SYNTHESIS OF PLATELET-AGGREGATING FACTOR BY HUMAN PLATELET MICROSOMES

P. P. K. Ho, C. P. Walters, and R. G. Hermann

Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46206

Received January 15,1976

SUMMARY: The microsomal fraction of human platelets catalyzed the conversion of arachidonic acid to an unstable factor that induced platelet aggregation. Formation of a polar product, similar in properties to thromboxane B_2, was also demonstrated. Indomethacin and fenoprofen (2-phenoxy-phenyl-propionic acid) inhibited this enzymatic activity, while preincubation with enzyme protein was necessary for aspirin to exert an inhibitory effect.

Hamberg and Samuelsson (1,2) reported the hemiacetal derivative of 8-(1-hydroxy-3-oxopropyl)-9, 12L-dihydroxy-5, 10-heptadecadienoic acid (thromboxane B_2)[1] and 12L-hydroxy-5, 8-10-heptadecatrienoic acid (HHT) as the major metabolites of prostaglandin G_2 (PGG_2) and arachidonic acid in suspensions of human platelets.

It was later demonstrated that an unstable intermediate, designated as thromboxane A_2 was found in the conversion of PGG_2 into thromboxane B_2 (3) and was responsible for irreversible platelet aggregation.

Since incubation of arachidonic acid with washed platelets also led to formation of thromboxane A_2, a fatty acid cyclo-oxygenase was suggested to be present in the platelet in addition to another enzyme protein that converts PGG_2 to thromboxane B_2. To understand the mechanism of these conversions, it is evident that a cell-free system for the synthesis of thromboxanes has

---

[1]Abbreviations: PGG_2, prostaglandin G_2; HHT, 12L-hydroxy-5,8,10-heptadecatrienoic acid, thromboxane B_2[8-(1-hydroxy-3-oxopropyl)9, 12L-dihydroxy-5,10-heptadecadienoic acid].

to be isolated. The present work demonstrates that arachidonic acid is oxygenated to thromboxane-like substances with a human platelet microsomal preparation.

MATERIALS AND METHODS

[5,6,8,9,11,12,14,15,^{3}H] Arachidonic acid (New England Nuclear, Boston, Mass.) was diluted with unlabeled arachidonic acid (Nu Chek Prep. Inc., Elysian, Minn.). The specific activity of the sample (20 μM) prepared in this way was 1 Ci/mmole. Bovine hemoglobin was purchased from Sigma Chemical Co., St. Louis, Missouri. Platelet rich plasma and washed human platelets were isolated from blood collected from donors according to the procedures described by Hamberg et al. (3). Platelet aggregation was monitored with a Chronolog aggregometer. The aggregometer tube contained 0.5 ml of suspension of platelets preincubated at 37°C for two min with 14 μM indomethacin. Thin-layer chromatography was performed with silical gel G. plates (LQ6D, Quantum Industries, Fairfield, New Jersey) and the organic layer of ethyl acetate-2,2,4-trimethylpentane-water (50:100:100 v/v/v) as solvent.

Proteins were measured using the method by Lowry et al. (4). Bovine serum albumin was used as the standard.

RESULTS

Preparation of Human Platelet Micromes

All the preparations were performed at 4°C. Platelet rich plasma (500 g) was centrifuged for 10,000 x g for 20 min, and the platelet pellet was suspended in 100 ml of 0.1 M potassium phosphate buffer, pH 8.0, by homogenization with a Teflon-glass homogenizer. The suspension was sonicated at maximum energy in a Bronson Sonifier (Heat System, Melville, New York) for three min. The sonicate was centrifuged at 10,000 x g for

15 min and the cloudy supernatant solution was further centrifuged at 100,000 x g for 90 min. The pellet (microsomal fraction) was suspended in 10 ml of 10 mM potassium phosphate buffer, pH 8.0, and the suspension was lyophilized to dryness. The dried powder (400 mg) was stored at -20°C. The protein concentration of this powder is 50% by weight.

