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IRREVERSIBLE PLATELET AGGREGATION DOES NOT DEPEND ON LIPOXYGENASE METABOLITES

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Previous investigations in our laboratory demonstrated the existence of an intrinsic mechanism, termed membrane modulation, capable of restoring sensitivity to aspirin treated platelets, resulting in irreversible aggregation in response to arachidonic acid (AA). The mechanism underlying correction of aspirin induced inhibition of platelet function, however, was not clear. In the present study we have evaluated the role of lipoxygenase (LO) metabolites of AA in securing irreversible aggregation of drug induced cyclooxygenase (CO) deficient platelets. Platelets treated with aspirin or Ibuprofen did not convert radiolabeled AA to thromboxane, but generated significant quantities of hydroxy acids via the LO pathway. However, drug exposed platelets, when stirred with epinephrine first and then challenged with AA, aggregated irreversibly. Eicosatetraynoic acid (ETYA 1, U53119) inhibited AA conversion by the LO pathway, whereas 5,8,11,14-eicosatetraynoic acid (ETYA 2) inhibited AA conversion by both CO and LO enzymes. Yet, at the inhibitory concentration these fatty acids failed to prevent AA induced irreversible aggregation of CO deficient, alpha adrenergic receptor stimulated platelets. Results of our studies show that the generation of LO metabolites of AA are not essential for securing irreversible aggregation of platelets.

Several studies have proposed a critical role for arachidonic acid (AA) metabolites in the induction of platelet shape change, release of granule contents and irreversible aggregation (1-7). Activation of platelets results in mobilization of AA from membrane phospholipids (8-11). AA thus released is rapidly converted to transient intermediates, prostaglandin endoperoxides (PGG_2 and PGH_2) by cyclooxygenase (CO) and further to thromboxane A_2 by thromboxane synthetase (12,13). Thromboxane A_2 is the major metabolite of AA in platelets (14). Aspirin treatment of platelets inactivates CO and prevents the conversion of AA to endoperoxides, thus blocking formation of thromboxane (15,16). Therefore, aspirin prevents AA induced secretion as well as irreversible aggregation of platelets, but does not prevent release of AA from mem-

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brane phospholipids in stimulated platelets nor its conversion to hydroperoxy (HPETE) and hydroxy acids (HETE) via the lipoxygenase (LO) pathway (17-21).

Recent studies by Dutilh et al have shown that LO products may play a role in securing irreversible aggregation of platelets (22). Their studies tried to correlate platelet stickiness with the process of hydroxy acid generation and elevated intracellular levels of cyclic guanosine monophosphate (cGMP). Based upon the results of their study, they suggested a role for AA metabolites of LO and fatty acids in irreversible platelet aggregation. However, their results on the critical role of LO metabolites of AA in irreversible aggregation are largely unconfirmed.

Studies from our laboratory have demonstrated the existence of an intrinsic mechanism, termed membrane modulation, capable of securing irreversible aggregation of aspirin treated platelets (23). Researchers from other laboratories also have demonstrated platelet aggregation independent of cyclooxygenase metabolites (19,24). Treatment of aspirin platelets with epinephrine restores the sensitivity of platelets to AA induced stimulation in the absence of detectable thromboxane formation (23,25). It has been shown that epinephrine can potentiate peroxidase activity of prostaglandin endoperoxide synthetase and promote formation of 11-HETE (26). In addition, aspirin treatment of platelets facilitates AA conversion by the LO pathway and promotes formation of large amounts of LO metabolites of AA.

In the present study we explored the role of LO metabolites in irreversible aggregation of CO deficient platelets. Specific inhibitors of CO and LO were employed to modulate the activity of the enzymes during these studies. Results of our study demonstrate that epinephrine potentiation of AA induced stimulation of aspirin platelets is not mediated by LO metabolites. Furthermore, studies with 5,8,11,14-eicosatetraynoic acid (ETYA, an inhibitor of both cyclooxygenase and lipoxygenase pathways) suggest that AA may be able to induce irreversible aggregation of these platelets without undergoing any conversion to various metabolites.

