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ON THE MECHANISM OF THE OXYGENATION OF ARACHIDONIC ACID BY HUMAN PLATELET LIPOXYGENASE

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Summary: Formation of 12L-hydroxy-5,8,10,14-eicosatetraenoic acid from [10L-^{3}H; 3-^{14}C]arachidonic acid in suspensions of human platelets occurred with extensive loss of tritium and was accompanied by an isotope effect. These experiments showed that there is an antarafacial relation between the elimination of hydrogen from C-10 and insertion of oxygen at C-12 by human platelet lipoxygenase, and that the hydrogen elimination probably occurs as the initial step of the conversion. (Endo)peroxide intermediates formed by the fatty acid cyclooxygenase pathway activated platelet lipoxygenase.

During previous studies on the mechanism of the soybean lipoxygenase catalyzed oxygenation of 8,11,14-eicosatrienoic acid it was found that the absolute configuration of the hydrogen removed from C-13 was "L" (pro-S) and that the absolute configuration of the hydroperoxy group at C-15 was "L" (1). Also in the biosynthesis of prostaglandin E_1 from 8,11,14-eicosatrienoic acid the "L" hydrogen at C-13 was selectively removed and oxygens having the "L" configuration were introduced at C-11 and C-15 (2). Subsequent studies using stereospecifically labeled linoleic acids confirmed that an antarafacial relation between hydrogen removal and oxygen introduction existed in the oxygenations catalyzed by soybean and corn lipoxygenase (3) and by fatty acid cyclooxygenase (4).

The present paper is concerned with the stereochemistry and activation of the oxygenation catalyzed by a recently discovered lipoxygenase, i.e. the lipoxygenase of human blood platelets (5).

The abbreviations used are: EPA, 5,8,11,14,17-eicosapentaenoic acid; 12-HPETE, 12L-hydroperoxy-5,8,10,14-eicosatetraenoic acid; 12-HETE, 12L-hydroxy-5,8,10,14-eicosatetraenoic acid; 12-HHT, 12L-hydroxy-5,8,10-heptadecatrienoic acid; 12-HEP, 12-hydroxy-5,8,10,14,17-eicosapentaenoic acid; 12-HHTE, 12-hydroxy-5,8,10,14-heptadecatetraenoic acid; TXB_2, thromboxane B_2; TXB_3, thromboxane B_3.

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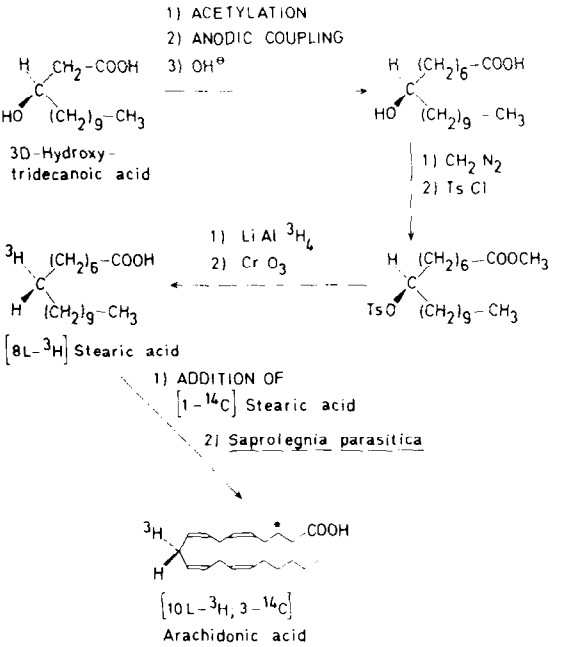


Fig. 1. Reactions used to prepare [10L-^{3}H; 3-^{14}C]arachidonic acid from 3D-hydroxytridecanoic acid. "Ts" = p-toluenesulfonyl.

MATERIALS AND METHODS

[10L-^{3}H; 3-^{14}C]Arachidonic acid was prepared from 3D-hydroxytridecanoic acid as outlined in Fig. 1. The radiochemical purity was better than 96% and the specific radioactivity was about 0.1 Ci/mol of ^{3}H and 0.03 Ci/mol of ^{14}C. The details of the preparation will be reported later.