Demonstration of Enzymatic Activity in Lyophilized Platelet Microsomal Powder

A reaction mixture contained 50 mM Tris·HCl buffer, pH 8.0, 5 mM L-tryptophan, 2 μM hemoglobin and 0.1 mg of lyophilized microsomal powder in a total volume of 0.2 ml. The reaction mixture was initiated by the addition of 10 μl of 20 μM [5,6,8,9,11,12,14,15-^{3}H] arachidonic acid. After 5 min incubation at 37°C, the reaction was terminated by the addition of 10 μl of 1 M citric acid. The mixture was then extracted twice with 4 ml of ethylacetate. The residue obtained after evaporation of the ethylacetate (70-80% of the added radioactivity) was treated with diazomethane and subjected to thin-layer chromatography. Four peaks of radioactivity appeared (Fig. 1). A polar substance (Compound III) with a similar Rf value of thromboxane B_2 was found. The formation of this polar product was inhibited by the addition of indomethacin or fenoprofen to the reaction mixture (Table 1), while aspirin had no effect.

Formation of Platelet Aggregation Factor

A reaction was performed with the above reaction components, except a larger amount of microsomal powder (1 mg) was used. The complete reaction mixture was preincubated at 37°C for 2 min before the addition of 10 μl of 0.3 mM arachidonic acid. After stirring for 5 sec, 0.1 ml was rapidly transferred to the aggregometer tube which contained 0.5 ml of platelet

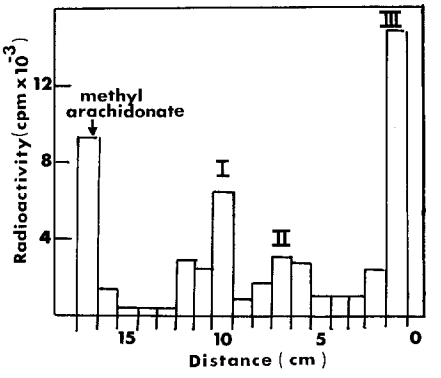

Fig. 1. Thin-layer radiochromatogram of product (methyl esters) obtained after incubation of [^{3}H]-arachidonic acid as described in the text. Solvent system = organic layer of ethylacetate-2,2,4-trimethylpentane-water (50:100:100, v/v/v).

Table 1

Effect of Inhibitors

Inhibitor	Amount (µg/ml)	Percentage Composition of Compound III	Percent Inhibition
Not present		22.2	-
Indomethacin	10	2.6	88
Indomethacin	1	6.9	68
Fenoprofen	10	2.7	88
Fenoprofen	1	11.7	47
Aspirin	100	20.6	<1
Aspirin	10	22.8	0

The reaction was performed without preincubation of drug with enzyme.

suspension preincubated with 14 µM of indomethacin.

As seen in Fig. 2, aggregation took place. It was clear that some aggregating factor was formed in our reaction mixture. In the absence of platelet microsomal preparation or arachidonic acid, no aggregation occurred.

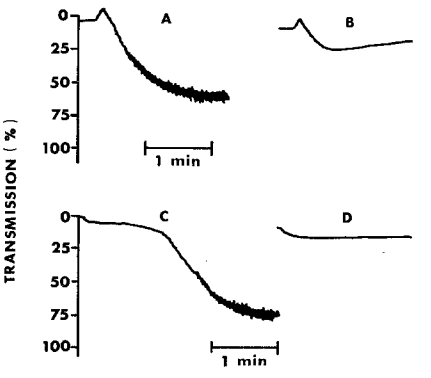

Fig. 2. Platelet aggregation induced by (a) 0.1 ml of reaction mixture incubated for 5 sec with 15 μM arachidonic acid, and (b) 0.1 ml of reaction mixture incubated for 5 sec with 1.5 μM arachidonic acid. All the reaction mixtures were preincubated for 2 min at 37°C before the addition of arachidonic acid. The aggregator tube contained 0.5 ml of platelet suspension that had been preincubated for 2 min at 37°C with 14 μM indomethacin. Reference curves of platelet aggregation induced by direct addition of 0.1 ml of 3 mM arachidonic acid, (c) in the absence of 14 μM indomethacin, and (d) in the presence of 14 μM indomethacin are also included.