MATERIALS AND METHODS

Materials. AA as the sodium salt was obtained from NuChek Prep, Elysian, Minnesota, and made up in 0.1 M Tris buffer of pH 7.4. Radiolabeled AA was from New England Nuclear (NEC-661) and injectable adrenalin from Parke-Davis Company. ETYA-1 (U53119; 4,7,10,13-eicosatetraynoic acid) was a gift from the Upjohn Company whereas ETYA-2 (5,8,11,14-eicosatetraynoic acid) was from Hoffman-La Roche. Unless otherwise stated, all other chemicals were from Sigma Chemical Company, St. Louis, Missouri.

Methods. Blood was drawn from volunteers who had not taken any medications two weeks prior to this study and mixed immediately with citrate-citric acid-dextrose, pH 6.5 (citrate 0.1 M, citric acid 7 mM, dextrose 0.14 M) in a ratio of 9 parts blood to 1 part anticoagulant (27). Platelet-rich plasma (PRP) was separated by centrifugation of whole blood at room temperature for twenty minutes at 100 x g. Platelet aggregation studies were carried out on a Payton dual channel aggregometer preset with PRP and platelet-poor plasma (PPP). Where needed, inhibitory drugs were added to PRP and incubated prior to testing the action of agonists. To obtain CO deficient cells, platelets were either obtained from donors who had ingested 650 mgs of aspirin 24 hours before blood drawing or normal platelets were incubated with 100 μM Ibuprofen for 30 minutes before testing. For measuring radiolabeled AA conversion to CO and LO metabolites, each reaction mixture, containing 1.5×10^9 cells suspended in 1 ml of Hank's Balanced Salt Solution (HBSS), was stirred on an aggregometer for 5 minutes at 37°C with 1 μg of labeled AA (28). Some samples were pretreated with inhibitors for 30 minutes before the addition of AA. At the end of the experiment, 1 ml of ethyl acetate was added to each reaction mixture and acidified to pH 3.5 with 0.5 M citric acid. After thorough mixing, the ethyl acetate layer was separated and the incubation mixture reextracted with an equal volume of ethyl acetate. Pooled ethyl acetate extract was concentrated with nitrogen and spotted on a thin layer plate (silica gel G). The solvent system used for the separation of AA metabolites was petroleum ether:diethyl ether:acetic acid (60:39:1 v/v)(29). Radioactive metabolites were monitored with a Berthold radiolabel scanner and quantitation was achieved by scraping radioactive spots from the thin layer plates and scintillation counting.

RESULTS AND DISCUSSION

Normal control platelets aggregated irreversibly when stimulated with epinephrine (5 μM) and arachidonate (0.45 mM), whereas platelets obtained from donors who had ingested aspirin, or those treated with Ibuprofen, did not respond to AA and only responded with a primary wave to epinephrine (Figure 1). On the other hand, exposure of aspirin/Ibuprofen treated platelets to epinephrine restored their sensitivity to a further challenge by AA (Figure 1). Neither aspirin treated platelets nor Ibuprofen treated platelets made significant quantities of thromboxane when incubated with radiolabeled AA (Table 1). Therefore, these cells could only make products of LO with AA. Normal control platelets converted significant quantities of radiolabeled AA via the LO and CO pathways (Figure 2). A specific inhibitor of lipoxygenase, ETYA 1

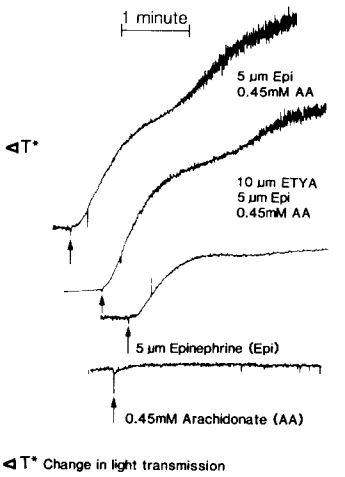

Figure 1. Platelets exposed to aspirin (in vivo, 650 mgs) or Ibuprofen (in vitro, 100 µM) did not respond to AA and responded only with a primary wave to the action of epinephrine. However, exposure of drug treated platelets to epinephrine restored their sensitivity to AA. Eicosatetraynoic acid (ETYA 1, U53119; ETYA 2, 5,8,11,14-eicosatetraynoic acid) at the inhibitory concentration (10 µM) did not prevent AA induced irreversible aggregation of epinephrine stimulated platelets previously treated with aspirin.

