A review of possible roles of the platelet 12-lipoxygenase

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THE 12-LIPOXYGENASE ENZYME of platelets was the first lipoxygenase to be discovered in animal tissue,[1] yet its physiologic significance remains far from clear. The enzyme catalyzes the reaction of arachidonic acid with molecular oxygen to form 12(*S*)-hydroperoxy-eicosatetraenoic acid (12-HPETE); under most conditions the majority of the hydroperoxide is reduced to the hydroxy form (12-HETE) and released from the cell. What is the function of this very simple transformation? At present we have no definitive answer. The pathways of arachidonic acid metabolism that are understood lead to the formation of potent biological mediators such as the prostaglandins and leukotrienes. However, there is no obvious analogy to these mediators in the 12-lipoxygenase pathway. For this reason, even the most elementary consequences of the platelet 12-lipoxygenase reaction are included in the following list of possible functions of the pathway:

(1) Reaction forms 12-HPETE or 12-HETE as a specific mediator?

(2) Reaction consumes molecular oxygen.

(3) Reaction consumes free arachidonic acid.

(4) Reaction forms a hydroperoxide.

(5) Specific mediators are formed from 12-HPETE (or 15-HPETE)?

(6) Involvement in cell-cell interactions?

Figure 1 illustrates the overall reaction in its most elementary form. Lipoxygenases react the polyunsaturated lipid substrate (LH in figure 1) with molecular oxygen to form a lipid hydroperoxide (L-OOH). The cyclooxygenase reaction involves two steps of lipoxygenase-like oxygenation. For comparison, figure 1 also shows the simple equation for p-450. This enzyme complex can be regarded both as a monooxygenase (formation of L-OH) and as a mixed-function oxidase (multiple cosubstrates are used in the reduction of molecular oxygen to water). Note that a reducing cofactor is required by p-450 but not by the lipoxygenase or cyclooxygenase.

In the following review of the 12-lipoxygenase, the possible physiologic functions are considered by reference to six consequences of the simple reaction:

$$LH + O_2 \rightarrow L\text{-}OOH$$

Reaction forms 12-HPETE or 12-HETE as a specific mediator? This section is a review of the evidence that 12(*S*)-HPETE or 12(*S*)-HETE functions as a specific biological agonist acting via specific receptors. (The possible importance of the hydroperoxide group of 12-HPETE will be considered later.) Certainly, it can be argued that specific receptors for such simple structures could exist. For example, specific radioimmunoassays have been developed for 12-HETE.[2] However, there is very little evidence to support the notion that the primary products of the 12-lipoxygenase pathway are specific agonists. Furthermore, there is no convincing precedent for this with related structural analogs such as the other HPETEs. Notably, submicromolar concentrations of 12-H(P)ETE are inactive in eliciting contraction or relaxation of smooth muscle. In contrast, the prostaglandins and leukotrienes each exhibit a distinct profile of myotropic activity in the nanomolar range. Although the very first report of chemotactic activity for leukocytes among the eicosanoids concerned 12-HETE,[3] and 12-HPETE was later found to be more active,[4] both products are now recognized as extremely weak agonists compared with LTB_4.[5] More recently, a series of studies by Nakao et al.[6] have suggested that 12-HETE induces migration of aortic smooth muscle cells at the astoundingly low concentration of 10^{-15} M. An unresolved question concerning these experiments is the fact that all incubations contained 5% to 10% fetal calf serum. Because bovine platelets synthesize 12-HETE,[7] the serum potentially could contain roughly 10^6 times more 12-HETE than is subsequently added to induce chemotaxis of the smooth muscle cells. However, if these findings are confirmed, they stand as an important exception to the current impression that 12-HETE is a biologically inactive compound.

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Supported by NIH grant GM 15431.

