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Prostaglandin Endoperoxides. A New Concept Concerning the Mode of Action and Release of Prostaglandins*

(platelet aggregation/prostaglandin G_2/endoperoxide metabolites/quantitative determination/aspirin and indomethacin)

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ABSTRACT **Methods were developed for quantitative determination of the three major metabolites of arachidonic acid in human platelets, i.e., 12L-hydroxy-5,8,10,-14-eicosatetraenoic acid (HETE), 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and 8-(1-hydroxy-3-oxopropyl)-9,12L-dihydroxy-5,10-heptadecadienoic acid (PHD). Aggregation of washed platelets by thrombin was accompanied by release of 1163–2175 ng/ml of HETE, 1129–2430 ng/ml of HHT, and 998–2299 ng/ml of PHD. The amount of PGG_2 (prostaglandin G_2) produced as calculated from the sum of the amounts of its metabolites (HHT and PHD) was 2477–5480 ng/ml. In contrast, the amounts of PGE_2 (prostaglandin E_2) and $PGF_{2\alpha}$ (prostaglandin $F_{2\alpha}$) released were approximately two orders of magnitude lower. In this system, the prostaglandins thus exert their biological action through the endoperoxides, which are almost exclusively metabolized to nonprostanoate structures and only to a small extent to the classical prostaglandins.**

Platelets from subjects given aspirin produced less than 5% of the above mentioned amounts of HHT and PHD, whereas the production of HETE was stimulated about 3-fold. This provides additional evidence for our earlier proposal [Hamberg, M., Svensson, J., Wakabayashi, T. & Samuelsson, B. (1974) *Proc. Nat. Acad. Sci. USA* 71, 345–349] that the anti-aggregating effect of aspirin is through inhibition of PGG_2 formation.

In a recent paper (1) we showed that the prostaglandin endoperoxides, PGG_2 and PGH_2, in low concentrations induce aggregation of human platelets and that they are released during thrombin-induced aggregation. Together with the known effects of inhibitors of prostaglandin biosynthesis on platelets, this indicated a physiological role of endoperoxides in platelet function and gave information on the mechanism of action of, e.g., aspirin (1, 2). With a similar technique, it was also demonstrated that aggregation induced by collagen, epinephrine, and arachidonic acid was accompanied by release of the intact endoperoxides (3).

We recently studied the transformation of [1-^{14}C]arachidonic acid by human platelets and isolated three metabolites, i.e., 12L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE), 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT), and the hemiacetal derivative of 8-(1-hydroxy-3-oxopropyl)-9,12L-dihydroxy-5,10-heptadecadienoic acid (PHD) (4). HETE was formed by the action of a novel lipoxygenase unrelated to the enzymes involved in prostaglandin biosynthesis, whereas HHT and PHD were formed by pathways involving the fatty acid cyclooxygenase in the initial step with PGG_2 as the intermediate (Fig. 1).

The present paper is concerned with quantitative determination of the three metabolites released during thrombin-induced aggregation and the effects of aspirin and indomethacin on the release. The results show that the prostaglandin synthesis stops almost completely at the endoperoxide stage and that instead of being converted to the classical prostaglandins (PGE_2 and $PGF_{2\alpha}$), the endoperoxides are transformed into other metabolites which have lost the basic prostanoate structure. The role of this pathway in the biological action of prostaglandins is discussed.

MATERIALS AND METHODS

[5,6,8,9,11,12,14,15-$^2H_8$]Arachidonic acid was prepared as previously described (5). [3,3,4,4-$^2H_4$]PGE_2 and [3,3,4,4-$^2H_4$]-$PGF_{2\alpha}$ were generously provided by Dr. U. Axen, The Upjohn Co., Kalamazoo, Mich. Thrombin (Topostasin®) was purchased from Hoffmann-La Roche Co.