TABLE 1

INFLUENCE OF DIFFERENT INHIBITORS
ON THE CONVERSION OF ^{14}C-ARACHIDONIC ACID TO
CYCLOOXYGENASE (HHT) AND LIPOXYGENASE (HETE) PRODUCTS

	Total counts recovered	Counts recovered as HETE	Counts recovered as HHT	% conversion HETE	% conversion HHT
Control	182.6 ± 24.7	29.9 ± 2.8	39.8 ± 2.4	10.4 ± 0.6	21.0 ± 1.8
Ibuprofen (100 µM)	183.3 ± 8.6	82.6 ± 7.1	5.2 ± 0.6	45.1 ± 2.8	2.8 ± 0.3
Aspirin (650 mgs oral)	198.7 ± 8.1	116.8 ± 8.5	4.4 ± 2.0	58.8 ± 8.9	2.2 ± 0.6
ETYA 1 (U53119) (10 µM)	194.6 ± 12.4	4.8 ± 0.4	38.6 ± 2.6	2.4 ± 0.1	19.8 ± 1.6
ETYA 2 (10 µM)	203.8 ± 17.8	3.5 ± 0.3	3.8 ± 0.2	1.7 ± 0.1	1.9 ± 0.1

Normal control platelets converted significant quantities of radio-labeled AA to its metabolites by the CO (HHT) and LO (HETE) pathways. A single oral dose of aspirin (650 mgs) as well as 100 µM Ibuprofen in vitro effectively blocked the conversion of AA by the CO pathway. Eicosatetraynoic acid (ETYA 1, U53119) blocked the conversion of arachidonate by the LO pathway, whereas ETYA 2 (5,8,11,14-eicosatetraynoic acid) prevented the conversion of AA by both pathways.

Mean and the standard error (n = 4)
HHT = 12L-5,8,10-heptadecatrienoic acid
HETE = 12L-hydroxy-5,8,10,14-eicosatetraenoic acid

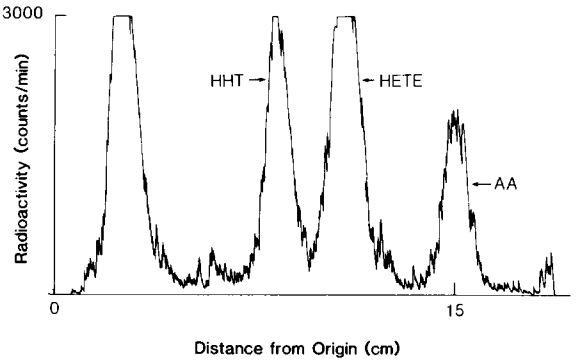

Figure 2. Conversion of radiolabeled AA by intact, washed platelets was followed. Normal control platelets converted significant quantities of AA to hydroxy acids via the lipoxygenase (HETE) and cyclooxygenase (HHT) pathways.

(U53119), did not inhibit AA induced aggregation of aspirin treated platelets (Figure 1) at the concentration (10 μM) in which it completely blocked the conversion of AA via the lipoxygenase pathway (Figure 3, Table 1). Furthermore, ETYA 2, an inhibitor of both the CO and LO pathways (Figure 4, Table 1), also did not prevent AA induced aggregation of aspirin treated platelets.