[1-^{14}C]5,8,11,14,17-Eicosapentaenoic acid (EPA) was obtained by incubation of [1-^{14}C]arachidonic acid with the fungus, Saprolegnia parasitica, as recently described (6).

[1-^{14}C]Arachidonic acid was purchased from The Radiochemical Centre, Amersham. Unlabeled arachidonic acid was obtained from Nu-Chek-Prep., Inc., Elysian, Minn.

Suspensions of human platelets (10^{6}/µl, in a medium consisting of 0.154 M NaCl, 25 mM tris(hydroxymethyl)aminomethane, and 0.2 mM disodium EDTA; pH 7.4) were prepared as previously described (6).

Incubation mixtures of labeled fatty acids with platelet suspensions were extracted, treated with diazomethane, and analyzed by thin layer radiochromatography. The percentage composition of the labeled material was determined using a Berthold Dünnschichtscanner II. In experiments with [10L-^{3}H; 3-^{14}C]arachidonic acid, the methyl esters of 12-HETE and 12-HHT were localized and recovered from the silica gel. ^{3}H/^{14}C ratios were determined using a Packard model 2425 Tri-Carb liquid scintillation spectrometer.

RESULTS AND DISCUSSION

Experiments with [10L-^{3}H; 3-^{14}C]Arachidonic Acid. Incubation of [10L-^{3}H; 3-^{14}C]arachidonic acid with platelet suspensions (20 µg/ml; 20 min at 37°) led to the formation of three labeled products, i.e. 12-HETE (lipoxygenase pathway) and TXB_2 and 12-HHT (cyclooxygenase pathway)(5). The two monohydroxy acids had lost the major part of the tritium label (12-HETE, 14% retention relative to precursor; 12-HHT, 5% retention). Loss of ^{3}H during the formation of 12-HHT was expected since carbons 9, 10, and 11 are expulsed as malonaldehyde (5,7).

The finding that formation of 12-HETE was also accompanied by loss of ^{3}H showed that the hydrogen of the "L" configuration at C-10 was selectively removed by the lipoxygenase. The hydroperoxy group at C-12 of 12-HPETE was previously shown to have the "L" configuration (5). Therefore, in the conformation shown in Fig. 2, there is an antarafacial relation between hydrogen abstraction and oxygen insertion. Such a relation has earlier been demonstrated for soybean lipoxygenase-1 (1,3), fatty acid lipoxygenase (2,4), and corn lipoxygenase (3) and may thus be a general feature of lipoxygenase reactions.

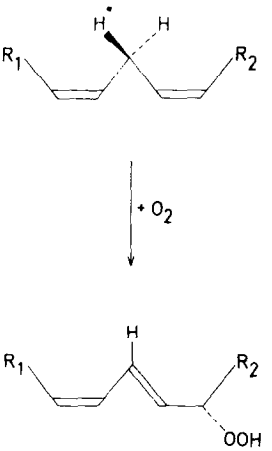


Fig. 2. Stereochemistry in the conversion of arachidonic acid into 12-HPETE by human platelet lipoxygenase. The hydrogen marked with an asterisk ("L" configuration) is removed in the initial step of the reaction. Oxygen is introduced stereospecifically, i.e. is present as a hydroperoxy group of the "L" configuration in the product. R_1, CH_2-CH=CH-$(CH_2)_3$-COOH; R_2, CH_2-CH=CH-$(CH_2)_4$-CH_3.

Table I

Tritium removal and isotope effect in the formation of 12-HETE from [10L-^{3}H; 3-^{14}C]arachidonic acid (20:4) by human platelet lipoxygenase [1].

Incubated 20:4 $^{3}H/^{14}C$ (%)	12-HETE $^{3}H/^{14}C$ (%)	20:4 remaining $^{3}H/^{14}C$ (%)	Conversion (%)
100	5	217	60
100	4	368	78
100	5	419	82

[1] The incubations were carried out in the presence of 50 µg/ml of indomethacin.