Quantitative Determination of 12L-Hydroxy-5,8,10,14-Eicosatetraenoic Acid (HETE). [1-^{14}C; 5,6,8,9,11,12,14,15-$^2H_8$]-HETE was prepared by incubation of deuterium-labeled arachidonic acid mixed with [1-^{14}C]arachidonic acid (specific radioactivity, 0.93 Ci/mol) with a suspension of washed human platelets followed by silicic acid chromatography (4). The mass spectrum of the Me_3Si derivative of the methyl ester showed ions of high intensity at m/e 399 (M-15; loss of $\cdot CH_3$), 383 (M-31; loss of $\cdot OCH_3$), 301 (M-113; loss of $\cdot CH_2$-C^{2}H=C^{2}H-$(CH_2)_4CH_3$), 232, 211, and 210 [301 − 90(Me_3SiOH) and 91 (Me_3SiO^2H), respectively], and 178 [301 − (91 + 32)]. The intensity of the ion at m/e 295 (protium form) was 0.75% of that of the ion at m/e 301 (deuterium form). Mixtures of unlabeled and deuterium-labeled HETE were prepared, converted into the methyl ester-Me_3Si derivatives, and subjected to multiple-ion analysis using an LKB 9000 instrument equipped with an accelerating voltage alternator. The intensity of the ions at m/e 295 and 301 was monitored. By plotting the ratio between the ions at m/e 295 and 301 on the y-axis against the ratio between the amounts of added unlabeled and deuterium-labeled HETE on the x-axis, a standard curve was obtained. A linear relationship was found

Abbreviations: PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; HETE, 12L-hydroxy-5,8,10,14-eicosatetraenoic acid; HHT, 12L-hydroxy-5,8,10-heptadecatrienoic acid; PHD, 8-(1-hydroxy-3-oxopropyl)-9,12L-dihydroxy-5,10-heptadecadienoic acid; Me_3Si, trimethylsilyl; TBA, thiobarbituric acid.

* This is paper No. VI in a series. Paper No. V is ref. 23.

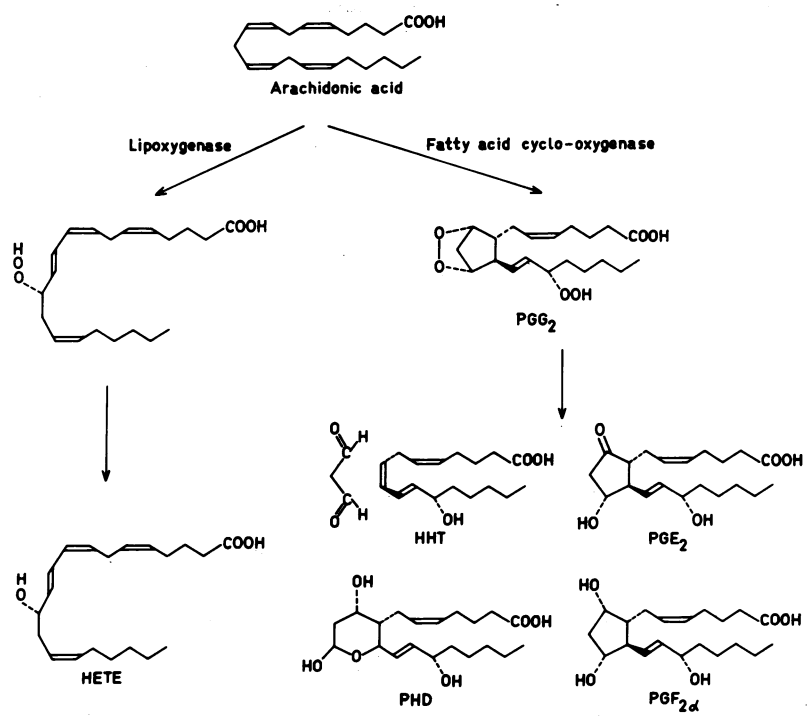

FIG 1. Transformations of arachidonic acid in platelets.

(ratios between protium and deuterium forms, 0–4; y-intercept, 0.0075; slope, 1.21).