Dutilh and associates showed that a product of AA metabolism via the LO pathway when endogenously formed directly influenced the AA induced platelet aggregation (22). They suggested a correlation between generation of 12-hydroxy acid, the process of platelet stickiness and activation of guanylate cyclase. Wilhelm et al established an ID_{50} of 0.46 μM for the inhibition of platelet LO by ETYA 1 (U53119)(30). In our study, we used 10 μM ETYA 1 and demonstrated that even at a high concentration the fatty acid was a specific

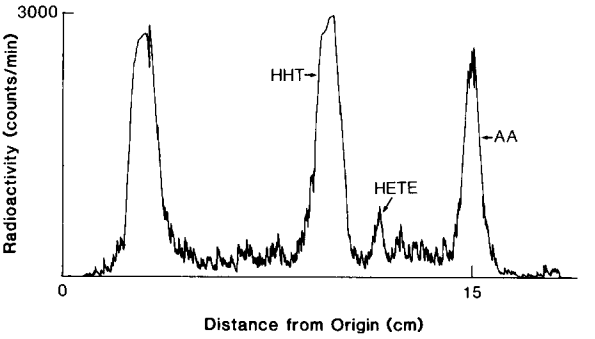

Figure 3. Eicosatetraynoic acid (ETYA 1, U53119), a specific inhibitor of LO, prevented the conversion of AA to hydroxy acid (HETE) via the LO pathway. However, it did not inhibit the formation of hydroxy acid (HHT) by the CO pathway.

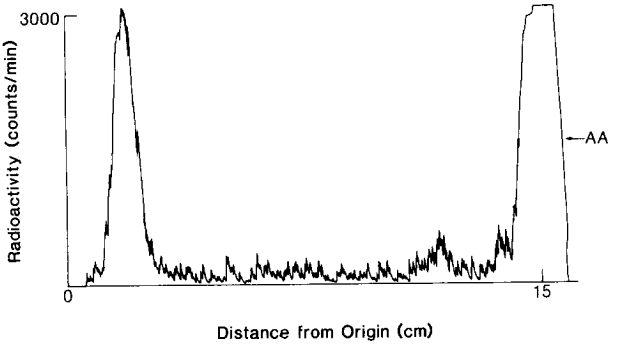

Figure 4. Eicosatetraynoic acid (ETYA 2, 5,8,11,14-eicosatetraynoic acid) blocked the conversion of AA to its metabolites by both the CO (HHT) and LO (HETE) pathways.

inhibitor of LO and did not affect the formation of CO products. Although ETYA 1 treated aspirin platelets could not convert AA to any metabolites, they aggregated irreversibly when challenged with epinephrine and AA.

Therefore, we sought to determine if ETYA 2, a competitive inhibitor of AA for both pathways, had any effect on platelet function in these studies. Dutilh et al showed that ETYA 2 at a concentration of 7 μM can inhibit both pathways of AA metabolism (22). In this study we used a final concentration of 10 μM and found no inhibitory effect on AA induced aggregation of aspirin/Ibuprofen treated platelets. Since both ETYA 1 and ETYA 2 did not inhibit AA induced stimulation of CO deficient platelets, it is reasonable to suggest that the generation of endogenous LO metabolites of AA are not essential for securing irreversible aggregation of platelets (31).

We did not explore the role of cGMP in these studies. However, a recent study has shown that unsaturated fatty acids, such as AA and linoleic acid, could exert their stimulatory effects on soluble guanylate cyclase without having to be converted to peroxides by other enzymes (32). Therefore, it is possible that cGMP generation or metabolism may have some role in platelet stickiness, but further studies are required to elucidate the existence of such a mechanism.

In conclusion, results of our studies show that exposure of aspirin/Ibuprofen treated platelets to epinephrine restores their sensitivity to AA. Arachidonate induced aggregation of cyclooxygenase deficient, alpha adrener-

gic receptor stimulated, platelets does not seem to require the generation of elevated levels of thromboxanes, nor the formation of endogenous metabolites of AA via the LO pathway. Furthermore, these studies suggest that AA may stimulate platelets under these conditions without undergoing conversion to other biologically active metabolites.

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