxides as potential substrates (or inhibitors) for the cyclooxygenase–thromboxane synthetase system, (ii) quantitative characterization of the endoperoxide and thromboxane generating systems, and (iii) comparison of the relative potency of the various endoperoxides and thromboxanes as vasoconstrictors and platelet aggregators.

The incubation of PG-precursor fatty acids C_{20} : 3 (*n*–6) (dihomo-γ-linolenate), C_{20} : 4 (*n*–6) (arachidonate), and C_{20} : 5 (*n*–3) with the cyclooxygenase from sheep seminal vesicle (SSV) microsomes resulted in the generation of the moderately potent aorta vasoconstrictors PGG_1-PGH_1, PGG_2-PGH_2, and PGG_3-PGH_3, respectively (Fig. 1, A, C, and H). Extraction of the reaction mixture of SSV with either ^{14}C-labeled arachidonate or ^{14}C-labeled dihomo-γ-linolenate indicated a 70 to 80 percent conversion to the endoperoxide mixture PGG_2-PGH_2 or PGG_1-PGH_1, respectively (Fig. 2, C and D). In contrast, no contractile substance was generated by incubating seminal vesicles with the fatty acids C_{22} : 4 (*n*–6) or C_{22} : 6 (*n*–3) (Fig. 1, F and G). All the above fatty acids produced a substrate-catalyzed destruction of the seminal vesicle cyclooxygenase (Fig. 1I) but did not antagonize platelet thromboxane synthetase (Fig. 1J). Auto-