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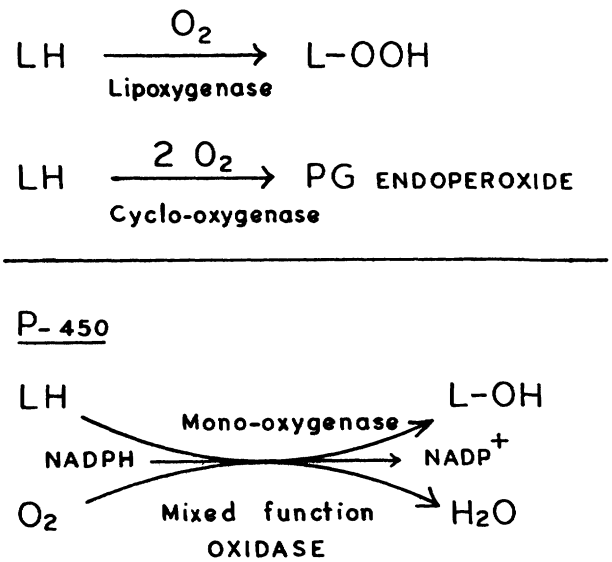

FIGURE 1. Oxygenation of lipid substrates (LH) by lipoxygenase, cyclooxygenase, and p-450.

Several studies have pursued the possibilities for specific interaction of 12-HETE on platelet function. Invariably, micromolar concentrations are required to elicit any effect, and this is a higher concentration than is likely to be achieved in vivo. Potentially, studies with 12-lipoxygenase inhibitors hold the key to understanding the role of the pathway. However, it has yet to be demonstrated that the available 12-lipoxygenase inhibitors are suitable for tests of cellular function. This problem is exemplified by the series of acetylenic analogs of arachidonic acid. The most widely used member of this series, 5,8,11,14-eicosatetraynoic acid (ETYA), is both a lipoxygenase and cyclooxygenase inhibitor, and it has been used to test platelet function in several studies.[7-9] Although ETYA will inhibit both these enzymes, there are analogs with selective lipoxygenase or cyclooxygenase inhibitory[10, 11] activities and therefore this problem can be circumvented. However, there are yet other analogs that inhibit neither enzyme, and these analogs are equipotent with the enzyme inhibitors in modifying indexes of platelet[12] and leukocyte function.[13] It remains to be seen whether this same problem will arise with the more recently developed 12-lipoxygenase inhibitors such as the flavonoids related to esculetin.[14]

Currently, the balance of evidence weighs heavily against 12-H(P)ETE acting as a specific agonist acting via specific receptors. There is no evidence for specific modification of the aggregation, adhesion, or secretory functions of platelets. This must be considered as one of the least likely functions of the platelet 12-lipoxygenase.

Reaction consumes molecular oxygen. Several years ago this was considered as a possible function of the lipoxygenases commonly found in plants,[15] although little evidence has been presented to substantiate the possibility. With regard to the 12-lipoxygenase of platelets, it is relevant to point out the potentially high capacity to oxygenate arachidonic acid and hence the corresponding capacity to deplete cellular levels of oxygen. In a well-stirred system such as the circulating blood or in platelet-rich plasma in vitro it is inconceivable that a significant depletion of oxygen could be attained. The only realistic possibility for oxygen depletion is within a mass of clumped platelets. For example, within a platelet plug on a blood vessel wall, the activity of the 12-lipoxygenase might render the system anaerobic.

Several articles have drawn attention to the fact that the 12-lipoxygenase is slow to be activated in the intact platelet[16, 17] and it remains active well beyond the time required to aggregate the cells.[2, 8] This is in contrast to the cyclooxygenase, which is inactivated after a short burst of activity. Although it may appear unlikely that depletion of oxygen is the physiologic function of the platelet 12-lipoxygenase, this possibility should not be rejected out of hand. Clearly, the activation and aggregation of platelets is followed by gross structural and metabolic changes within the cell. There are no data one way or the other to indicate whether forced depletion of oxygen by lipoxygenase activity could contribute to these changes. Nevertheless, if there is significant enzymatic activity in a closed system such as a clump of aggregated cells, then it may well be worth calculating the rate of oxygen depletion by the lipoxygenase and the rate of replenishment by diffusion from the surrounding fluids.

