

Formation of an Intermediate in Prostaglandin Biosynthesis and Its Association with the Platelet Release Reaction

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ABSTRACT A compound that could be converted to prostaglandin $F_{2\alpha}$ by mild chemical reduction was formed by human platelets in response to arachidonic acid, collagen, or L-epinephrine. It was present in maximal amounts at about 1 min after addition of arachidonic acid or collagen to platelet-rich plasma. Its initial formation appeared to precede platelet aggregation by these agents and was closely correlated with the release of adenine nucleotides and radioactive 5-hydroxytryptamine from platelets. Moreover, the compound was itself found outside the platelets. This compound is probably an endoperoxide intermediate in prostaglandin biosynthesis and may be a trigger for the platelet release reaction.

INTRODUCTION

Hamberg and Samuelsson (1) have described the detection and isolation of the endoperoxide intermediate that gives rise to prostaglandin (PG)¹ E_2 and $PGF_{2\alpha}$ during incubation of arachidonic acid with a preparation of sheep seminal vesicles. Since it has been established that human platelets form PGE_2 and $PGF_{2\alpha}$ during blood clotting (2, 3) and during platelet aggregation in response to ADP, L-epinephrine, or collagen (4, 5), it was of interest to determine whether they also form this intermediate. Recently, it was shown that the precursor of PGE_2 and $PGF_{2\alpha}$, arachidonic acid, induces platelets to form prostaglandins and aggregate (6, 7). The latter effect appears to be specific for arachidonic acid, since it does not occur in response to a number of related fatty acids (8). The present studies show that the link be-

tween prostaglandin formation and aggregation is the platelet release reaction (see reference 9) and suggest that the endoperoxide intermediate may be its trigger.

METHODS

Platelet aggregation. Human blood was collected from the antecubital veins of healthy subjects into 0.1 vol of 3.8% trisodium citrate and was centrifuged to prepare platelet-rich plasma (PRP) as previously described (4). Platelet aggregation was initiated by addition of up to 0.16 ml of the agent under study to 2 ml PRP stirred at 1,000 rpm in siliconized test tubes at 37°C. Aggregation was monitored by the continuous recording of light transmission (aggregometer from Chrono-Log Corp., Broomall, Pa.). Aggregating agents investigated were L-epinephrine hydrochloride (Sigma Chemical Co., St. Louis, Mo.), collagen suspension (bovine tendon [10] from Sigma Chemical Co.), and 99%-pure arachidonic acid (Hormel Institute, Austin, Minn.). The latter compound was converted to a clear 50 mM solution by dissolution in 0.1 M sodium carbonate about 1 h before use.

Release of 5-hydroxytryptamine and nucleotides. A portion (generally 20 ml) of the PRP under study was incubated at 20–22°C for 30 min with [$3\text{'-}^{14}\text{C}$]5-hydroxytryptamine creatinine sulphate, sp act 57 mCi/mmol, (Amersham/Searle Corp., Arlington Heights, Ill.) at a final concentration of 0.5 μM . At appropriate times after initiating aggregation as described above, samples (1.5 ml) were rapidly decanted into 0.15 ml ice-cold, 0.1 M EDTA (pH 7.4) and mixed. The samples were further cooled in crushed ice and centrifuged for 1 min at 15,000 g (Eppendorf microfuge, Brinkmann Instruments, Inc., Westbury, N. Y.), and 0.2-ml samples of the supernatant fluid were taken to determine the release of adenine nucleotides and radioactivity (11). Aggregation recordings in radioactive PRP were identical to those obtained with the same concentration of aggregating agent in nonradioactive PRP.

Preparation of samples for radioimmunoassay. At appropriate times after addition of an aggregating agent or saline, the contents of the aggregometer tube (2 ml PRP) were rapidly decanted into 10 ml ethanol, containing 0.1

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¹Abbreviations used in this paper: PG, prostaglandin; PGD_2 , 11-dehydro- $PGF_{2\alpha}$; PRP, platelet-rich plasma.

pmol [^3H]PGF $_{1\alpha}$, sp act 67 Ci/mmol (New England Nuclear, Boston, Mass.) as an internal standard for recovery. Alternate tubes of ethanol also contained 50 mg stannous chloride to reduce the endoperoxide intermediate to PGF $_{2\alpha}$ (1). Duplicate samples, both with and without stannous chloride treatment, were extracted for prostaglandins, essentially as described by Unger, Stamford, and Bennett (12). The prostaglandins were separated into fractions containing E or F types by column chromatography on silicic acid (13). These fractions were blown to dryness at 40°C under a stream of nitrogen, suspended in 0.5 ml Tris-saline (0.015 M Tris-HCl, pH 7.5, 0.14 M NaCl) and kept for up to 1 wk at -30°C.