Quantitative Determination of 12L-Hydroxy-5,8,10-Heptadecatrienoic Acid (HHT). The mass spectrum of the Me_3Si derivative of the methyl ester of HHT showed an ion of high intensity at m/e 295 (4). Accordingly, the deuterated HETE prepared as described above could be used as internal standard in quantitative determination of HHT.

Known amounts (0–35.0 nmol) of HHT were added to 17.9 nmol of deuterated HETE, the mixtures converted into the methyl ester-Me_3Si derivatives and subjected to multiple-ion analysis. The intensity of the ion at m/e 295 appearing with a retention time corresponding to an equivalent chain length of C-19.3 (HHT derivative) was divided by the intensity of the ion at m/e 301 at C-21.3 (deuterated HETE derivative) and the ratios plotted against the ratios between added HHT and deuterated HETE. The standard curve obtained was linear and had a y-intercept = 0.00 and a slope = 0.56.

Quantitative Determination of 8-(1-Hydroxy-3-oxopropyl)-9,-12L-Dihydroxy-5,10-Heptadecadienoic Acid (PHD). [1-^{14}C; 5,6,8,9,11,12,14,15-$^2H_8$]PHD was prepared by incubation of deuterium- and ^{14}C-labeled arachidonic acid (specific radioactivity, 0.93 Ci/mol) with a suspension of washed human platelets followed by silicic acid chromatography (4) and thin-layer chromatography TLC (solvent, ethyl acetate–2,2,4-trimethylpentane–acetic acid, 80:20:0.5, v/v/v; R_F = 0.41). Mass spectrometric analysis of the Me_3Si derivative of the methyl ester showed a base peak at m/e 260 ([Me_3-SiO-C^{2}H=C^{2}H-CH$_2$-C^{2}H=C^{2}H-(CH$_2$)$_3$COOCH$_3$]$^+$); m/e 256 in the corresponding unlabeled derivative. Known mixtures of unlabeled and deuterated PHD, in ratios 0–1.2, were esterified and converted into the Me_3Si derivatives and subjected to multiple-ion analysis. The standard curve relating the ratios between the intensities of the ions at m/e 256 and 260 to the ratios between added unlabeled and deuterated molecules was linear and had a y-intercept = 0.034 and a slope = 1.00.

Analytical Method. For determination of HETE, HHT, and PHD in 1–2 ml of platelet suspensions, 4.48 nmol of deuterated HETE and 4.94 nmol of deuterated PHD were added in 10 ml of 90% ethanol. The mixture was diluted with water, acidified to pH 3 and extracted twice with diethyl ether. The residue obtained after evaporation of the ether was esterified and subjected to TLC with the organic layer of ethyl acetate–2,2,4-trimethylpentane–water, 75:75:100 (v/v/v), as solvent. The positions of the methyl esters of added HETE and PHD were determined by a Berthold Dünnschichtscanner II (R_F = 0.73 and 0.09, respectively). The methyl ester of HHT almost coincided with cholesterol extracted from the platelets (R_F = 0.67). The band of cholesterol was located by spraying with 2′,7′-dichlorofluorescein and viewing by UV. The zone containing the methyl esters of HETE and HHT, and that containing the methyl ester of PHD, were scraped off and eluted with diethyl ether and ethyl acetate, respectively. Material obtained from the former zone was converted into the Me_3Si derivatives and subjected to multiple-ion analysis (m/e 295 and 301) with a column of 1% OV-1 and a column temperature of 200° (Fig. 2). The Me_3Si derivative of cholesterol appeared with a retention time about 6 times longer than that of the methyl ester-Me_3Si derivative of HETE. Accordingly, after five consecutive injections of the derivatives of HHT and HETE, it was necessary to interupt the injections so that the cholesterol derivatives could be eluted. Material in the zone of PHD was also converted into Me_3Si derivatives and subjected to multiple-ion analysis (m/e 256 and 260) using a column of 1% OV-1 at 240°.

Determination of TBA-positive Material. Thiobarbituric acid positive material was assayed by the method of Flower *et al.* (6).

