Formation of Prostaglandins during the Aggregation of Human Blood Platelets

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ABSTRACT Prostaglandins E_2 and $F_{2\alpha}$ were formed in response to ADP, L-epinephrine, or collagen by human platelets suspended in plasma containing citrate anticoagulant and stirred at 37°C. The prostaglandins formed by platelets in response to collagen were rapidly released and the amounts formed were proportional to the amount of collagen added. The formation of the prostaglandins was associated with the single wave of aggregation induced by collagen or the second wave of aggregation induced by epinephrine. The above findings are discussed with reference to published studies on the biochemical changes occurring during platelet aggregation. It is suggested that the formation and release of prostaglandins is associated with the secretion of endogenous ADP and 5-hydroxytryptamine.

INTRODUCTION

Aggregation of platelets by exposed subendothelial collagen is generally considered to be a very early stage in hemostasis (1). Studies in vitro indicate that such aggregation is the consequence of the release of endogenous ADP (2, 3). Endogenous ADP is also released during aggregation of platelets by epinephrine or by exogenous ADP, and after treatment of platelets with thrombin (4). Since prostaglandins E_2 and $F_{2\alpha}$ are known to be formed and released from platelets by thrombin (5, 6), we investigated whether prostaglandins were produced by collagen, epinephrine, or ADP.

METHODS

Platelet aggregation. Human blood was collected from the antecubital vein of healthy volunteers (who had not taken aspirin within the previous week) into 0.1 vol of 3.8% trisodium citrate. Platelet-rich plasma (PRP)[1] was prepared by centrifuging the blood at 250 *g* for 15 min at 20–22°C, and the cell content was determined using a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.) (average 4.5×10^8 platelets, 9.3×10^6 red cells, and 8.5×10^2 white cells/ml). Aggregation of the platelets was studied photometrically in siliconized test tubes at 37°C with the continuous recording of light transmission (Aggregometer, Chrono-Log Corp., Broomall, Pa.). Routinely, 2.5-ml samples of PRP were used, and 1 ml ethanol was added at the end of the incubation period. ADP sodium salt (Sigma Chemical Co., St. Louis, Mo.) and epinephrine hydrochloride (Sigma) were stored as 1-mM solutions and thrombin (Parke, Davis & Co., Detroit, Mich., Bovine) as a 100 U/ml solution for up to 2 mo at −20°C. Collagen suspensions were prepared essentially as described elsewhere (7) and stored for up to 2 wk at 4°C.

Extraction and determination of prostaglandins. The contents of two aggregometer tubes, each of which contained 2.5 ml PRP and 1 ml ethanol, were decanted into a glass-stoppered tube containing 5 ml saline and 8 ml ethanol. The pH was adjusted to 3 with 9.2% formic acid, the mixture shaken with 25 ml chloroform, and then centrifuged. The lower chloroform phase containing the prostaglandins was removed and evaporated to dryness *in vacuo,* and the formic acid-free residue was kept overnight under nitrogen (modified from reference 8). Prostaglandins in the residue were partially purified by column chromatography and measured as PGE_2 by bioassay on the rat fundus strip or as $PGF_{2\alpha}$ by radioimmunoassay. Recoveries through the above procedure of [^{3}H]PGE_2 or [^{3}H]$PGF_{2\alpha}$ (New England Nuclear, Boston, Mass.) were 71.8 (3.6 SD) or 76.5% (3.5 SD), respectively. The methods of bioassay, radioimmunoassay, column chromatography, and thin-layer chromatography (TLC) have been described previously (6).

RESULTS

Fig. 1A shows typical aggregation recordings obtained with the PRP of 10 donors in response to 40 μM ADP, 40 μM epinephrine, or 200 μl collagen suspension: aggregation by ADP was rapid in onset and continued as one wave for 3 min; aggregation by epinephrine was

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[1] *Abbreviation used in this paper:* PRP, platelet-rich plasma.