Radioimmunoassay of prostaglandins. Samples containing F type prostaglandins were assayed in duplicate by employing a rabbit antiserum generated against PGF $_{2\alpha}$ and using nitrocellulose membranes (14). The sensitivity of this assay is illustrated in Fig. 1, and the conditions are given in the legend. Prostaglandin levels were determined by interpolation from standard curves. PGE $_2$ was assayed indirectly, using the PGF $_{2\alpha}$ antiserum, after reduction of PGE to PGF with sodium borohydride (15).

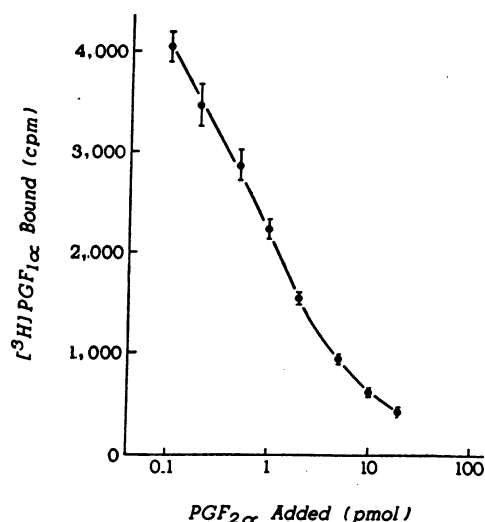


FIGURE 1 Standard curve for PGF $_{2\alpha}$ radioimmunoassay. Each point represents the average (\pm SD) of 10 determinations (five different assays). Varying amounts of unlabeled PGF $_{2\alpha}$ in 0.1 ml Tris-saline were incubated at 37°C for 2 h with 0.1 ml PGF $_{2\alpha}$ antiserum (1:1,500 dilution of serum) and 0.1 ml [^3H]PGF $_{1\alpha}$ suspended in the same medium. Since there was little difference in the binding of PGF $_{1\alpha}$ and PGF $_{2\alpha}$ by the antiserum, [^3H]PGF $_{1\alpha}$ was employed because of its higher specific activity (0.27 pmol, sp act 67 Ci/mmol). The antibody-PGF complex was isolated on a nitrocellulose membrane, 0.45- μm pore size, 25 mm diameter (Schleicher and Schuell, Inc., Keene, New Hampshire), washed with 12 ml ice-cold Tris-saline, and transferred to a scintillation vial. The membrane was dissolved in 10 ml of dioxane-based phosphor (1 g 2,5-diphenyloxazole, 20 g naphthalene, 200 ml dioxane, 2.0 ml H $_2\text{O}$), and radioactivity was determined in a Packard 3320 liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Total binding in the absence of added prostaglandin was approximately 50% (4,800 cpm).

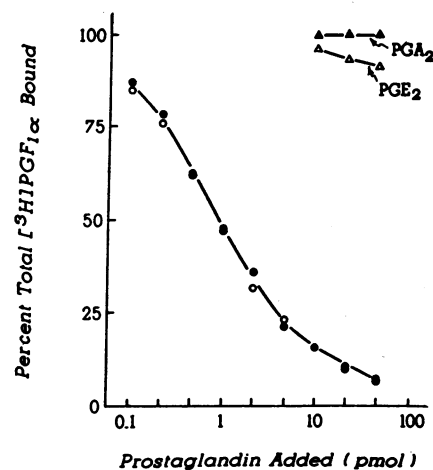


FIGURE 2 Validation of PGE $_2$ assay by using PGF $_{2\alpha}$ antiserum. Curves show inhibition of [^3H]PGF $_{1\alpha}$ -anti-PGF $_{2\alpha}$ binding by PGA $_2$ (\blacktriangle), PGE $_2$ (\triangle), PGF $_{2\alpha}$ (\bullet), and PGF $_{2\alpha}$ produced by sodium borohydride reduction of PGE $_2$ * (\circ). Each point is the mean of duplicate incubations. Varying amounts of PGE $_2$ in 0.5 ml Tris-saline were incubated at 22°C with 5 μl sodium borohydride (60 mg/ml water) for 30 min. Excess borohydride was removed by acidification (10 μl 0.66 M citric acid), with subsequent neutralization (10 μl 2 M NaOH). Portions were taken for radioimmunoassay, essentially as described in the legend to Fig. 1.