Reaction consumes arachidonic acid. Potentially, arachidonic acid is a far more potent mediator than 12-HETE. By conversion to prostaglandins, thromboxane A_2, and leukotrienes it forms highly active agonists. In contrast, no highly active metabolites of 12-H(P)ETE have been described. Therefore, there emerges the very real possibility that the function of the 12-lipoxygenase pathway is to inactivate free arachidonic acid and thus prevent the generation of biologically active products.

There is another potentially significant consequence of the metabolism of arachidonic acid to 12-H(P)ETE. In the unactivated platelet, the cell membranes are relatively rich in esterified arachidonic acid. During cell activation the structure of the cell membrane is altered to permit change in shape, adhesion, and secretion. The 12-lipoxygenase eliminates free arachidonic acid, and as stated earlier it continues to function well

after the immediate events of platelet activation. Therefore it is quite possible that the 12-lipoxygenase could act to eliminate arachidonic acid and hence, either facilitate structural changes that accompany the adhesion and secretory process and/or prevent the reversal of structural changes in the membrane.

The idea that the platelet lipoxygenase functions as a metabolic sink for free arachidonic acid may not be very exciting, but it remains a very real possibility that is in accord with our current understanding of arachidonic acid metabolism and the events that accompany platelet activation.

Reaction forms a hydroperoxide: effects on cellular metabolism. The platelet 12-lipoxygenase forms 12-HPETE, but under most experimental conditions it is the hydroxy analog 12-HETE that is released from the cells. Reduction of the hydroperoxide involves a sequence of redox reactions, and ultimately the reducing equivalents are generated at the expense of glucose. As the hydroperoxide is reduced, glutathione is oxidized and then replenished with equivalents from NADPH, which is itself regenerated via the hexose monophosphate shunt. It is therefore apparent that the formation of a hydroperoxide per se could influence cellular metabolism in the platelet and thus modulate cellular function. Notably, lipoxygenases generate hydroperoxides in a reaction that needs no cofactors; in contrast, cellular formation of hydrogen peroxide requires the reduction of molecular oxygen at the expense of NAD(P)H.

The quantitative relationship between 12-HPETE formation, hydroperoxide reduction, and platelet redox balance was examined by Bryant et al.[18] They found that the synthesis and subsequent reduction of 3 to 10 nmol (1 to 3 μg) of 12-HPETE by 3×10^8 platelets required the consumption of 10 times more reduced glutathione (GSH) than was present in the resting cell. In other words, a very rapid regeneration of GSH is required to enable the cells to deal with this amount of hydroperoxide.

Could a temporary depletion of GSH exert (indirect) effects on cellular function? Bosia et al.[19] have shown that chemical agents that deplete cellular GSH cause inhibition of platelet aggregation and secretion. Cross-binding of disulfide proteins was also shown to accompany GSH depletion and this may be related to the antiaggregatory effects.[19] If these findings have any relevance to the formation of 12-HPETE in platelets, then 12-HPETE and other lipid hydroperoxides should inhibit aggregation and secretion. In fact there are conflicting reports in the literature. There is some evidence to suggest that an intact 12-lipoxygenase pathway is required for the normal process of platelet aggregation in the rat[8] and in man.[9] This idea is supported by reports that 12-HPETE can enhance cyclooxygenase metabolism.[16, 20] In contrast, Aharony et al.[21] and Vericel and Lagarde[22] found that 12-HPETE and 15-HPETE exerted an antiaggregatory and antisecretory effect on human platelets with median inhibitory concentrations in the range of 2 to 6 μM; these effects are compatible with the concept of GSH depletion by hydroperoxide, leading to an inhibitory effect on platelet function. However, the hydroxy analogs (12-HETE and 15-HETE) also exhibited significant activity (median inhibitory concentration 10 μM[21]), and therefore GSH depletion can only be a partial explanation of these findings.

