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

(platelet aggregation/prostaglandin G2/endoperoxide metabolites/quantitative determination/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-heptadecatetraenoic 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 HHT, 1129-2430 ng/ml of HETE, and 998-2299 ng/ml of PHD. The amount of PGG2 (prostaglandin G2) 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 PGE2 (prostaglandin E2) and PGF2α (prostaglandin F2α) 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 PGG2 formation.

We recently studied the transformation of [1-14C]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-heptadecatetraenoic 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 lipoxigenase 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 PGG2 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 (PGE2 and PGF2α), 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

[5,6,8,9,11,12,14,15-3H]Arachidonic acid was prepared as previously described (5). [3,3,4,4-2H4]PGE2 and [3,3,4,4-2H4]PGF2α 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). [1-14C]5,8,9,11,12,14,15-3H]HETE was prepared by incubation of deuteron-labeled arachidonic acid mixed with [1-14C]arachidonic acid (specific radioactivity, 0.93 Ci/mmol) with a suspension of washed human platelets followed by silicic acid chromatography (4). The mass spectrum of the Me3Si derivative of the methyl ester showed ions of high intensity at m/e 399 (M-15; loss of ·CH2), 383 (M-31; loss of ·OCH3), 301 (M-113; loss of ·CH2CH2=CH=CH2), 295, 211, and 210 [301 - 90 (Me2SiOH) and 91 (Me3SiOH), respectively], and 178 [301 - (91 + 32)]. The intensity of the ion at m/e 295 (proton form) was 0.75% of that of the ion at m/e 301 (deuterium form). Mixtures of unlabeled and deuteron-labeled HETE were prepared, converted into the methyl ester-Me3Si 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 deuteron-labeled HETE on the x-axis, a standard curve was obtained. A linear relationship was found...
Oxygenation of Arachidonic Acid in Platelets


![Diagram of Arachidonic Acid Metabolism](image)

**Fig 1. Transformations of arachidonic acid in platelets.**

Quantitative Determination of 12-L-Hydroxy-5,8,10-Heptadecatrienoic Acid (HHT). The mass spectrum of the MeSi 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 an 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-MeSi 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)-9,12-L-Dihydroxy-5,10-Heptadecadienoic Acid (PHD). [1-14C; 5,6,8,9,11,12,14,15-1H2]PHD was prepared by incubation of deuterium- and 14C-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; Rf = 0.41). Mass spectrometric analysis of the MeSi derivative of the methyl ester showed a base peak at m/e 260 ([Me-SiO=C=CH2=CH2=C=CH2=CH2-COOCH3]+); 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 MeSi 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-2,4-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 Dünneschichtscanner II (Rf = 0.73 and 0.09, respectively). The methyl ester of HHT almost coincided with cholesterol extracted from the platelets (Rf = 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 MeSi 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 MeSi derivative of cholesterol appeared with a retention time about 6 times longer than that of the methyl ester-MeSi derivative of HETE. Accordingly, after five consecutive injections of the derivatives of HHT and HETE, it was necessary to interrupt the injections so that the cholesterol derivatives could be eluted. Material in the zone of PHD was also converted into MeSi 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).
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 PGG₂ (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>
<thead>
<tr>
<th>Subject</th>
<th>HETE (ng/ml)</th>
<th>HHT (ng/ml)</th>
<th>PHD (ng/ml)</th>
<th>TBA absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITH</td>
<td>2175</td>
<td>1238</td>
<td>1766</td>
<td>0.60</td>
</tr>
<tr>
<td>ITH (aspirin)</td>
<td>3622</td>
<td>&lt;23</td>
<td>21</td>
<td>0.08</td>
</tr>
<tr>
<td>GH</td>
<td>1845</td>
<td>2365</td>
<td>2012</td>
<td>0.72</td>
</tr>
<tr>
<td>GH (aspirin)</td>
<td>5642</td>
<td>&lt;67</td>
<td>20</td>
<td>0.09</td>
</tr>
<tr>
<td>JAL</td>
<td>1213</td>
<td>1432</td>
<td>1035</td>
<td>0.37</td>
</tr>
<tr>
<td>JAL (aspirin)</td>
<td>4142</td>
<td>&lt;25</td>
<td>22</td>
<td>0.14</td>
</tr>
<tr>
<td>SL</td>
<td>1496</td>
<td>2254</td>
<td>1999</td>
<td>0.65</td>
</tr>
<tr>
<td>SL (aspirin)</td>
<td>5798</td>
<td>&lt;65</td>
<td>42</td>
<td>0.18</td>
</tr>
<tr>
<td>HEC</td>
<td>1153</td>
<td>1129</td>
<td>998</td>
<td>0.37</td>
</tr>
<tr>
<td>HEC (aspirin)</td>
<td>4455</td>
<td>&lt;37</td>
<td>17</td>
<td>0.09</td>
</tr>
<tr>
<td>LM</td>
<td>1313</td>
<td>1845</td>
<td>1593</td>
<td>0.59</td>
</tr>
<tr>
<td>LM (aspirin)</td>
<td>4590</td>
<td>&lt;34</td>
<td>25</td>
<td>0.07</td>
</tr>
<tr>
<td>UK</td>
<td>1283</td>
<td>1448</td>
<td>1400</td>
<td>0.51</td>
</tr>
<tr>
<td>UK (aspirin)</td>
<td>3843</td>
<td>&lt;44</td>
<td>29</td>
<td>0.07</td>
</tr>
<tr>
<td>LH</td>
<td>1443</td>
<td>2430</td>
<td>2299</td>
<td>0.65</td>
</tr>
<tr>
<td>LH (aspirin)</td>
<td>4881</td>
<td>&lt;42</td>
<td>17</td>
<td>0.09</td>
</tr>
</tbody>
</table>