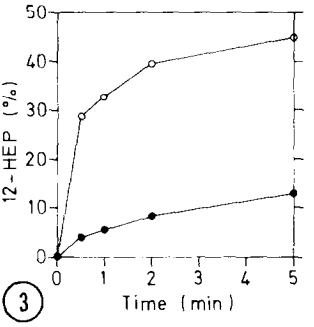
Subsequent studies with [10L-^{3}H; 3-^{14}C]arachidonic acid were designed to elucidate whether an isotope effect accompanied the conversion into 12-HPETE. In these experiments $^{3}H/^{14}C$ ratios of arachidonic acid remaining unconverted at different percentage conversions were measured. Indomethacin, an inhibitor of fatty acid cyclooxygenase in e.g. platelets (5), was added in order to avoid interference by the cyclooxygenase pathway. The results are given in Table I. As seen, 12-HETE lost most of the tritium label (4-5% retention relative to precursor), thus confirming the above mentioned results obtained in the absence of indomethacin. More significantly, a conversion-dependent enrichment of tritium in the arachidonic acid remaining unconverted was observed. The extent of enrichment was comparable to that previously found in the soybean lipoxygenase and fatty acid cyclooxygenase catalyzed oxygenations of [13L-^{3}H; 3-^{14}C]8,11,14-eicosatrienoic acid (1,2). The mechanistic implication of the finding is that elimination of the "L" hydrogen from C-10 probably is the initial step in the conversion of arachidonic acid into 12-HPETE.

Activation of Platelet Lipoxygenase. During studies on the oxygenation of EPA by platelet suspensions (6) it was found that the percentage conversion of the acid into cyclooxygenase products (TXB_3 and 12-HHTE) was low but relatively constant (2-5%). On the other hand, conversion into the lipoxygenase product (12-HEP) was larger but showed great variation (7-59%).

It seemed possible that the variable yield of 12-HEP might be explained by different degree of activation of the lipoxygenase by peroxide intermediate(s) formed from the precursor acid by the cyclooxygenase and/or lipoxygenase pathways. Soybean lipoxygenase is known to be activated in the presence of its products (8) and recently platelet lipoxygenase was reported to be activated in a similar way, i.e. in the presence of 12-HPETE (9).

In order to study the mode of activation of platelet lipoxygenase in intact platelets [1-^{14}C]EPA, a poor substrate for platelet cyclooxygenase (6), was incubated with platelet suspensions pretreated for 15 sec with 1 μg/ml of (unlabeled) arachidonic acid, a good substrate for platelet cyclooxygenase. As seen in Fig. 3, "sparking" the platelets with a low concentration of arachidonic acid led to a markedly increased rate of conversion of the labeled EPA into 12-HEP (5-8 fold stimulation at 30 sec incubation time; 5 exp.).

In other experiments [1-^{14}C]arachidonic acid was incubated with platelet suspensions pretreated with 140 μM indomethacin, an inhibitor of platelet cyclooxygenase (5) and of the enzyme(s) responsible for the conversion of 12-HPETE into 12-HETE (12-HPETE peroxidase; ref. 10). As seen in Fig. 4A, a lag phase in the conversion of the labeled arachidonic acid into 12-HETE appeared. This lag phase could not be explained by inhibition of the 12-HPETE peroxidase since accumulation of 12-HPETE was not observed. In fact, if accumulated, 12-HPETE would be expected to activate the platelet lipoxygenase (9) rather than to induce a lag phase as observed. It seemed likely that the lag phase was due to deficient formation of (endo)peroxide intermediates (cyclooxygenase pathway) activating the lipoxygenase. Support for this idea was provided by experiments in which [1-^{14}C]arachidonic acid was incubated with platelet suspensions in the presence of 2 mM sodium salicylate. This drug has no or insignificant effects on the cyclooxygenase but an inhibiting effect on the 12-HPETE peroxidase (10). As seen in Fig. 4B, formation of 12-HETE from arachidonic acid was not significantly affected by sodium salicylate, in agreement with its lack of inhibitory effect on endoperoxide formation. A transient