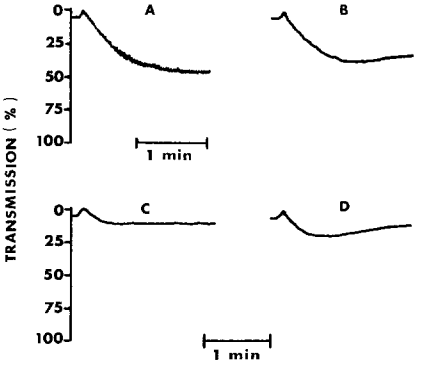

Fig. 3. Platelet aggregation induced by 0.1 ml of reaction mixture incubated for various times with 15 μM arachidonic acid; (a) 30 sec, and (b) 1 min. Platelet aggregations induced by 0.1 ml of reaction mixture incubated for 5 sec with 15 μM arachidonic acid in the absence of (c) hemoglobin, and (d) L-tryptophane are also shown. All the reaction mixtures were preincubated for 2 min at 37°C. The platelets in the aggregometer tubes were preincubated for 2 min at 37°C with 14 μM indomethacin.

A dose-response relationship was obtained with lower concentrations of arachidonic acid. This factor could not be arachidonic acid, since the platelets in the recipient tube were made totally unresponsive toward the aggregating effect of arachidonic acid by preincubation with indomethacin.

Fig. 3 shows that the aggregating factor was very unstable. A rapid loss of activity was observed after 1 min of incubation. In this experiment, transformation of arachidonic acid to the aggregating factor was shown to be dependent upon hemoglobin and L-tryptophane as cofactors.

DISCUSSION

The present paper demonstrates the presence of an enzymatic activity that synthesizes platelet-aggregating factor. This factor has the characteristic of thromboxane A_2 because of its short half-life. The half-life of the endoperoxides at 37°C was about 5 min, whereas, that of thromboxane A_2 and our unstable factor was below 1 min. It is interesting to note that platelet enzyme shows similar cofactor requirements as that observed with fatty acid cycloxygenase isolated from bovine vesicular gland (5).

The finding that indomethacin and fenoprofen, inhibitors of prostaglandin biosynthesis (6), blocked formation of the polar substance, gave independent support that the product was synthesized via the fatty acid cyclo-oxygenase pathway. However, the chemical identity of the product awaits further isolation and characterization.

As to the effect of aspirin, inhibition could be shown if aspirin was preincubated with the enzyme at 37°C (Table 2). The results suggest that aspirin inhibits platelet fatty acid cyclo-oxygenase via chemical interaction with the enzyme protein

Table 2

Effect of Aspirin Upon Preincubation

Time of Pre-incubation (min)	Control	With Aspirin (100 µg/ml)	Percent Inhibition
5	28.9	17.2	41
10	24.4	8.2	66
15	20.0	7.5	62

Aspirin was incubated with reaction mixture containing enzyme at 37°C before the addition of arachidonate, and the reaction was performed as described in Results. The rate of reaction is expressed as percentage composition of Compound III.

molecule, a finding in agreement with the results reported by Roth et al. (7).

The lyophilized human platelet microsomes should therefore provide opportunity for purification and for the study of the fatty acid cyclo-oxygenase and other enzyme systems involved in the conversion of PGG_2 to thromboxane A_2 and B_2.

REFERENCES

1. Hamberg, M. and Samuelsson, B. (1974) Proc. Nat. Acad. Sci. USA 71,3400-3404.

2. Hamberg, M., Svensson, J., and Samuelsson, B. (1974) Proc. Nat. Acad. Sci. USA 71,3824-3828.

3. Hamberg, M., Svensson, J., and Samuelsson, B. (1975) Proc. Nat. Acad. Sci. USA 72,2994-2998.

4. Lowry, O. H., Rosebrough, N. J., Fan, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193,265-275.

5. Miyamoto, T., Yammoto, S., and Hayaishi, O. (1974) Proc. Nat. Acad. Sci. USA 71,3645-3648.

6. Ho, P. P. K. and Esterman, M. (1974) Prostaglandins 6,107-113.

7. Roth, G. J., Stanford, N. and Majerus, P. W. (1975) Proc. Nat. Acad. Sci. USA 72,3073-3076.