The important question in terms of a cellular control mechanism is whether 12-lipoxygenase activity could significantly deplete cellular GSH under physiologic conditions. This must remain doubtful. There is every indication that platelets are very well equipped to eliminate peroxide; one important index of this is the fact that it is very difficult to isolate the hydroperoxide 12-HPETE from platelet suspensions. Extreme circumstances are required before the cellular redox balance is seriously disturbed. These extreme circumstances might include (1) absence of glucose in the medium, (2) dietary deficiency of selenium,[23] (3) the rapid formation of large amounts of 12-HPETE from an exogenous source of arachidonic acid, or (4) the possible contribution of unknown factors that might strain the GSH regeneration system. These conditions have to be contrived intentionally in the laboratory and it seems unlikely they would arise in vivo. The balance of evidence does not favor the concept that generation of a hydroperoxide per se is the function of the platelet 12-lipoxygenase.

Specific mediators are formed from 12-HPETE (or 15-HPETE)? Arachidonic acid can be converted to endoperoxides, epoxides, and diH(P)ETEs under the influence of the platelet 12-lipoxygenase. However, there is no evidence that any of these products have biological activity. Furthermore, the compounds are formed in extremely low yield. In fact, they have been demonstrated to be formed only under very contrived conditions in vitro. Their mechanisms of biosynthesis can be categorized as: (1) Nonenzymatic decomposition of 12-HPETE; this gives epoxy-hydroxy and trihydroxy products.[24–26] There is evidence that the epoxy-hydroxy compounds are formed via an unstable endoperoxide reaction intermediate.[27] (2) Formation of 15(*S*)-HPETE and further conversion to 15-series leukotrienes. There is evidence that these reactions are

enzymatic and that the 12-lipoxygenase may be the enzyme responsible for the transformation.[28] It has also been shown that bradykinin is a specific stimulus for the synthesis of these products in human platelets.[29]

Formation of the first group of products, the nonenzymatic breakdown products of 12-HPETE, depends on accumulation of the hydroperoxide in the cells. Selenium deficiency (glutathione peroxidose deficiency) is one extreme circumstance known to promote this condition. The demonstration of product formation has also always depended on addition of exogenous arachidonic acid. This is necessary to increase the yield of 12-HPETE and thus to overcome the very efficient mechanisms for reduction to 12-HETE within the cell. The nonenzymatic breakdown products are not formed from 12-HETE.

The second group of products, the 15-series dihydroxy leukotrienes, also require the addition of exogenous substrate to form detectable amounts of the compounds. The yields from exogenous arachidonic acid are very low; addition of 15-HPETE improves the yield,[28] but this is a very artificial condition. The preliminary report that bradykinin stimulates biosynthesis of these products certainly deserves further attention. However, our current state of knowledge tends to suggest that all the products considered in this section are no more than biochemical artifacts formed under extreme conditions in vitro.

Involvement in cell-cell interactions

Inactivation of prostaglandin (PG)I_2 synthase. Prostacyclin synthesis is very sensitive to inhibition by fatty acid hydroperoxides.[30] Consequently it has been suggested that the 12-lipoxygenase activity of platelets could result in inhibition of PGI_2 synthesis in the vascular endothelium. However, there are at least two major problems with this simple hypothesis. First, an exogenous source of arachidonic acid is needed to generate large amounts of 12-HPETE. Second, it is questionable whether the hydroperoxide 12-HPETE can escape from the platelet without being reduced to 12-HETE. This particular point was studied by Turk et al.[31] Indomethacin-treated platelets were stirred together with aortic microsomes. In effect, the PGI_2 synthase activity of the microsomes served as an assay for the escape of platelet-derived 12-HPETE into the extracellular medium. It was found that the synthesis of PGI_2 from PGH_2 was not compromised by prior stimulation of the 12-lipoxygenase pathway in intact platelets. Thus it could be inferred from these results and the appropriate controls that 12-HPETE was reduced to 12-HETE before its release into the medium.