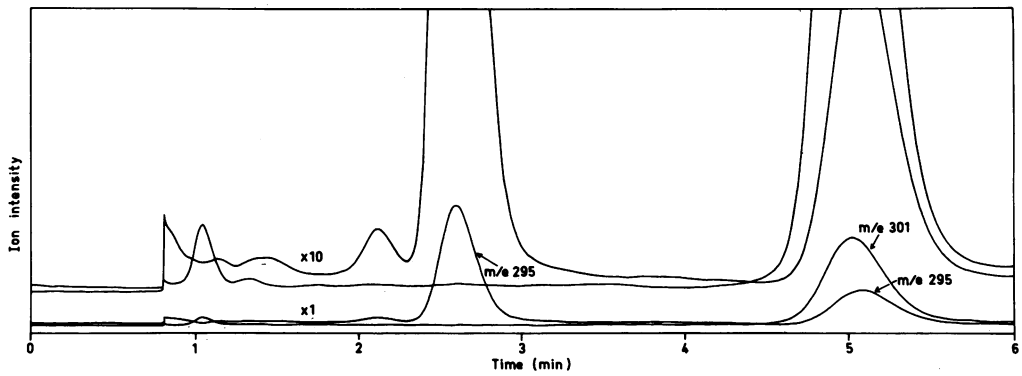

FIG. 2. Multiple-ion analysis recording obtained in quantitative determination of HHT and HETE. The more rapidly eluting peak (m/e 295) is due to the Me_3Si derivative of the methyl ester of HHT, whereas the other peaks (m/e 301 and 295) are due to the Me_3Si derivative of the methyl ester of deuterium labeled and unlabeled HETE. Column, 1% OV-1; column temperature, 200°. Electron energy, 22.5 eV, trap current, 60 μA.

Platelet Preparation. Washed human platelets were prepared as previously described (1).

RESULTS

Table 1 gives amounts of HETE, HHT, and PHD released during thrombin-induced aggregation (5 U/ml; 20 min) of washed platelets (288,000–756,000/μl) obtained from eight healthy subjects before and 2 hr after ingestion of two tablets of aspirin (648 mg of acetylsalicylic acid). The ranges found were: HETE, 1163–2175 ng/ml (before aspirin) and 3622–5798 ng/ml (after aspirin); HHT, 1129–2430 ng/ml (before aspirin) and less than 23–67 ng/ml (after aspirin); PHD, 998–2299 ng/ml (before aspirin) and 17–42 ng/ml (after aspirin). The ranges of PGG_2 (calculated from the sum of the amounts of HHT and PHD after correction for the different molecular weights) were 2477–5480 ng/ml (before aspirin) and less than 51–127 ng/ml (after aspirin). Also given in Table 1 are absorbancies recorded in the TBA reaction.

TABLE 1. *HETE, HHT, and PHD (ng/ml) released during thrombin-induced aggregation of washed platelets and A_{532} recorded for TBA reaction**

Subject		HETE	HHT	PHD	TBA reaction
ITH	(♀)	2175	1238	1766	0.60
ITH (aspirin)		3622	<23	21	0.08
GH	(♂)	1845	2365	2012	0.72
GH (aspirin)		5642	<67	20	0.09
JÅL	(♂)	1213	1432	1035	0.37
JÅL (aspirin)		4142	<25	22	0.14
SL	(♀)	1496	2254	1959	0.65
SL (aspirin)		5798	<65	42	0.18
HEC	(♂)	1163	1129	998	0.37
HEC (aspirin)		4455	<37	17	0.09
LM	(♂)	1313	1846	1593	0.59
LM (aspirin)		4590	<34	25	0.07
UK	(♂)	1283	1448	1400	0.51
UK (aspirin)		3843	<44	29	0.07
LH	(♀)	1443	2430	2299	0.65
LH (aspirin)		4881	<42	17	0.09

* The concentrations and absorbancies given are corrected for different platelet counts in suspensions obtained from the different subjects; they are expressed as ng and absorbancy units, respectively, per ml of suspension containing 500,000 platelets per μl.