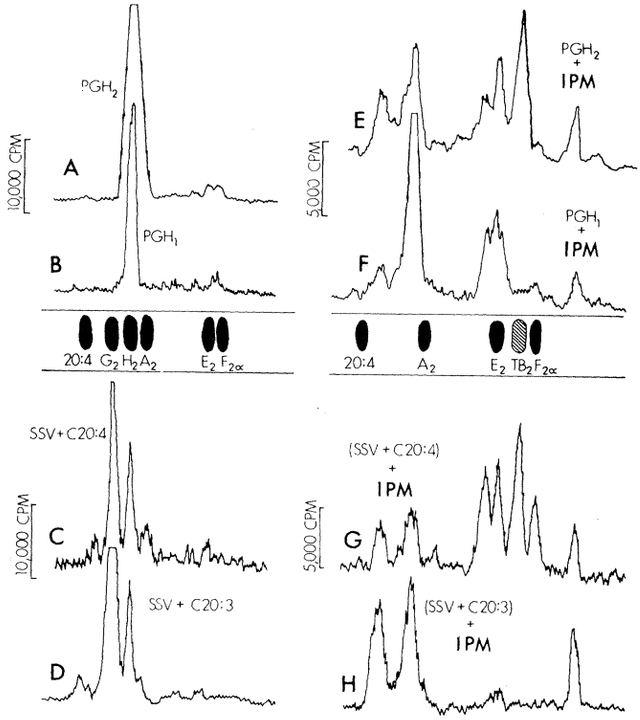


Fig. 1 (left). Bioassay of rabbit aorta contracting substances. Spiral strips of rabbit thoracic aorta were superfused at 10 ml/min with Krebs solution at 37°C containing a mixture of antagonists and indomethacin (1 μg/ml). (A, C, and F to H). These indicate the contractile response produced by testing 50 μl of the incubation mixture (22°C, 1 minute) of sheep seminal vesicle (SSV) microsomes (25 μl, 250 μg of protein) in a tube containing 50 μl of 50 m*M* phosphate (*p*H 7.8) with various fatty acid sodium salts. The tube was then placed on ice, and 25 μl of indomethacin-treated human platelet microsomes (IPM) (*10*) was added; 2 minutes later 50 μl of the reaction mixture was tested. The purified endoperoxide (the number below the tracing indicates the number of nanograms tested) was added to a tube and dried in a stream of nitrogen prior to the addition of 100 μl of the phosphate buffer; the 0°C reaction was started by the addition of IPM (*16, 17*). To standardize the assay tissues, 9-epoxy-CEE (E), the stable cyclic ether endoperoxide, 9α,11α-epoxymethano-15 hydroxyprosta-5,13-dienoic acid was used. Fig. 2 (right). The chromatographic separation of prostaglandins, endoperoxides, and thromboxane. (A to D) The scans of these radioactive endoperoxides were obtained from silica gel G plates (–20°C) with an ether, petroleum ether solvent system (4:1). (E to H) Scans of these endoperoxides were especially selected for the clear separation of thromboxane B, and were obtained in solvent system C of Nugteren and Hazelhof (*18*). Scans (E) and (F) illustrate the conversion of purified endoperoxides (*16*) by IPM's to prostaglandin products. The incubation was performed as described in the legend to Fig. 1. Scans (G) and (H) illustrate the enzymatic (SSV microsomes) generation of endoperoxides (C and D), as well as the coupling of the endoperoxide generating system to platelet microsomes for thromboxane production (G) and (H), under the incubation conditions described in the legend to Fig. 1. Each experiment was repeated at least three times with the same results.

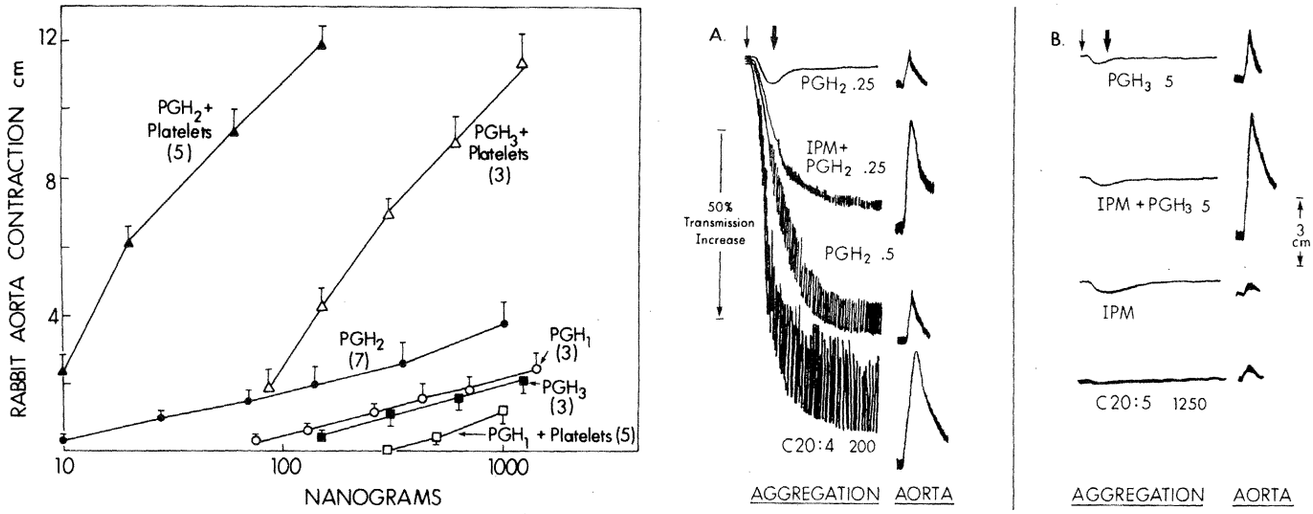


Fig. 3 (left). The comparative potency of prostaglandin endoperoxides and thromboxanes. The incubation of varying concentrations of endoperoxides and IPM are described in Fig. 1. The values represent the mean ± standard error, and the number in parentheses represents the number of aorta strips tested. Fig. 4 (right). Aggregation of human platelet-rich plasma and generation of rabbit aorta contractile activity. The thin arrow designates the addition of the fatty acids to the human platelet-rich plasma (*11*). All the other reactions were started by addition of the platelet-rich plasma to the cuvette, which contained either the endoperoxide (in 40 μl of phosphate buffer) or the preformed thromboxane. The thromboxanes were generated by incubating the endoperoxides with 15 μl of IPM for 2 minutes at 0°C. The cuvette was placed in the aggregometer (Payton single module) and 0.4 ml of platelets with plasma being added immediately. The thick arrow denotes removal of a sample for immediate rabbit aorta bioassay shown adjacent to its appropriate aggregation curve.

destruction of the cyclooxygenase by fatty acids has been reported (*9*).

