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Physiological Role of an Endoperoxide in Human Platelets: Hemostatic Defect Due to Platelet Cyclo-Oxygenase Deficiency*

(lipoxygenase/arachidonic acid/platelet aggregation/prostaglandins/release reaction)

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ABSTRACT **The endoperoxide prostaglandin G_2 (PGG_2) induced platelet aggregation as well as the platelet release reaction (release of ADP and serotonin) when added to human platelet-rich plasma. Formation of a metabolite of PGG_2 [8-(1-hydroxy-3-oxopropyl)-9,12*L*-dihydroxy-5,10-heptadecadienoic acid] and a lipoxygenase product [12*L*-hydroxy-5,8,10,14-eicosatetraenoic acid] accompanied the release reaction caused by aggregating agents such as collagen, ADP, epinephrine, and thrombin. Indomethacin inhibited the release reaction and PGG_2 formation induced by these agents but had no effect on PGG_2-induced release reaction. The aggregating effect of PGG_2 was abolished by furosemide, which is a competitive inhibitor of ADP-induced primary aggregation. These data indicate that the aggregating effect of PGG_2 is due to release of ADP and that PGG_2 synthesis is required for induction of the release reaction by various aggregating agents.**

A subject with a hemostatic defect due to abnormal release mechanism [decreased aggregation with epinephrine (second wave) and collagen and normal platelet ADP] had a deficiency of the cyclo-oxygenase that catalyzes formation of PGG_2. Normal aggregation and release reaction were obtained with added PGG_2.

It is concluded that the endoperoxide (PGG_2) is essential in normal hemostasis because of its role in initiating the release reaction required for aggregation by collagen and the second wave of aggregation caused by, e.g., ADP.

Prostaglandins (PG) E_2 and $F_{2\alpha}$ are formed during aggregation of human platelets induced by various agents (2, 3). Neither PGE_2 nor $PGF_{2\alpha}$ cause aggregation, but PGE_2 has been reported to inhibit the first wave and enhance the second wave of aggregation induced by ADP (4–6). Conflicting results showing inhibition of both ADP- and collagen-induced aggregation by PGE_2 have also appeared (7). The role of these classical prostaglandins in platelet aggregation therefore seemed uncertain.

Recent work from this laboratory demonstrated that the two endoperoxides derived from arachidonic acid, PGG_2 and PGH_2, are very potent in inducing rapid and irreversible aggregation of human platelets (8, 9). Since aspirin, an inhibitor of endoperoxide formation, inhibits the second wave of aggregation, it was suggested that the endoperoxides play a role in the release reaction (9). The formation of material reducible with stannous chloride to $PGF_{2\alpha}$ during aggregation induced by various agents also supported this view (9, 10). Arachidonic acid induces aggregation when added to human platelets (11, 12) and aggregating material is formed from this acid when it is incubated with preparations of sheep vesicular gland (13). The latter material has not been conclusively identified but might, in view of the work described above (9), be a mixture of PGG_2 and PGH_2 (14).

The importance of the endoperoxide system has recently become apparent through the discovery of novel transformations of the endoperoxide (15). When incubated with washed human platelets, arachidonic acid was transformed via two main pathways (Fig. 1). The initial step in one of them was catalyzed by a lipoxygenase and a hydroxyeicosatetraenoic acid (HETE) was formed as end product. In the second pathway arachidonic acid was transformed by a cyclo-oxygenase into the endoperoxide PGG_2, which was almost exclusively converted into two nonprostanoate structures, namely a C_{17}-hydroxy-trienoic acid (HHT) and a hemiacetal derivative (PHD) (Fig. 1). Additional studies demonstrated that aggregation of washed platelets by thrombin was accompanied by release of large amounts of end products from both pathways of arachidonic acid metabolism (16). In this system intact endoperoxides and PGE_2 and $PGF_{2\alpha}$ only accounted for a very small part of the transformation via the cyclo-oxygenase pathway, the main products being HHT and PHD. The new concept resulting from this study was that arachidonic acid can exert its biological action through the endoperoxide instead of the classical prostaglandins and that the endoperoxide may be metabolized to and subsequently released almost exclusively as nonprostanoate structures.

In the present work the mechanism of action of the endoperoxide in platelet aggregation has been further studied. We have also established a physiological role of the endoperoxide in platelets, since a hemostatic defect in a patient was found to be due to platelet cyclo-oxygenase deficiency and thus deficient formation of the endoperoxide PGG_2.