Fig. 3 shows the effect of preincubation of platelets, with aspirin or indomethacin, on the synthesis of HETE and HHT. Formation of HHT was inhibited in the presence of both agents, whereas formation of HETE was stimulated. As can be seen in Fig. 3, indomethacin in high concentrations appeared to have a weak inhibitory effect on the formation of HETE although the amount of HETE formed even in the presence of 100 μg/ml of indomethacin was higher than that formed in the absence of indomethacin.

Fig. 4 shows two experiments in which HETE, HHT, PHD as well as PGE_2, $PGF_{2\alpha}$ and the endoperoxides, PGG_2 and PGH_2, were determined at different times in the incubation of two platelet suspensions with thrombin. The time courses for the release of HHT and PHD appeared to be identical and different from that observed for the release of HETE. In a separate experiment, 47 μg of [1-^{14}C]arachidonic acid were incubated with 2 ml of platelet suspensions (10^6 platelets per μl) for different times. The amounts of labeled HETE, HHT, and PHD were determined by thin-layer radiochromatography (4). Again, the time courses for the formation of HHT and PHD were identical and faster than that observed for the formation of HETE.

In Fig. 4, the amounts of PGE_2 (34 and 28 ng/ml at 1 min; 43 and 37 ng/ml at 5 min; upper and lower panels, respectively) and $PGF_{2\alpha}$ (16 and 9 ng/ml at 1 min; 22 and 10 ng/ml at 5 min; upper and lower panels, respectively) were very small when compared to the amounts of HHT and PHD. The amounts of intact endoperoxide(s) (PGG_2 and/or PGH_2) present after 1 min of incubation was 40 (upper) and 49 (lower) ng/ml as judged from the difference between the amounts of $PGF_{2\alpha}$ present in samples treated with ethanol, with or without 0.5% stannous chloride (ref. 1).

DISCUSSION

The present paper is concerned with quantitative determination of the three major oxygenated metabolites formed from arachidonic acid when it is incubated with washed human platelets; these metabolites are HETE (12L-hydroxy-5,8,10,-14-eicosatetraenoic acid), HHT (12L-hydroxy-5,8,10-heptadecatrienoic acid) and PHD [8-(1-hydroxy-3-oxopropyl)-9,-12L-dihydroxy-5,10-heptadecadienoic acid] (4). The methods were based upon multiple-ion analysis with [5,6,8,9,11,12,-14,15-$^2H_8$]HETE and [5,6,8,9,11,12,14,15-$^2H_8$]PHD as internal standards. Since the mass spectrum of the Me_3Si derivative of the methyl ester of HHT had an ion of high