Washed human platelets convert arachidonic acid to a potent rabbit aorta contracting substance (*1*). We therefore incubated human platelets with indomethacin (2 μg/ml) prior to preparation of the 100,000*g* (*10*) pellet [indomethacin-treated platelet microsomes (IPM)]. This treatment inhibits the cyclooxygenase reaction and selectively establishes the IPM as the source of thromboxane synthetase, reacting only with the endoperoxides to form TA_2. Incubation of IPM at 0°C for 2 minutes with purified PGH_2 (or PGG_2) or with the SSV-arachidonate reaction mixture resulted in the formation of the potent rabbit aorta contracting substance, TA_2 (Fig. 1, A and B). An aorta contraction was produced during the incubation of SSV with C_{20} : 5 fatty acid (the precursor for PGE_3) (*11*) (Fig. 1H). Addition of IPM to this mixture, or to purified PGH_3 (*12*), produced a much more potent contractile substance (Fig. 1H). This substance is presumably thromboxane A_3 (TA_3) because of (i) its enhanced potency when compared to its endoperoxide (Fig. 1, G and H), (ii) the parallelism between the aorta contractile dose-response curves for TA_3 and TA_2 (Fig. 3), (iii) its extraction characteristics coinciding with TA_2 (that is, neutral ether extraction with a 20 to 40 percent recovery), and (iv) the aqueous instability of TA_3 coinciding with that of TA_2.

Parallel dose-response curves were generated when we compared the aorta contractile potency of the endoperoxides (PGH_2, PGH_1, and PGH_3 to each other) and the thromboxanes (A_2 compared to A_3). The contractile response of superfused rabbit thoracic aorta strips increased in a parallel linear fashion with increasing concentrations of the endoperoxides PGH_1, PGH_2, and PGH_3 (Fig. 3). The PGH_2 is about four times more potent as a vasoconstrictor than PGH_1 or PGH_3. Both PGG_2 and PGH_2 have been reported to be five to ten times more potent in constricting superfused rabbit aorta strips than PGG_1-PGH_1 (*13*). Thromboxane A_2 appeared to be about 100 times more potent than the PGH_2 alone (Fig. 3). This potency ratio could be as high as 300 : 1 since only 29 percent of the original endoperoxide was converted by IPM to TB_2 (Fig. 2, E and G). Prostaglandin H_2 and TA_2, respectively, appeared to be about five to ten times more potent than PGH_3 and TA_3 as rabbit aorta contractile substances (Fig. 3). In sharp contrast to PGH_2 or PGH_3, when PGH_1 (or PGG_1, not shown) was incubated with IPM there was no enhancement of its blood vessel contractile activity (Figs. 1D and 3). Similar negative results obtained when the IPM's were incubated with the reaction mixture of SSV and C_{20} : 3 (Fig. 1C). The lack of evidence for a biologically active TA_1 is supported by the absence of a distinct radioactive TB_1 peak (Fig. 2, F and H). In parallel experiments it was readily possible to demonstrate that the platelets converted labeled PGH_2 into TB_2 (Fig. 2, E and G). If TB_1 is formed it is less than 2 percent of the original radioactive endoperoxide; and, if produced, it exerts no contractile effect on vascular smooth muscle.