Novel eicosanoid products derived via platelet-leukocyte interactions. The consecutive actions of oxygenases in separate cell types can lead to the formation of novel products. This aspect of cell interactions is the subject of another contribution to this symposium[32] and is reviewed very briefly here. The first of the double oxygenation products to be identified, 5(*S*),12(*S*)-DiHETE, was originally detected as a product of porcine leukocytes.[33] White cells of this species contain both 5-lipoxygenase and 12-lipoxygenase activities. Subsequently, the formation of 5(*S*) 12(*S*)-DiHETE was studied in mixtures of human platelets and leukocytes.[34, 35] More recently, the ω-oxidation metabolite of 12-HETE, namely 12,20-DiHETE, was detected in platelet-leukocyte mixtures.[36, 37] As yet, these products have not been shown to exhibit marked biological activity.

Platelet lipoxygenase products and vascular relaxation. We have investigated the biological activity of lipoxygenase-derived products in causing relaxation of vascular smooth muscle. It seemed possible that a 12-lipoxygenase or 15-lipoxygenase product could account for the biological activity known as "endothelial cell derived relaxing factor," EDRF.[38] We have taken two approaches to testing the ability of lipoxygenase products to relax arterial smooth muscle. First, we have tested the biological activity of authentic standards. These included 14,15-LTA_4 and its derivatives, which were prepared in our laboratory. In this way we were able to discount the involvement of all the prostaglandins and leukotrienes. The 15-lipoxygenase products tested included 14,15-LTA_4,14,15-LTC_4, 14,15-DiHETE, 8,15-DiH(P)ETES, and 5,15-DiHPETE. None were active in concentrations up to 5 μM.*

Our second approach was to encourage the generation of unstable lipoxygenase products and by-products in situ in the bioassay tissue bath. This was accomplished with hemoglobin to catalyze the autoxidation of authentic HPETEs added to the buffer. Hemoglobin is well known to promote the autoxidative breakdown of HPETEs to hydroxy, epoxy, and peroxy derivatives.[39] In this way unstable products can be formed in the tissue bath and their biological activity measured. However, the results proved to be negative. As shown in figure 2 (*top*), addition of 15-HPETE (5 μM) and then hemoglobin (50 μg/ml) to a ring of rabbit aorta precontracted with 10^{-7} M norepinephrine led to an immediate relaxation. This relaxation is an artifact caused by the autoxidative destruction of the norepinephrine; however, this does indicate that the expected autoxidation takes place when 15-HPETE

*Murray JJ, Brash AR: Unpublished observations.

and hemoglobin are mixed together in the buffer. When a stable agonist was used to precontract the rabbit aorta ($PGF_{2\alpha}$, 10^{-5} M), it can be seen that the autoxidation catalyzed by the mixture of 15-HPETE and hemoglobin had no effect on the tone of the vascular smooth muscle (figure 2, bottom). Subsequent addition of acetylcholine (10^{-6} M) effected a pronounced relaxation typical of the endothelial cell–dependent response.

Further work is in progress on the possible involvement of lipoxygenase products in the endothelial cell–dependent relaxation of blood vessels. This topic is also addressed by Vanhoutte and Houston.[40]