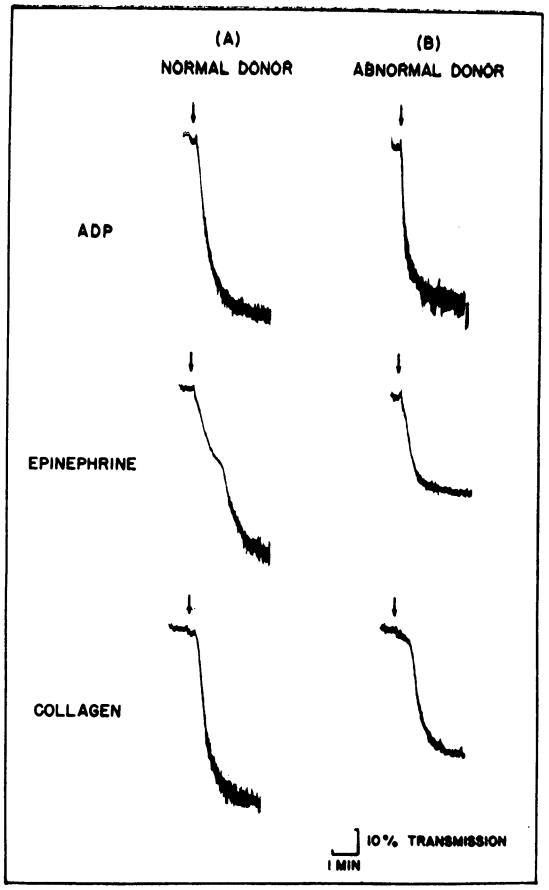

FIGURE 1 Photometric recordings of platelet aggregation induced by 40 μM ADP, 40 μM epinephrine, or 200 μl collagen suspension. Agents were added at (↓) to 2.5 ml PRP stirred at 37°C. Recordings show normal response of 10 donors (A) and "abnormal" response of 1 donor (B).

also rapid in onset, but a second wave of aggregation commenced at about 1 min and continued up to 3 min; aggregation by collagen was preceded by a lag phase of about 20 s and then continued as one wave. Recordings obtained with plasma from the 11th donor (Fig. 1B) were different from those of the other donors: the extent of aggregation in response to ADP was somewhat reduced; no second wave of aggregation was seen in response to epinephrine; the rate and extent of aggregation in response to collagen were markedly reduced.

Fig. 2 shows that platelets produced prostaglandins during aggregation with the above agents. Prostaglandins were not detected by biological assay in the prostaglandin fraction of total lipid extracts of saline-treated PRP, and therefore their basal levels in platelets must be very low. However, measurable amounts of prostaglandin were found in 9 of the 11 PRP treated with ADP and in 10 of the 11 PRP treated with epinephrine, collagen, or thrombin. With each of 10 donors, more prostaglandins were formed in response to collagen than to epinephrine or ADP. The donor with atypical aggregation responses did not produce prostaglandins in response to any agent.

To identify the biologically active prostaglandins formed by platelets, the partially purified extracts from 15 ml of collagen-treated PRP were submitted to TLC on silver-impregnated plates (which separate PGE_1, PGE_2, $PGF_{1\alpha}$, and $PGF_{2\alpha}$). Biological activity was confined to zones corresponding to PGE_2 and $PGF_{2\alpha}$ as previously reported for serum (6). Further evidence that the platelets synthesize $PGF_{2\alpha}$ was obtained by radioimmunoassay. After TLC of a partially purified extract from 10 ml of collagen-treated PRP, material which inhibited the binding of [^{3}H]$PGF_{2\alpha}$ to its antibody was found mainly in the zone corresponding to authentic $PGF_{2\alpha}$. The values obtained by radioimmunoassay of partially purified extracts of PRP treated with saline (< 1 pmol $PGF_{2\alpha}$/ml), 40 μM ADP (3.0 pmol/ml), 40 μM epinephrine (4.4), collagen suspension (6.4), and 1 U/ml thrombin (9.8) suggest that this prostaglandin is produced by platelets in response to a variety of agents.

Prostaglandins E_2 and $F_{2\alpha}$ were released from platelet by collagen. Table I shows the results of experiments in which the amounts of prostaglandins in plasma, centrifuged free of aggregated platelets, were compared with the amounts present in PRP before centrifugation. When increasing amounts of collagen suspension were added to PRP, proportionately greater amounts of prostaglandins E_2 (from 7.1 pmol/ml with 50 μl collagen to 12.3 with 200 μl) and $F_{2\alpha}$ (from 3.6 pmol/ml with 50 μl collagen to 9.1 with 200 μl) were produced.

The time course of prostaglandin formation induced by collagen was compared with corresponding photometric recordings of aggregation (Fig. 3*a*). Prostaglandins were synthesized apparently as aggregation commenced. Synthesis stopped after 5 min incubation with collagen (not shown). In similar experiments with 40 μM epinephrine, the formation of detectable amounts of prostaglandins was associated with a second wave of aggregation (Fig. 3*b*).