We performed experiments to qualitatively and quantitatively compare the aggregation induced by arachidonate, PGG_2-PGH_2, and TA_2 to C_{20} : 5, PGG_3-PGH_3, and TA_3. Arachidonate produced concentration-dependent irreversible aggregation of human platelet-rich plasma and simultaneously generated a potent rabbit aorta contracting substance (Fig. 4A). The PGH_2 was considerably more potent in producing concentration-dependent aggregation. Surprisingly, bioassay (rabbit aorta) of the reaction mixture of platelet-rich plasma and a high dose of PGH_2, taken at the point of maximum aggregation, did not appear to contain the potent vasoconstrictor. The TA_2, preformed in the cuvette, rapidly aggregated platelet-rich plasma (Fig. 4A). In sharp contrast, the fatty acid C_{20} : 5, the endoperoxides PGG_3 and PGH_3, or TA_3 did not produce an aggregatory response (Fig. 4B). These results are supported by the observation that incubation of C_{20} : 5, C_{20} : 3, or C_{22} : 6 to platelet-rich plasma did not result in aggregation (*2*). In addition, administration of these fatty acids to rabbits did not produce platelet thrombi in the pulmonary circulation, whereas arachidonate was lethal (*14*).

The apparent lack of TA_2 generation

during aggregation of platelet-rich plasma with PGH_2 (Fig. 4A) (*3*), as well as the inability of the potent vasoactive TA_3 to induce aggregation (Fig. 4B), indicate that the two properties of thromboxanes—that is, the powerful rabbit aorta contraction and platelet aggregation—are dissociated. Some pertinent observations have been reported. Addition of PGG_2 to platelet-rich plasma caused aggregation but little if any thromboxane production (*3*). In contrast, incubation of arachidonate or PGG_2 with washed platelets resulted in irreversible aggregation, generation of a potent rabbit aorta contractile factor, and in recovery of TB_2 (*7*).

The biosynthesis of thromboxanes is apparently not essential for platelet function. For example, primary aggregation, the release reaction, and irreversible aggregation induced by physiological concentrations of thrombin are not abolished by aspirin (*15*). In addition, a genetic deficiency of platelet cyclooxygenase resulted (in one patient) in a mild hemostatic defect associated only with a modest prolongation of bleeding time (*3*). The implication from our data and the earlier reports is that TA_2 formation is not an essential process in platelet aggregation. The primary physiological function of TA_2 is presumably as a potent localized vasoconstrictor that enhances hemostasis primarily by sharply reducing the blood vessel lumen, and perhaps secondarily by augmenting aggregation.

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10. Human platelet-rich plasma was incubated with indomethacin (2 μg/ml) for 1 hour (0°C), and the platelets were centrifuged (2000*g*, 15 minutes) and lysed by freezing and thawing (three times). The 8000*g* supernatant was centrifuged at 100,000*g* (60 minutes), and the pellet was suspended in phosphate buffer (50 m*M*, *p*H 7.8) at a protein concentration of 14 mg/ml.
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12. Radioactive C_{20} : 5 is not available. ^{14}C-Labeled dihomo-γ-linolenic acid was used as the tracer in the synthesis (SSV), isolation, and quantitation of PGG_3 and PGH_3. The ^{14}C-labeled PGH_1 did not interfere with the vasoconstrictor experiments since no TA_1 was formed. In addition, PGH_1 is reported not to inhibit PGH_2 platelet aggregation (*13*). No difference was observed when the PGH_3 was prepared without the ^{14}C-labeled C_{20} : 3.
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16. Endoperoxides PGH_2-PGG_2 and PGH_1-PGG_1 were prepared by incubating 175 μg of the ^{14}C-labeled (4×10^6 count/min) fatty acid (C_{20} : 4 or C_{20} : 3) with 100 mg of SSV acetone-pentane microsomal powder (*17*). Incubation was performed in 0.025*M* tris-phosphate buffer, *p*H 8.1, at 22°C for 2 minutes with stirring. The reaction mixture was added to a mixture, cooled to −5°C, of ethyl acetate (10 ml) and 0.2*M* citric acid (1.2 ml), the resulting *p*H of the aqueous phase being 3.3. Isolation and purification of the endoperoxides was according to method described by Nugteren and Hazelhof (*18*).
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19. We thank A. Wyche, S. L. Key, and S. E. Denny for technical assistance and the Upjohn Company for the endoperoxide 9-epoxy-CEE. Supported by SCOR HL-17646, RCDA HL-19586, HE 14397, HEW surgical training grant 55552, and American Heart Association grant-in-aid.

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