MATERIALS AND METHODS

Thrombin (Topostasin®) was purchased from Hoffman-La Roche Co., ADP and epinephrine from Sigma Chemical Co., arachidonic acid from Nu Chek Prep. Inc., Elysian, Minn., collagen from Stago Laboratoire, Asnières, France, and furosemide (Lasix®) from Farbwerke Hoechst AG.

Platelet Preparations. Blood from healthy donors who had not taken aspirin for 1 week was collected from the antecubital vein with 0.13 volume of 0.1 M trisodium citrate solution and

Abbreviations: PG, prostaglandin; HETE, 12*L*-hydroxy-5,8,10,-14-eicosatetraenoic acid; HPETE, 12*L*-hydroperoxy-5,8,10,14-eicosatetraenoic acid; HHT, 12*L*-hydroxy-5,8,10-heptadecatrienoic acid; PHD, 8-(1-hydroxy-3-oxopropyl)-9,12*L*-dihydroxy-5,10-heptadecadienoic acid; PRP, platelet-rich plasma; 20:4, arachidonic acid; PCO, platelet cyclo-oxygenase.

* This is paper no. 8 in a series on prostaglandin endoperoxides. Paper no. 7 is ref. 1.

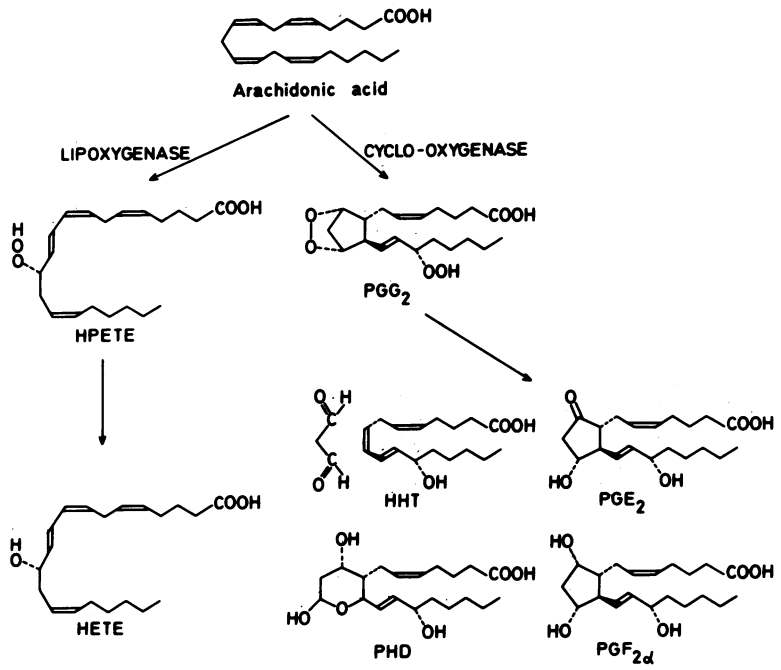

FIG. 1. Transformations of arachidonic acid in human platelets.

centrifuged at 200 × *g* for 15 min at room temperature. The upper layer of platelet-rich plasma (PRP) was removed and kept at room temperature. Calcium chloride (30 μl, 0.25 M solution/ml of PRP) was added and samples were preincubated for 2 min at 37° before use. Washed human platelets were prepared as previously described and suspended in Krebs-Henseleit medium without calcium (9).

Aggregation was monitored by continuous recording of light transmission (Aggregometer, Chrono-Log Corp., Broomall, Pa.).

Release of [^{14}C]Serotonin and ADP. The PRP was incubated at room temperature for 30 min with 0.5 μM [2-^{14}C]serotonin creatinine sulfate (55 Ci/mol, The Radiochemical Centre, Amersham, England). One and a half milliliter samples were decanted into 0.2 ml ice-cold 0.1 M EDTA, pH 7.4, with rapid mixing. After centrifugation aliquots were removed for determination of radioactivity and of ADP (17).

Formation of Metabolites (PHD and HETE) of Arachidonic Acid. The quantitative measurements were carried out as previously described using deuterated carriers and quantitative mass spectrometry (16).

Transformation of [1-^{14}C]Arachidonic Acid. The formation of metabolites after incubation with washed platelets was determined as previously described (15).