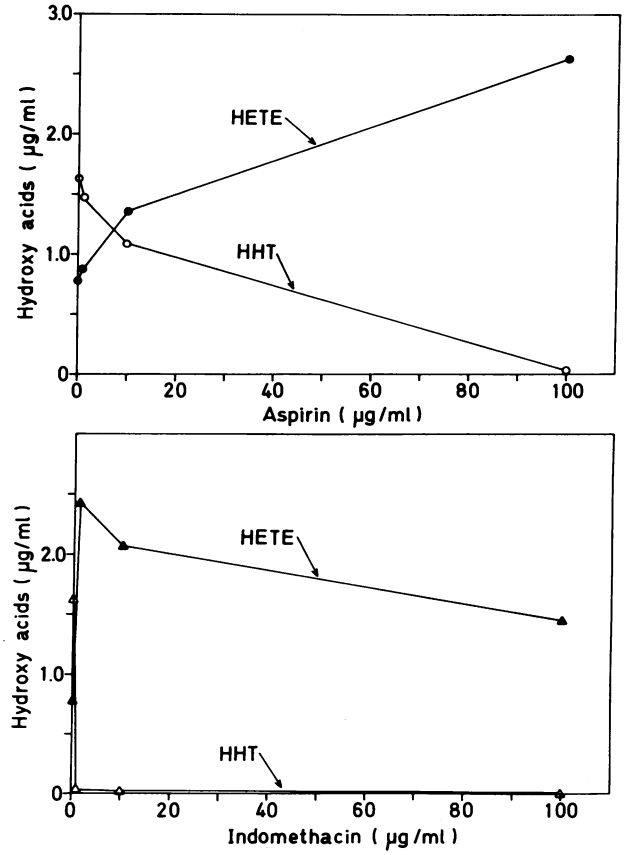

FIG. 3. Effects of aspirin (upper panel) and indomethacin (lower panel) on the release of HETE and HHT during thrombin-induced aggregation. One ml suspensions of platelets (10^8 platelets per μl) were stirred for 2 min at 37° with or without inhibitor and subsequently treated with 5 U of thrombin. After 1 min of stirring, 10 ml of ethanol containing 4.48 nmol of deuterated HETE was added and the mixture further treated as is described in the *text*. ●, HETE and aspirin; ○, HHT and aspirin; ▲, HETE and indomethacin; △, HHT and indomethacin.

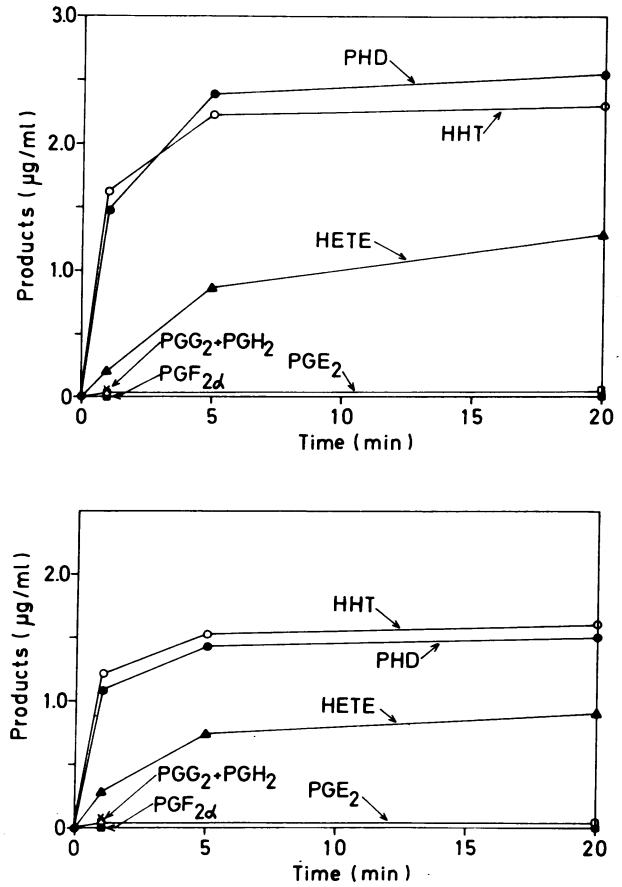

FIG. 4. Release of products of arachidonic acid during thrombin-induced aggregation of human platelets. One ml suspensions (upper, 422,000/μl; lower, 680,000/μl) were stirred for 2 min at 37° and subsequently treated with 5 U of thrombin. Stirring was continued for 1, 5, and 20 min at which times 10 ml of ethanol containing either 4.48 nmol of deuterated HETE plus 4.94 nmol of deuterated PHD, 14.9 nmol of deuterated PGE_2, or 11.2 nmol of deuterated $PGF_{2\alpha}$ were added. HETE, HHT, and PHD were determined as described in the *text* whereas PGE_2 and $PGF_{2\alpha}$ were determined as described by Grėen *et al.* (9). ▲, HETE; ○–○ HHT; ●, PHD; □, PGE_2; ■, $PGF_{2\alpha}$; ×, $PGG_2 + PGH_2$.

intensity at m/e 295, as was the case with the corresponding derivative of HETE (4), the deuterated HETE standard could be used for determination of HHT as well as HETE.

