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COMMENTARY

Is There a Role for 15-Lipoxygenase in Atherogenesis?

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ABSTRACT. 15-Lipoxygenase has been suggested to play a role in atherogenesis. The proposed action of this enzyme is to oxidize low density lipoprotein (LDL) to the extent that LDL becomes a ligand for the macrophage scavenger receptor. 15-Lipoxygenase and oxidized LDL are co-localized in atherosclerotic lesions; antioxidant drugs that block the lipoxygenase also block oxidation of LDL and the progression of experimental atherosclerosis. Biochemical experiments have demonstrated that the lipoxygenase can be induced by cytokines and/or another factor(s) associated with hypercholesterolemia. However, molecular biological work has shown that induction of the enzyme alone is not sufficient to induce lesion formation. Furthermore, the mechanism of action of 15-lipoxygenase in atherogenesis remains unclear. Predictions of the stereochemistry of enzyme-oxidized linoleate products appear to conflict with the available data. In fact, most studies have discovered substantial levels of racemic 13-hydroxyoctadecadienoic acid (13-HODE) in arterial lesions rather than the stereochemically pure 13(S)-HODE expected from purified enzyme. The possibility that the generation of products of 15-lipoxygenase metabolism must occur in a specific cellular location and during a brief time window in the development of the disease has been discussed. It is also possible that the true function of the linoleate metabolites is to modulate gene expression and regulate mitogenesis, and that oxidation of LDL may play a secondary role. The advent of transgenic species that both develop atherosclerosis and either fail to express or overexpress the lipoxygenase presents an opportunity to clarify some of these issues in the near future. BIOCHEM PHARMACOL 54;9:953–959, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. atherosclerosis; lipoxygenase; macrophage; endothelium; low density lipoprotein; oxidation

Atherosclerosis is a complex disease process that develops in stages, beginning with the deposition of lipid in the vessel wall and progressing to the accumulation of lipid-laden foam cells. Later stages of the disease are marked by increasing accumulation of extracellular lipid, smooth muscle cell proliferation, and alterations in the extracellular matrix. The development and consequences of this process have been reviewed extensively [1, 2]. In this commentary, we focus on the induction of the 15-lipoxygenase enzyme and its putative role in the oxidation of plasma LDL§ and in atherogenesis. Generation of oxidized LDL occurs early in the development of vascular lesions and appears to be critical in altering the normal flux of LDL through the vascular wall [2]. Recent findings suggest that oxidized LDL binds with higher affinity than native LDL to extracellular matrix [3], and this may play a role in the lipid retention and over-accumulation characteristic of the developing atherosclerotic lesion. Although oxidation of LDL has been a major research focus, the possibility that lipoxygenase-derived products may have direct effects on the cell biology of the vessel wall must be considered in parallel. Biologically active lipids have been implicated as regulators of adhesion molecule expression, control of smooth muscle cell proliferation, and modulators of intracellular kinases.

The initial accumulation of lipid in developing lesions occurs in the macrophages. These cells do not take up native LDL to an appreciable extent due to a low level of LDL receptor expression. In contrast, macrophages express high levels of scavenger receptors, which readily mediate the internalization of oxidized LDL, although this event alone may not be sufficient to induce foam cell formation. The importance of the oxidation of LDL to this process is underscored by the preponderance of circumstantial evidence that links oxidized LDL to atherosclerotic lesions. Epitopes of oxidized LDL are readily identified in vascular lesions by immunohistochemical methods [4, 5], and pharmacologic interventions that reduce the apparent oxidation of LDL also retard the development of atherosclerotic lesions (reviewed in Ref. 6). Lipoprotein extracted from lesions has an altered electrophoretic mobility that is similar to LDL that has been oxidized *in vitro* [7]. Recently, direct evidence has shown that specific, surface-located

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§ *Abbreviations*: DAG, diacylglycerol(s); ETYA, eicosatetraynoic acid; HNE, 4-hydroxy-2-nonenal; HODE, hydroxyoctadecadienoic acid; HPODE, hydroperoxyoctadecadienoic acid; ICAM, intercellular cell adhesion molecule; IL, interleukin; LDL, low density lipoprotein; MAPK, mitogen-activated protein kinase; MM-LDL, minimally modified LDL; PKC, protein kinase C; TNFα, tumor necrosis factor-α; VCAM, vascular cellular adhesion molecule.

histidines of apoB-100 form HNE adducts during copper-mediated oxidation of human LDL [8]. HNE has been shown to arise from the radical-based decomposition of linoleate and arachidonate (reviewed by Ref. 9). It will be most interesting to see this finding extended to cell-mediated oxidations. Whether or not oxidized LDL leads directly to foam cell formation or mediates this process through the induction of cytokines or biologically active oxidized lipid products has not been resolved.