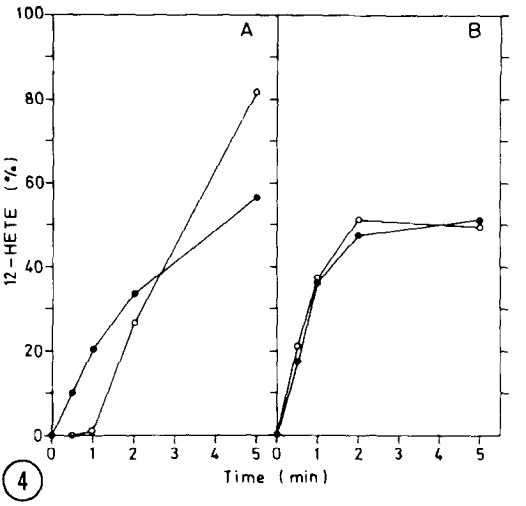


Fig. 3. Activation of platelet lipoxygenase by (endo)peroxide intermediates generated from arachidonic acid.
Suspensions of human platelets were stirred with 2 µg/ml of [1-^{14}C]EPA in the absence (-●-●-●-●-) or in the presence (-o-o-o-o-) of 1 µg/ml of arachidonic acid. The percentage conversion of EPA into 12-HEP was determined by thin layer radiochromatography using the following solvent system: diethyl ether - light petroleum (3:7; v/v).

Fig. 4. Rates of conversion of [1-^{14}C]arachidonic acid into 12-HETE in the presence of indomethacin (A) and of sodium salicylate (B).
Suspensions of human platelets were stirred with 20 µg/ml of [1-^{14}C]arachidonic acid in the absence of inhibitors (-●-●-●-) or in the presence of 50 µg/ml of indomethacin (A; -o-o-o-o-) or 2 mM sodium salicylate (B; -o-o-o-o-). The percentage conversion into 12-HETE was determined by thin layer radiochromatography.

accumulation of small amounts of 12-HPETE (1-3%; in the 30- and 60-sec incubations) was observed, in line with the reported inhibitory effect of sodium salicylate on 12-HPETE peroxidase (10).

Finally, the importance of (endo)peroxides and thus the cyclooxygenase pathway for the activation of platelet lipoxygenase was demonstrated using aspirin *in vivo*. In these experiments platelet suspensions were prepared before and after oral administration of 1.5 g of aspirin to healthy subjects. The suspensions were incubated with [1-^{14}C]arachidonic acid and the percentage conversions into 12-HETE and 12-HHT were determined by thin layer radiochromatography (Fig. 5). The data obtained confirmed previous findings that formation of cyclooxygenase products in the absence of aspirin is very rapid (11) and that peroral administration of aspirin results in virtually complete

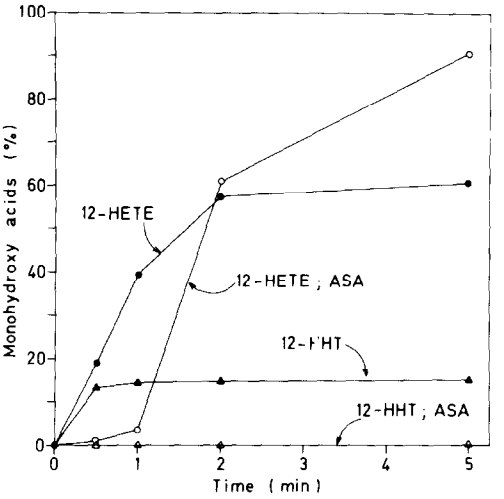


Fig. 5. Rates of formation of 12-HETE and 12-HHT from [1-^{14}C] arachidonic acid (20 µg/ml) by platelets collected before and 3 hours after oral administration of 1.5 g of aspirin. 12-HETE, before aspirin: -●-●-●- ; 12-HETE, after aspirin: -o-o-o-o- ; 12-HHT, before aspirin: -▲-▲-▲- ; 12-HHT, after aspirin: -Δ-Δ-Δ- . "ASA" = aspirin.

inhibition of cyclooxygenase activity (11). Furthermore, the presence of a lag phase in the conversion of arachidonic acid into 12-HETE in the incubation where the cyclooxygenase pathway was inhibited (Fig. 5) indicated a new functional role of platelet (endo)peroxides, i.e. to activate platelet lipoxygenase.

ACKNOWLEDGMENTS

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