* Since PGE $_2$ is reduced to 50% PGF $_{2\alpha}$ and 50% PGF $_{2\beta}$ (which does not cross react), the amount originally present as PGE $_2$ is twice that shown.

Validation of methods. After addition of 0.9 pmol [^3H]PGE $_2$, sp act 49 Ci/mmol, (New England Nuclear) to PRP, with extraction and chromatography as described above, 87.6% (SD 2.4) of the radioactivity eluted from the columns was present in the PGE fraction and 3.2% (SD 1.7) was in the PGF fraction (20 determinations). After addition of 7.5 pmol [^3H]PGF $_{2\alpha}$, sp act 8.8 Ci/mmol, (New England Nuclear), 94.0% (SD 1.7) of the radioactivity was present in the PGF fraction, and 3.0% (SD 1.1) in the PGE fraction (18 determinations). These distributions of radioactivity were not altered by incubation of the prostaglandins in PRP for 15 min at 37°C, confirming that E and F types of PG are relatively stable in plasma.

The validity of sodium borohydride reduction for the measurement of pure PGE $_2$ by radioimmunoassay is illustrated in Fig. 2, and the conditions of the reduction are given in the legend. In crude extracts from biological sources, this method would also measure 11-dehydro-PGF $_{2\alpha}$, which is almost exclusively converted to PGF $_{2\alpha}$ by sodium borohydride reduction (1). However, 11-dehydro-PGF $_{2\alpha}$ (PGD $_2$) elutes before PGE $_2$ from silicic acid columns (16) and therefore should not interfere in our estimations of PGE $_2$ formation. This assumption was confirmed by the finding that radioimmunoassay and bioassay gave essentially the same values for PGE $_2$ formation (cf. references 4 and 8).

Addition of known amounts of PGF $_{2\alpha}$ to PRP yielded corresponding increases upon radioimmunoassay (Fig. 3).

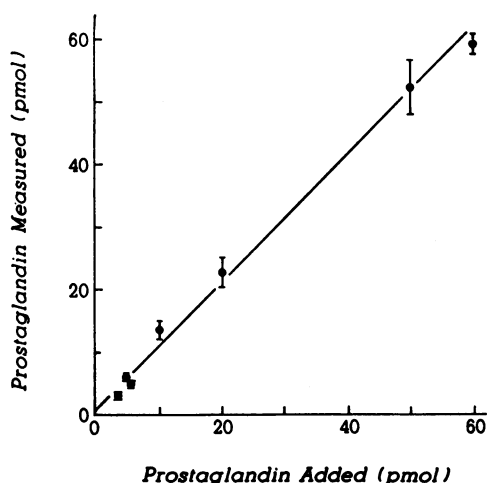


FIGURE 3 Relation between added $\text{PGF}_{2\alpha}$ and that measured by radioimmunoassay. Known amounts of $\text{PGF}_{2\alpha}$ were added to 2 ml PRP and extracted with and without stannous chloride treatment as described in Methods. Each point is the mean (\pm SD) of at least four determinations. Linear regression analysis of the data revealed a correlation coefficient of 0.993. The equation of the "added" versus "measured" plot is $Y = 1.00x + 1.19$.

The correlation coefficient between the amount added and the amount found was 0.993, and the slope of the regression line was 1.00. Basal levels of $\text{PGF}_{2\alpha}$ in PRP were less than 0.5 pmol/ml. These levels were not increased when 1,000 pmol PGE_2 was added to 2 ml PRP and extracted as described above. This demonstrates that PGE_2 does not interfere with the estimation of $\text{PGF}_{2\alpha}$ and that PGE_2 is not reduced to $\text{PGF}_{2\alpha}$ by treatment with stannous chloride. Addition of varying amounts of PGE_2 to PRP, followed by extraction, borohydride reduction, and radioimmunoassay yielded the expected increases.

RESULTS

When arachidonic acid was added to PRP, it induced irreversible platelet aggregation and the formation of PGE_2 and $\text{PGF}_{2\alpha}$, which continued during the 5-min observation period (Fig. 4). Values for $\text{PGF}_{2\alpha}$ in PRP decanted into ethanolic stannous chloride at 30 s or 1 min were significantly higher ($P < 0.005$, t test, six different subjects) than values for corresponding samples decanted into ethanol. Since the endoperoxide intermediate that gives rise to PGE_2 and $\text{PGF}_{2\alpha}$ is chemically reduced to $\text{PGF}_{2\alpha}$ with stannous chloride, the difference between these values is presumably endoperoxide intermediate. Fig. 4 shows that the amount of endoperoxide intermediate was maximal at about 1 min after addition of arachidonic acid. When 1 mM aspirin was added 30 s before the arachidonic acid in three experiments, it prevented aggregation and blocked the formation of endoperoxide (< 0.1 pmol/ml PRP). Neither aggregation nor endoperoxide formation was detected when a non-

precursor fatty acid, 11, 14, 17-eicosatrienoic acid, was incubated with PRP.