Application of the methods to platelets revealed that large amounts of HETE, HHT, and PHD were released during thrombin-induced aggregation. Washed platelets obtained from eight healthy subjects released 1163–2175 ng/ml of HETE, 1129–2430 ng/ml of HHT, and 998–2299 ng/ml of PHD after incubation at 37° for 20 min with thrombin. The calculated production of PGG_2, the precursor of HHT and PHD, was 2477–5480 ng/ml. It was also found that thiobarbituric acid-positive material was produced during thrombin-induced aggregation. Although this material was not identified, its appearance together with HHT indicated that it was identical with malonaldehyde (compare refs. 7 and 8, and Fig. 1). Intake of two tablets of aspirin (648 mg) inhibited formation of HHT and PHD by more than 95%. In another experiment, aspirin and indomethacin were preincubated with platelet suspensions and were found to inhibit synthesis of HHT and PHD (Fig. 3). This corroborated our earlier results concerning the inhibitory effect of these drugs on the formation of labeled HHT and PHD from [*1*-^{14}C]arachidonic acid in platelets by blockade of the fatty acid cyclo-oxygenase (4).

As expected, aspirin also strongly suppressed formation of TBA-positive material (Table 1). Accordingly, the TBA reaction may be used as a convenient assay method in the evaluation of new drugs impairing platelet function. The release of HETE was increased about 3-fold in platelets obtained from subjects given aspirin (Table 1). Similarly, preincubation of platelet suspensions with aspirin (100 μg/ml) and indomethacin (1 μg/ml) resulted in a 3.1- to 3.4-fold stimulation of the release of HETE (Fig. 3). This effect on the production of HETE was also seen in the previous study (4) in which [*1*-^{14}C]arachidonic acid was used. It is probable that on treatment with thrombin, a limited pool of free arachidonic acid suddenly becomes available to the lipoxygenase and the fatty acid cyclo-oxygenase involved in the synthesis of HETE and of HHT plus PHD, respectively (Fig. 1). Blockade of the fatty acid cyclo-oxygenase by aspirin and indomethacin would make more arachidonic acid available to the lipoxygenase and result in increased formation of HETE.

Aspirin inhibits the second wave of platelet aggregation *in vitro* (10–12) and has an antihemostatic effect *in vivo* (13,

14). Its effects on platelets include acetylation of proteins (15) and inhibition of prostaglandin formation (16). However, among the classical prostaglandins, PGE_1 has an inhibitory effect (17), $PGF_{2\alpha}$ no effect (17) and PGE_2 a weak inhibitory (first phase) and stimulatory (second phase) effect (18) on platelet aggregation. Inhibition of the production of the prostaglandins PGE_2 and $PGF_{2\alpha}$, earlier known to be released during platelet aggregation, did not, therefore, satisfactorily explain the effect of aspirin on this process. Reports on the synthesis and blockade of arachidonic-acid-induced formation of an unidentified factor with aggregating properties have also appeared. These studies did not provide any structural information on the mechanism involved in the antiaggregating effect of aspirin (19–21). The aggregating properties of the endoperoxides, PGG_2 and PGH_2, their release during aggregation, and the inhibition of their formation by aspirin led us to propose that the mechanism of action of aspirin was through inhibition of the platelet cyclooxygenase responsible for formation of PGG_2 (1). The evidence for this mechanism is strongly amplified by the results reported here which demonstrate considerable release of endoperoxide metabolites during aggregation and inhibition of the release by aspirin.

The new concept emerging from the present study is that the prostaglandins can exert their biological action through the endoperoxides, and that these compounds may be metabolized almost exclusively to nonprostanoate structures and only to a small extent to the classical prostaglandins. We have also found that the endoperoxide metabolites described here can be released from several other tissues† and that the endoperoxides, in addition to their effects on platelets, have unique biological actions on, e.g., airway and vascular smooth muscle (22) and adipocyte ghosts (23). Additional work is required to establish if this new model of prostaglandin action and release, demonstrated for human platelets, occurs more generally in regulation of various cell functions.

† M. Hamberg and B. Samuelsson, unpublished observations.

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