RESULTS

Platelet Aggregation. PGG_2 caused rapid aggregation of platelets in PRP (Fig. 2). The threshold concentration of PGG_2 was about 0.5 μM. A linear dose (log)–response relationship was seen up to a concentration of 1 μM. Higher concentrations inhibited the aggregation. The potency of PGG_2 was 4–10 times higher than that of ADP.

Release of [^{14}C]Serotonin and ADP. The release of [^{14}C]-serotonin from platelets in PRP was studied with various aggregating agents. Addition of PGG_2 caused very rapid release of [^{14}C]serotonin followed by re-uptake. Maximum release was seen already after 5 sec. The release of [^{14}C]-serotonin by ADP, collagen, or thrombin reached maximum after 3–5 min. Fig. 3 shows the release with various aggregating agents.

PGG_2 also caused release of platelet ADP in PRP. The results together with corresponding data for collagen are given in Table 1.

Formation of Metabolites of Arachidonic Acid and [^{14}C]-Serotonin Release. Effect of Indomethacin. In some cases, the release of [^{14}C]serotonin and the formation of PHD and HETE were determined in the same samples of PRP after addition of various aggregating agents and indomethacin. The results are summarized in Table 2. Addition of PGG_2 resulted in release of [^{14}C]serotonin and small amounts of PHD and HETE. Indomethacin did not have any effect on these parameters. The PHD formed apparently originated in the added PGG_2. Collagen and ADP both released [^{14}C]-serotonin and caused formation of PHD and HETE. Indomethacin inhibited both the release of [^{14}C]serotonin and the formation of PHD. With thrombin as aggregating agent, indomethacin only gave partial inhibition of both the release of [^{14}C]serotonin and the formation of PHD.

Effect of Furosemide on PGG_2- and ADP-Induced Aggregation. Aggregation was induced in PRP by either ADP (4 μM) or PGG_2 (0.8 μM) and followed in the aggregometer. Addition of furosemide 2 min prior to the aggregating agent inhibited the aggregation. The concentration of furosemide required for 50% inhibition was 10 mM for ADP and 0.8 mM for PGG_2. Complete inhibition of PGG_2-induced aggregation was obtained at a concentration of 3 mM.

Characterization of Subject with Platelet Fatty Acid Cyclooxygenase Deficiency (PCO-Deficiency). B.A. is a 30-year-old man with some tendency to easy bruising and a suspect

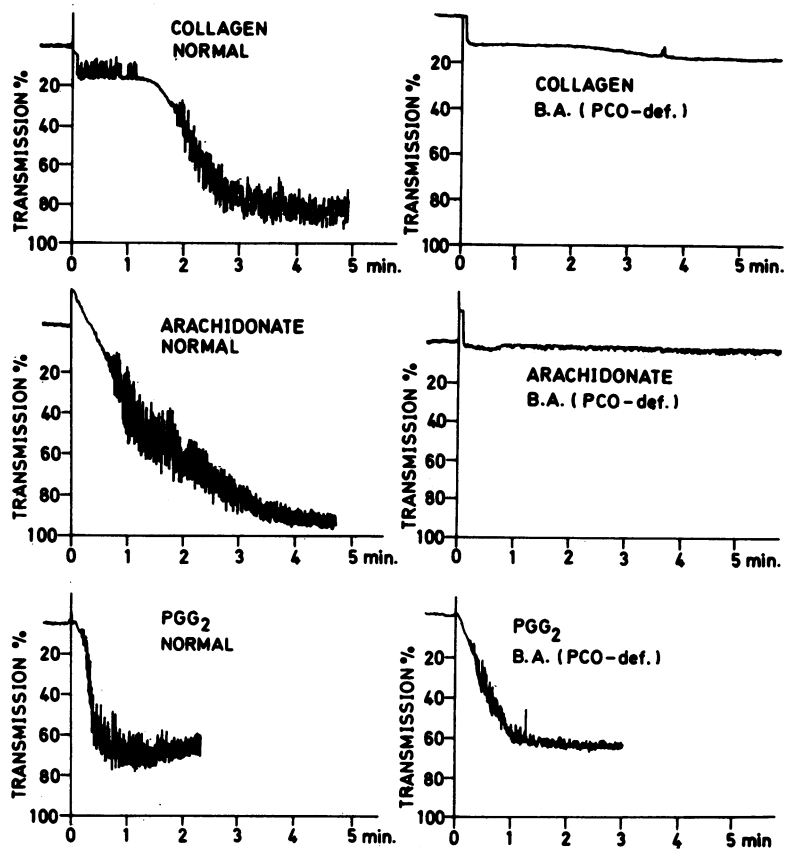

FIG. 2. Effects of aggregating agents on PRP from a normal subject and a case (B.A.) of platelet cyclo-oxygenase-deficiency (PCO-def.). Collagen: 20 μg/ml; arachidonate: 0.2 mM; PGG_2: 0.6 μM. The aggregating agents were added at 0 min.