Addition of collagen suspension to PRP induced changes similar to those noted with arachidonic acid (Fig. 5a). Although the amounts of PGE_2 and endoperoxide intermediate formed were relatively small, values for the endoperoxide intermediate were significant at 15 and 30 s ($P < 0.001$, t test, four different subjects).

In the case of epinephrine, which induced its characteristic biphasic aggregation pattern, the endoperoxide intermediate was not detected until the second wave of aggregation (Fig. 5b). Also, the amounts of endoperoxide found during the second wave were quite variable (four subjects).

With arachidonic acid and collagen, the initial formation of endoperoxide intermediate appeared to precede platelet aggregation. However, with epinephrine, endoperoxide formation was seen only during the second wave of aggregation. These results suggested that endoperoxide formation is associated with the platelet release reaction. Table I shows that whereas arachidonic acid and collagen released considerable amounts of

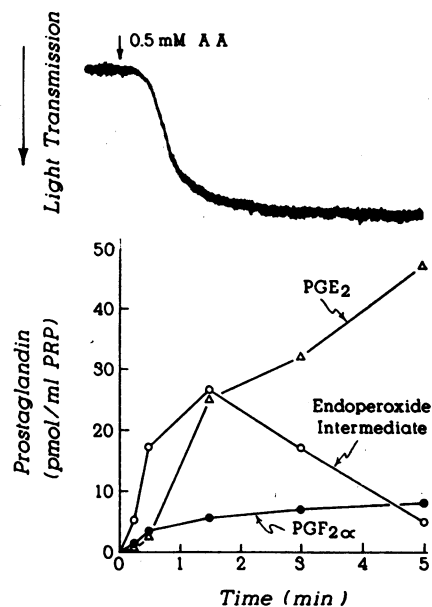


FIGURE 4 Platelet aggregation, prostaglandin and endoperoxide formation induced by 0.5 mM arachidonic acid (AA). A representative experiment is shown. Symbols represent PGE_2 (Δ), $\text{PGF}_{2\alpha}$ (\bullet), and endoperoxide intermediate reducible to $\text{PGF}_{2\alpha}$ (\circ). Incubation of PRP with saline for 5 min at 37°C yielded control values of less than 1 pmol PGE_2 or $\text{PGF}_{2\alpha}$ /ml PRP, while incubation of arachidonic acid with platelet-poor plasma for 5 min at 37°C yielded values of 5.9 pmol PGE_2 /ml and 1.0 pmol $\text{PGF}_{2\alpha}$ /ml. Results were corrected for the latter values and for recoveries of $[^3\text{H}]\text{PGF}_{2\alpha}$ (which ranged between 55 and 70%). The plasma contained 4.2×10^8 platelets/ml.

[3'-¹⁴C]5-hydroxytryptamine, ATP, and ADP by 1 min, epinephrine released very little. We also observed that some release of these substances appeared to have occurred as early as 15 s after addition of collagen to PRP and at 2 min after epinephrine. Therefore, the formation of the endoperoxide intermediate is closely correlated with the platelet release reaction (9).

In previous studies, newly formed PGE₂ and PGF_{2α} were found outside the platelets 3 min after addition of collagen to PRP (4). Experiments similar to the above showed that about 90% of the endoperoxide intermediate was present outside the platelets 1 min after collagen.

DISCUSSION

The distinguishing feature of the endoperoxide intermediate described by Hamberg and Samuelsson (1) is its ready convertibility to prostaglandins. Thus, while the compound spontaneously rearranges to PGE₂ and 11-dehydro-PGF_{2α}, its mild reduction with stannous chloride yields PGF_{2α}. The methods developed for measuring endoperoxide intermediate in the present study should be generally useful in investigations of its biological significance.

Our results show that an intermediate compound, reducible to PGF_{2α} with stannous chloride, was formed not only when platelets were incubated with the prostaglandin precursor, arachidonic acid (Fig. 4), but also when they were aggregated by collagen (Fig. 5). During collagen-induced aggregation, ADP is released from platelets, as shown by other workers (17, 18) and by Table I. In addition, it has been shown that, although it does not inhibit primary ADP-induced aggregation, aspirin inhibits the release of ADP and aggregation induced by collagen (19). These findings indicate that ADP release is necessary for aggregation by collagen. ADP is also released from platelets during the second wave of aggregation by epinephrine (11, and this paper).