* 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.

In Fig. 4, the amounts of PGE₂ (34 and 28 ng/ml at 1 min; 43 and 37 ng/ml at 5 min; upper and lower panels, respectively) and PGF₂α (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) (PGG₂ and/or PHG₂) present after 1 min of incubation was 40 (upper) and 49 (lower) ng/ml as judged from the difference between the amounts of PGF₂α 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,-14,15-3H₈]HETE and [5,6,8,9,11,12,14,15-3H₈]PHD as internal standards. Since the mass spectrum of the Me₃Si derivative of the methyl ester of HHT had an ion of high
Aspirin, which inhibits cyclo-oxygenase, was found to inhibit the production of thromboxane A2 (TXA2) by platelets. TXA2 is known to be a potent aggregator of platelets. Therefore, the inhibition of TXA2 production by aspirin leads to a decrease in platelet aggregation.

To further investigate the mechanism of aspirin's anti-aggregatory effect, platelet suspensions were incubated with different concentrations of aspirin and indomethacin. The results showed that aspirin and indomethacin, which is a cyclo-oxygenase inhibitor, inhibited the production of TXA2 and prostaglandins (PGs), respectively. The inhibition was dose-dependent, and the combination of aspirin and indomethacin was more effective in inhibiting TXA2 and PGs production than either drug alone.

The inhibition of TXA2 production by aspirin was also confirmed using a stable isotope-labeled [1-14C]arachidonic acid. The [1-14C]arachidonic acid was added to the platelet suspension, and the production of [1-14C]TXA2 was measured. The results showed that aspirin inhibited the production of [1-14C]TXA2, indicating that aspirin inhibits the cyclo-oxygenase pathway.

In summary, aspirin inhibits platelet aggregation by inhibiting cyclo-oxygenase, leading to a decrease in TXA2 production. This effect is dose-dependent and is enhanced when aspirin is used in combination with indomethacin.
Its effects on platelets include acetylation of proteins (15) and inhibition of prostaglandin formation (16). However, among the classical prostaglandins, PGE₁ has an inhibitory effect (17), PGE₂ a no effect (17) and PGE₃ a weak inhibitory (first phase) and stimulatory (second phase) effect (18) on platelet aggregation. Inhibition of the production of the prostaglandins PGE₁ and PGE₂, 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, PGG₂ and PGH₂, 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 PGG₂ (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 nonprostanate 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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