retinal bleeding at age 19. No complications followed hand surgery at age 27. There is no history of bleeding either in parents or a sister, whereas an uncle had excessive bleeding twice in connection with surgery. These relatives showed normal aggregation with collagen, ADP, and PGG_2.

B.A. had prolonged bleeding time (8.5 and 9.0 min, determined on two different occasions) (normal mean $\pm$ 2 SD = 3–7 min). Normal values were found for platelet count, prothrombin time, partial thromboplastin time, and plasma fibrinogen. These analyses were kindly carried out by Drs. M. Blombäck and I. Norén. B.A. had not taken any drugs at least 10 days prior to any of the investigations reported here, which were carried out on several different occasions.

PRP from B.A. was treated with various aggregating agents. Some of the results are shown in Fig. 2, together with a typical normal response. There was no aggregation of PRP from B.A. with collagen or arachidonic acid but normal aggregation with the endoperoxide, PGG_2. Only primary aggregation was seen with ADP or epinephrine, whereas normal subjects also gave secondary aggregation.

In comparison with normals, [^{14}C]serotonin release with PRP from B.A. was almost abolished when induced by ADP

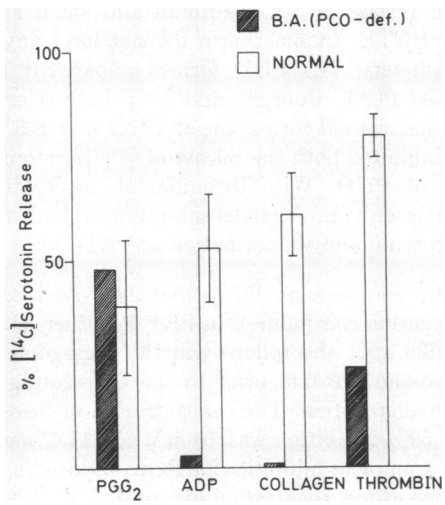

FIG. 3. Release of [^{14}C]serotonin from platelets in PRP by various aggregating agents in normal subjects ($\pm$SD, n = 5) and a case of PCO-deficiency. PGG_2: 0.6 μM; ADP: 10 μM; collagen: 20 μg/ml; thrombin: 1 U/ml.

TABLE 1. *Release of ADP by PGG_2 and collagen*

	ADP(μmol/10^{11} platelets)			
	Normal ($\bar{X}$ $\pm$ SD, n = 4)		B.A.	
	Collagen	PGG_2	Collagen	PGG_2
Resting platelets	2.2 $\pm$ 0.4	2.2 $\pm$ 0.4	1.8	1.8
Stimulated platelets	1.0 $\pm$ 0.2	0.8 $\pm$ 0.7	1.6	0.3
Released to plasma	1.3 $\pm$ 0.4	1.3 $\pm$ 0.5	0.4	1.6

The ADP content was determined 45 sec and 5 min after addition of PGG_2 (0.6 μM) and collagen (20 μg/ml), respectively.

TABLE 2. *Formation of PHD and HETE and [^{14}C]serotonin release induced by addition of various aggregating agents to PRP. Effect of indomethacin (IM).*

Aggregating agent	Inhibitor	PHD (ng/ml)				HETE (ng/ml)				[^{14}C]Serotonin release (%)			
		Normal subjects			PCO-def.	Normal subjects			PCO-def.	Normal subjects			PCO-def.
		C.M.	E.H.	C.L.	B.A.	C.M.	E.H.	C.L.	B.A.	C.M.	E.H.	C.L.	B.A.
Vehicle	None	1	2	1	0	0	—	4	2	0	0	0	0
PGG_2 (0.6 μM)	None	3	7	3	5	6	—	8	2	38	20	22	28
PGG_2 (0.6 μM)	IM	4	6	—	—	6	—	—	—	31	18	—	—
Collagen (20 μg/ml)	None	17	149	39	2	4	—	15	1	61	40	82	0
Collagen (20 μg/ml)	IM	1	3	—	—	5	—	—	—	5	7	—	—
ADP (10 μM)	None	10	32	21	2	10	—	16	0	66	50	35	0
ADP (10 μM)	IM	0	3	—	—	5	—	—	—	2	5	—	—
Thrombin (1 U/ml)	None	52	218	50	0	24	—	24	23	87	67	77	70
Thrombin (1 U/ml)	IM	21	19	—	—	61	—	—	—	58	34	—	—
Epinephrine (0.6 μM)	None	—	—	11	3	—	—	16	4	—	—	45	0