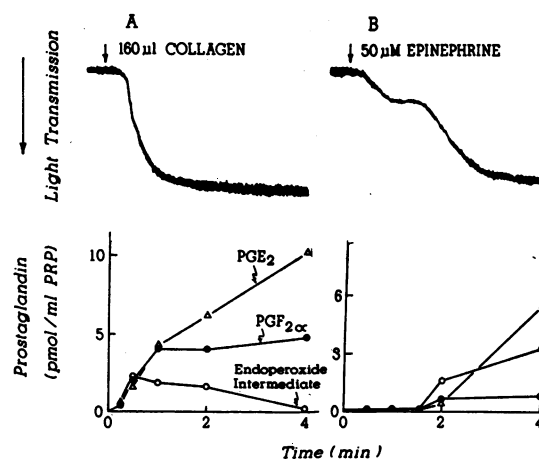


FIGURE 5 Time-courses for aggregation, prostaglandin and endoperoxide formation induced by 160 μ l collagen suspension (A) or 50 μ M epinephrine (B). Symbols as for Fig. 4. Values were corrected for recoveries of [³H]PGF_{2α} and for control levels. The latter were estimated by incubation of PRP with saline alone for 5 min at 37°C and were less than 0.5 pmol PGF_{2α}/ml PRP (experiments A and B), 0.98 pmol PGE₂/ml PRP (experiment A) and 0.93 pmol PGE₂/ml PRP (experiment B). The plasma contained 5.7×10^8 platelets/ml in experiment A and 3.3×10^8 platelets/ml in experiment B.

Both collagen and epinephrine induce the formation of PGE₂ and PGF_{2α} (4). The present studies indicate that this formation, like the release of ADP, begins before aggregation induced by collagen, but not until the second wave of aggregation by epinephrine. Therefore, the formation of prostaglandins is very closely related to the release of ADP.

Although PGE₂ and PGF_{2α} cause neither the release of ADP nor platelet aggregation (20), their precursor, arachidonic acid, does (Table I and Fig. 4). This suggests that some intermediate or byproduct of their bio-

TABLE I
Release of Adenine Nucleotides and of [3'-¹⁴C]5-Hydroxytryptamine during Platelet Aggregation

Aggregating agent	Increase in light transmission	Release		
		5-HT*	ADP	ATP
	%		%	
Arachidonic acid (0.5 mM)	47 \pm 1	55.1 \pm 1.6	25.2 \pm 1.8	22.9 \pm 1.0
Collagen suspension (120 μ l)	46 \pm 1	50.3 \pm 0.9	41.6 \pm 7.3	20.9 \pm 2.7
Epinephrine (50 μ M)	17.5 \pm 0.3	1.1 \pm 0.9	0.1 \pm 0.1	0.6 \pm 0.5

Samples of 1.5 ml PRP, previously incubated with radioactive 5-hydroxytryptamine, were stirred at 37°C for 1 min after the addition of the aggregating agent. At the end of the incubation, the platelets were removed by centrifugation and samples of the plasma were taken for measurement of the release of radioactivity and nucleotide content. Each value is the mean (\pm SEM) of four determinations with PRP from two individuals. The average platelet count was 3.8×10^8 platelets/ml plasma and there was, on the average, 6.8 nmol ATP and 5.1 nmol ADP/ 10^8 platelets.

* 5-HT, 5-hydroxytryptamine.

synthesis, if not arachidonic acid itself, may be a trigger for the platelet release reaction. The intermediate described in this communication was found outside platelets 1 min after addition of collagen. Its formation and platelet aggregation were abolished by aspirin. Therefore, we propose that the endoperoxide intermediate induces the release of ADP and causes platelet aggregation. Confirmation of this hypothesis will require studies of the effects of the pure endoperoxide.

Willis and Kuhn (21) have recently described the formation of a "labile aggregating-stimulating substance" during incubation of arachidonic acid with a sheep seminal vesicle preparation. They found that arachidonic acid alone did not cause platelet aggregation, but this may be because their vehicle for arachidonic acid contained excess amounts of antioxidants. The aggregating factor they detected may be the endoperoxide intermediate.

There is now considerable evidence showing that tissues form and release prostaglandins in response to a variety of stimuli that disturb cell membranes (see reference 22). Our results suggest that the endoperoxide intermediate would also be released in response to such stimuli. Could this intermediate in prostaglandin biosynthesis be a previously unrecognized platelet-aggregating factor released from damaged tissues?

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