In experiments in which low concentrations (1–4 μM) of ADP were used, so that a second wave of aggregation could be distinguished, prostaglandin formation could not be demonstrated. Also carrageenin (100 μg/ml), which enhanced the second wave of aggregation induced by ADP (9), did not produce detectable amounts of prostaglandins nor enhance the amounts formed in response to ADP.

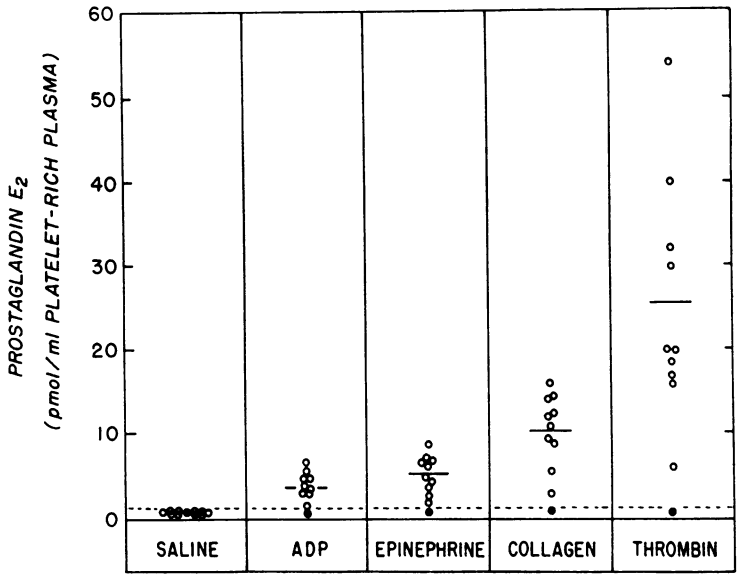

FIGURE 2 Prostaglandin contents of partially purified extracts of PRP stirred at 37°C for 3 min with saline, ADP, epinephrine, collagen, (aggregation recordings shown in Fig. 1) or 1 U/ml thrombin (which induced clotting). Prostaglandins were determined by biological assay and the broken line indicates the detection limit. Each circle depicts the mean value of duplicate incubations with an average variation from the mean of 17%. The horizontal lines indicate the means for the normals. The closed circle refers to the donor with abnormal aggregation patterns (Fig. 1).

DISCUSSION

We examined prostaglandin formation induced by collagen in some detail because of its possible physiological importance. The prostaglandins detected after treatment of PRP with collagen were the result of synthesis because prostaglandins could not be detected in saline-treated PRP. Their production was proportional to the amount of collagen added to PRP. Moreover, the prostaglandins were found to be entirely extracellular 3 min after the addition of collagen. Therefore, these prostaglandins may act either within the platelet before release or outside the platelet after release. However, the question of positive or negative feedback is complicated. Although exogenous prostaglandin E_2 enhances the single wave of ADP-induced aggregation of rat or pig platelets in concentrations of greater than 5 pmol/ml (10), it inhibits the first wave and enhances the second wave of aggregation of human platelets (9).

Aggregation by collagen and second wave of aggregation by epinephrine or ADP are generally believed to be caused by endogenous ADP extruded from the platelets into plasma (4). Therefore, the temporal association between prostaglandin formation and the single wave of collagen-induced aggregation or the second wave of epinephrine-induced aggregation indicates that synthesis of prostaglandins parallels the release of ADP. Although we were unable to detect prostaglandin formation in response to concentrations (1–4 μM) of ADP that caused two waves of aggregation, we could detect their formation with 40 μM ADP. More sensitive assay methods will be required to determine whether prostaglandins are formed during the second wave of ADP-induced aggregation.

TABLE I
Release of Prostaglandins by Collagen

	A	B
	Extracts of plasma containing aggregated platelets	Extracts of supernate containing no platelets
	pmol/ml	*pmol/ml*
PGE_2	7.0±0.6	7.3±0.6
$PGF_{2\alpha}$	4.1±0.2	4.5±0.2

2.5-ml samples of PRP were stirred at 37° C for 2.5 min with 100 μl collagen in aggregometer tubes. 0.1 ml EDTA (0.1 M) was added and the tubes were removed from the aggregometer. The B samples were centrifuged to remove platelets (20 s, 15,000 *g*, Eppendorf microfuge) and produce supernatant plasma. At 3 min, 2.5 ml PRP (A samples) or 2.5 ml supernatant plasma (B samples) was extracted with ethanol. Results show mean (picomoles/milliliter plasma) and standard error of four determinations. PRP not incubated with collagen contained less than 1 pmol/ml PGE_2 or $PGF_{2\alpha}$.