and collagen and considerably decreased with thrombin but was normal when induced by PGG_2 (Fig. 3). Release of ADP by collagen was decreased but the content of ADP and the release of ADP by PGG_2 were normal with PRP from B.A. (Table 1).

The formation of the endoperoxide metabolite PHD and the metabolite of the lipoxygenase-catalyzed reaction, HETE, were monitored in PRP from B.A. following addition of various aggregating agents (Table 2). Neither collagen, ADP, thrombin, or epinephrine caused any significant formation of PHD. Normal amounts of HETE were formed with thrombin as aggregating agent.

The transformation of [^{14}C]arachidonic acid by washed platelets from B.A. and from a normal subject is shown in Fig. 4. Only trace amounts of the endoperoxide metabolites PHD and HHT were formed with platelets from B.A., whereas transformation into HETE was facilitated. The identities of the compounds indicated in Fig. 4 were verified by mass spectrometry (15).

DISCUSSION

The work leading to the concept that arachidonic acid can exert its biological action through the endoperoxide instead of the classical prostaglandins is summarized in the introduction. The present work is concerned with the mechanism of action and the physiological significance of the endoperoxide system in human platelets.

On a molar basis the endoperoxide, PGG_2, was 4 to 10 times more active than ADP in inducing platelet aggregation in platelet-rich plasma. The aggregation induced by PGG_2 was accompanied by initiation of the release reaction studied by the release of ADP and [^{14}C]serotonin. The PGG_2-induced release of [^{14}C]serotonin was very rapid compared with that induced by ADP or collagen. Indomethacin, an inhibitor of endoperoxide synthesis, inhibited the release reaction due to collagen and ADP but had no effect on PGG_2-induced release. An inhibitor of primary ADP-induced aggregation, furosemide (18), also inhibited PGG_2-induced aggregation. This compound has no effect on the release reaction but inhibits the ADP effect in a competitive manner (18). The results therefore indicated that PGG_2 causes aggregation through release of ADP.

The aggregating agents collagen, ADP, epinephrine, and thrombin, in addition to inducing the release reaction, studied by [^{14}C]serotonin release, also caused formation of the endoperoxide metabolite PHD and the lipoxygenase product HETE. Inhibition of the release reaction by indomethacin was accompanied by inhibition of PHD formation and thus also endoperoxide synthesis. Together with the results described above on the effects of PGG_2 these data strongly indicated that PGG_2 synthesis is an essential step in the release of ADP and serotonin induced by various aggregating agents.

Our present and previous results on the endoperoxide system have been summarized in a scheme shown in Fig. 5. Hydrolysis (*1*) induced by the aggregating agents (*2*) yields free arachidonic acid, which is transformed by reaction *7* to give the hydroperoxy-acid HPETE and by reaction *4* to give the endoperoxide PGG_2. PGG_2 through reaction *11* induces release of ADP and serotonin, a process which leads to aggregation. In accordance with this scheme the release reaction and aggregation induced by, e.g., collagen (*2*) and arachidonic acid (*3*), were inhibited by cyclo-oxygenase inhibitors (i.e., aspirin and indomethacin) acting on reaction *4*, whereas PGG_2 induced aggregation (*5*) was not susceptible to inhibition by these agents. The formation of PGG_2 can be monitored by analyzing the release of PHD and HHT (reactions *6* and *9*).