WHAT IS THE LINK BETWEEN LIPOXYGENASE AND LDL OXIDATION?

15-Lipoxygenase converts some polyunsaturated fatty acids (principally linoleate and arachidonate) to hydroperoxides by the insertion of molecular oxygen [10, 11]. Interestingly, this enzyme, unlike most other lipoxygenases, will metabolize fatty acid esters including cholesteryl linoleate, the major neutral lipid component of LDL and atherosclerotic lesions [12, 13]. *In vitro* incubation of LDL with purified lipoxygenase leads to oxidation of the lipoprotein [14] as does incubation of LDL with the hydroperoxide generated from arachidonic acid by 15-lipoxygenase. These observations coupled with the ability of antioxidant drugs to block LDL oxidation, as well as to inhibit lipoxygenase, suggested a possible link between the lipoxygenase enzymes and modification of LDL.

Early studies by Steinberg and co-workers [15] suggested that endothelial cells could oxidize LDL and that the alteration of the lipoprotein could be blocked by antioxidant lipoxygenase inhibitors. 15-Lipoxygenase, although suspected of playing a role in this oxidation, was not shown to exist in these cells. Sparrow and Olszewski [16], on the other hand, failed to note any correlation between 15-lipoxygenase and LDL-oxidizing activities in the cells they examined. In addition, the reliance upon inhibitors of the lipoxygenase, which clearly have independent antioxidant or radical scavenging activity (such as NDGA or ETYA [17]), makes little contribution toward understanding the role of cellular lipoxygenases in this process.

Recently, a 12-lipoxygenase knockout mouse has been described [18] (12-lipoxygenase is the rodent homologue of human 15-lipoxygenase). Macrophages isolated from these mice were tested for the ability to oxidize LDL. Basal oxidative activity between the knockout and wild-type mice did not differ, indicating that non-lipoxygenase mechanisms (perhaps mediated by superoxide anion or thiols in the presence of transition metals [19]) are responsible for the modification of LDL under resting conditions. However, when the macrophages were activated with opsonized zymosan, wild-type cells oxidized LDL significantly more than cells from the knockout mice and significantly more than unstimulated macrophages under similar conditions. Furthermore, studies with cultured fibroblasts that overexpress 15-lipoxygenase have shown the rapid accumulation of lipid hydroperoxides in coincubated LDL particles [20, 21], and fibroblasts overexpressing human 15-lipoxygenase alter LDL so that it is more readily oxidized and taken up by macrophages [21]. Thus, the circumstantial evidence linking cellular 15-lipoxygenase to LDL oxidation is strong.

Under normal conditions, expression of 15-lipoxygenase in the vasculature, particularly in the endothelium, is minimal. However, 15-lipoxygenase message and protein have been detected in early developing lesions, in either cholesterol-fed or Watanabe rabbits. The enzyme was localized at the sites of macrophage accumulation and appeared to be induced in both macrophages [5, 22] and the endothelium [23]. Control tissues did not express detectable levels of this enzyme. A possible explanation of the localized induction of the 15-lipoxygenase comes from the work of Sigal and his collaborators [24] who have cloned 15-lipoxygenase and showed that expression of the enzyme is regulated by IL-4. It is now known from studies in IL-4-knockout mice that this cytokine is not absolutely required for the induction of 15-lipoxygenase [25], suggesting that other cytokines may help regulate expression of this enzyme. In fact, IL-13 has been found recently to induce the lipoxygenase [26]. The expression of 15-lipoxygenase also co-localized with the expression of epitopes of oxidized LDL [5]. These data support observations made as early as 1985, when Henriksson *et al.* [27] observed that aortae from cholesterol-fed rabbits expressed 15-lipoxygenase activity, whereas no lipoxygenase enzyme activity was detectable in control animals. Similarly, vessels isolated from Watanabe rabbits show expression of this enzyme [28], as do a number of other tissues in the cholesterol-fed rabbit [29]. Thus, some factor(s) associated with hypercholesterolemia appears to be able to induce expression of 15-lipoxygenase in the vasculature.