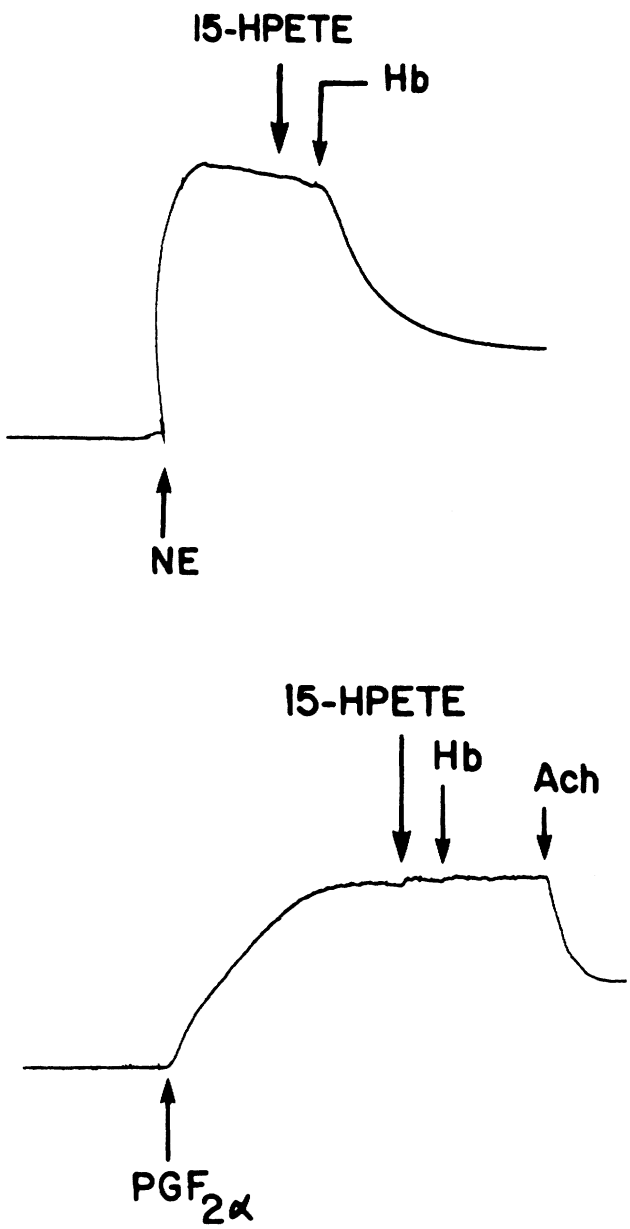

FIGURE 2. Effect of autoxidized 15-HPETE on vascular tone. *Top*, Rabbit aorta precontracted with 10^{-7} M norepinephrine then treated with 5 μM 15-HPETE and 50 μg/ml hemoglobin. *Bottom*, Precontraction with 10^{-5} M $PGF_{2\alpha}$, treatment with same concentrations 15-HPETE and hemoglobin and then 10^{-6} M acetylcholine.

Conclusion. The problem of assigning a physiologic role to the platelet 12-lipoxygenase is somewhat similar to the unsolved question of the role of lipoxygenases in plants. In the case of the plant lipoxygenases, the main substrate is linoleic acid, the simplest member of the series of polyunsaturated fatty acids. The plant enzymes convert linoleic acid either to its 9-hydroperoxy or 13-hydroperoxy derivative. Because linoleic acid has only two double bonds, there is no possibility for its further metabolism to prostaglandin or leukotriene-like structures. Thus, in plant tissues and in platelets, both rich in lipoxygenase activity, polyunsaturated fatty acid is converted to a simple hydroperoxy derivative. In neither case does it appear that the objective is to form biological mediators analogous to the prostaglandins and leukotrienes.

Six potential consequences of platelet 12-lipoxygenase activity have been considered in this review. Undoubtedly some of these are farfetched. Nevertheless it is worth adding up the evidence for and against before rejecting the hypothesis. It does seem improbable that 12-HPETE or any of its minor dihydroxy or other derivatives acts as a specific mediator or agonist. Furthermore, the concept that lipoxygenase activity could deplete cellular oxygen or cellular gluthathione demands that there is an exceptionally high turnover of the enzyme. In fact, it is difficult to make a convincing case that the 12-lipoxygenase of platelets is involved in the rapid series of events that results in adhesion, aggregation, and secretion of the granular contents of the cell. Rather, the evidence points to the fact that the 12-lipoxygenase continues to turnover well after the cells are fully aggregated. This may be related to some aspect of cell-cell interaction, or possibly it marks the metabolism of the potent biological mediator arachidonic acid to the relatively innocuous product 12-H(P)ETE.

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Circulation. 1985;72:702-707
doi: 10.1161/01.CIR.72.4.702
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7322. Online ISSN: 1524-4539