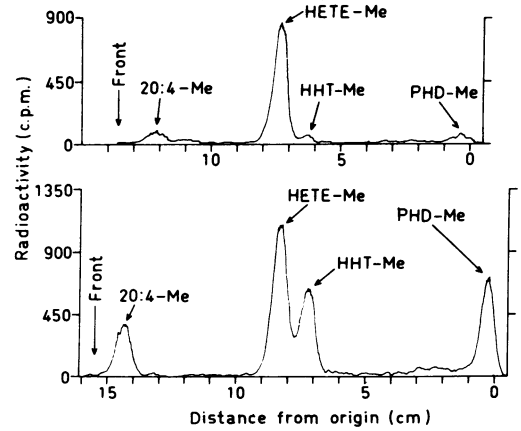

FIG. 4. Thin-layer radiochromatogram of products (methyl esters) obtained after incubation of 38 μg of [*1*-^{14}C]arachidonic acid (20:4) with 2 ml of platelet suspension at 37° for 5 min (compare ref. 15). Lower panel: Normal subject. Upper panel: PCO-deficiency (B.A.).

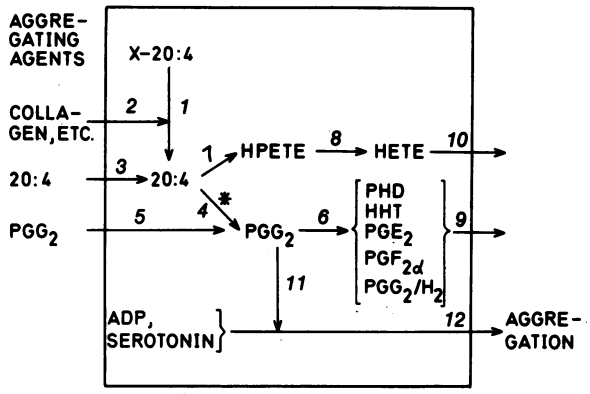

FIG. 5. Scheme of the role of the endoperoxide PGG_2 in human platelets. The asterisk marks the failing reaction in PCO-deficiency.

The function of the lipoxygenase reaction (7) is not yet clear.

Since aggregation of human platelets by collagen is a result of release of ADP and since an early stage in hemostasis is considered to be due to aggregation of platelets by exposed subendothelial collagen, a role of the endoperoxide PGG_2 in hemostasis also seemed feasible. We have now obtained direct evidence for such a role of PGG_2.

A subject with a mild hemostatic defect, who fulfilled the critical characteristics of a platelet disorder due to an abnormal release mechanism (17, 19–21), was found to have a deficiency of the cyclo-oxygenase enzyme (reaction 4 in Fig. 5). Platelets from this subject showed decreased aggregation with collagen and epinephrine (second wave) but had, in contrast to the findings in storage pool disease (17, 19–21), normal content of ADP. The new characteristics of the disorder are given in Table 3 (compare 21). Aggregation could not be induced by arachidonic acid but the platelets responded in a normal fashion to PGG_2 both with respect to aggregation and ADP or [^{14}C]serotonin release. Only traces of labeled PHD were formed from [^{14}C]arachidonic acid, whereas there was no decrease in the conversion to HETE. This was also reflected in the release of these two metabolites by aggregating agents. The findings that the cyclo-oxygenase was deficient and that the product of the deficient reaction, PGG_2 induced a normal response demonstrated that the abnormality is due to the enzyme deficiency. We, therefore, propose the name platelet cyclo-oxygenase (PCO) deficiency for this platelet disorder.

Another platelet release abnormality, storage pool disease, shows reduced platelet content of ADP and so called dense bodies (17, 19–21). This abnormality was recently found to be associated with reduced formation of PGE_2 and $PGF_{2\alpha}$ in response to aggregating agents (22). The relevance of this finding to the cause of the abnormality is not obvious since these prostaglandins do not seem to play a critical role in platelet aggregation (see introduction). It should be of interest to investigate endoperoxide synthesis and endoperoxide response in platelets from subjects with this disorder, since the primary defect might rather be decreased content of ADP and dense bodies.

TABLE 3. *New characteristics of platelet disorder due to abnormal release mechanism (21) (PCO-deficiency)*

Aggregation:	
Arachidonic acid	Abnormal
Prostaglandin G_2	Normal
Release of ADP or [^{14}C]serotonin:	
Prostaglandin G_2	Normal
Release of metabolite (PHD) of prostaglandin G_2 by collagen, ADP, or thrombin	Abnormal
Transformation of [^{14}C]arachidonic acid into:	
PHD	Abnormal
HETE	Normal

The present results establishing a role of the endoperoxide PGG_2 in platelet aggregation and more specifically in the release reaction seem to open new approaches to more detailed studies of these processes both under normal and pathological conditions. Furthermore, the endoperoxide system might be used as a tool in the development and evaluation of new therapeutic agents.

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