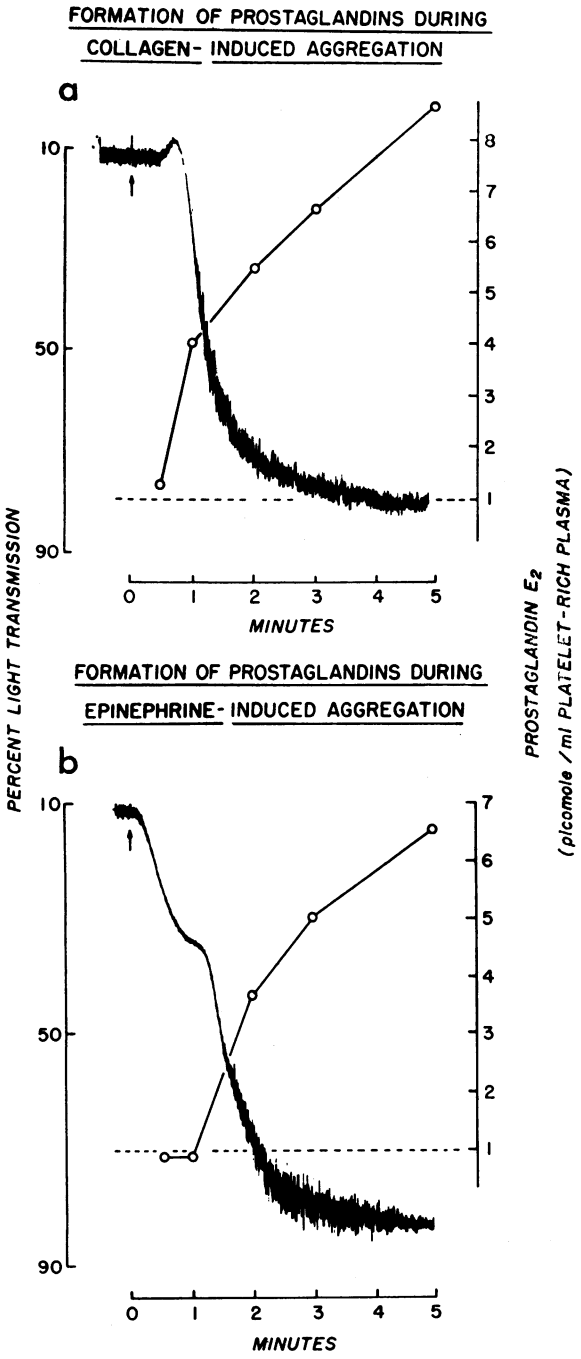

FIGURE 3 Time course of aggregation and prostaglandin formation when 100 μl collagen suspension (*a*) or 40 μM (final concentration) epinephrine (*b*) was added (↑) to 2.5 ml PRP stirred at 37°C. Representative experiments are shown. Broken lines indicate the detection limit of the assay. In four experiments with PRP from different donors measurable amounts of prostaglandins were present at 1 min after collagen (*a*) but not at 1 min after epinephrine (*b*). In all cases measurable amounts of prostaglandins were found 2 min after epinephrine.

The platelets from one donor did not produce prostaglandins, even in response to thrombin, and showed abnormal patterns of aggregation consistent with diminished release of endogenous ADP. Regardless of the reason for the abnormalities, their concurrence reinforces the association between prostaglandin formation and the release of ADP. Both abnormalities are virtually identical with those caused by aspirin ingestion (11, 12), but we have taken great care in screening our donors and feel reasonably confident that in this case they were not due to any drug. O'Brien (12) has also noted the absence of second wave aggregation with epinephrine in PRP from some donors who have not taken aspirin.

It has now been shown that prostaglandins are formed during clotting (6), by the action of thrombin on washed platelets (5), and, as we show here, during aggregation by collagen, epinephrine, or ADP. A common feature of all these reactions is that they involve the selective release of stored 5-hydroxytryptamine, ATP, and ADP from intracellular platelet organelles (13, 14). The relevance to this release of the finding that 5-hydroxytryptamine can act as a coenzyme for prostaglandin biosynthesis (15) remains to be established.

We have previously suggested that prostaglandins are formed by platelets during hemostasis and thrombosis (6). The findings presented here support this proposal since they show that prostaglandins are formed during collagen-induced platelet aggregation, certainly an early event in hemostasis.

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