On the other hand, overexpression of the lipoxygenase alone does not appear to be enough to initiate the development of atherosclerotic lesions. For example, the 15-lipoxygenase gene has been overexpressed in sections of rabbit iliac artery after retroviral transfer. The presence of excess lipoxygenase had no obvious effect in normal rabbits. However, overexpression of 15-lipoxygenase in the presence of moderate hypercholesterolemia caused a marked increase in the presence of epitopes of oxidized LDL in the arterial wall [30], confirming that the lipoxygenase plays a role *in vivo*. Further direct evidence for an *in vivo* role of the 15-lipoxygenase is provided by pharmacologic studies with a novel lipoxygenase inhibitor devoid of antioxidant activity, PD146176 [23]. Cholesterol-fed rabbits treated with this compound developed no lesions, did not accumulate cholesteryl ester in the vessel wall, and had no detectable accumulation of lipid-laden macrophages in the aortic intima. All of these results were in stark contrast to the changes observed in the aortae of untreated cholesterol-fed rabbits. These data further support a link between lipoxygenase and modification of LDL but only in situations where plasma cholesterol is elevated.

Not only is elevation of 15-lipoxygenase alone insufficient to initiate the development of atherosclerotic lesions, but the cellular localization of the induced enzyme appears

to play a key role as well. Data from two different transgenic species have suggested that it is induction of 15-lipoxygenase in the endothelium, and possibly not macrophages, that contributes to the disease process. For example, when overexpression of 15-lipoxygenase is targeted to the endothelium (driven by the preproendothelin promoter [31]), transgenic mice, which also lack the LDL receptor, developed significantly more lesions in their aortae than did the control mice [32]. On the other hand, in high fat, high cholesterol-fed Watanabe rabbits, which overexpress the 15-lipoxygenase in macrophages (driven by the lysozyme promoter [33]), there appeared to be a decrease in lesion development [34]. The reported protective effect of 15-lipoxygenase overexpression in rabbit macrophages is an unexpected result and thus needs careful evaluation. Three possibilities seem reasonable. First, these experiments may suggest that 15-lipoxygenase-mediated oxidation of LDL is unrelated to the development of atherosclerotic lesions, but this conclusion is seen as unlikely even by the authors of the study. Second, it is possible that abnormal sustained activity of the lipoxygenase may alter macrophage function in ways unrelated to LDL oxidation. Third, it is possible that insertion of the transgene itself alters macrophage function in some way not linked to lipoxygenase at all. Unfortunately, a variety of concerns makes it difficult to interpret the results in this report. For example, *in vitro*, the overexpressing cells generate levels of lipoxygenase products that are no higher than those generated by IL-4-activated macrophages. In the absence of *in vivo* data, there is no indication that there are any differences in lipoxygenase activity in the vessel walls of the transgenic and control cholesterol-fed animals. In addition, there is no independent evaluation of the function of the overexpressing macrophages. Thus, the protective effect could result from secondary changes in macrophage function due to either constant exposure of the macrophages to biologically active lipids or trivially from the insertion of the transgene. For example, the transgenic macrophages may accumulate more slowly in the vessel wall due to alterations in chemotaxis or adhesion, which may be unrelated to the lipoxygenase function. These critical control measurements appear to have been omitted. In addition, earlier studies of these transgenic macrophages demonstrated unusual properties of the lipoxygenase activity including altered and variable positional specificity of the enzyme [33], raising questions about the value of this genetic alteration in the study of the role of the lipoxygenase in atherogenesis. Nonetheless, the differences between overexpression of 15-lipoxygenase in macrophages versus endothelium may have uncovered a critical dependence on the localization of the induced enzyme. Since the lipoxygenase appears to be induced in both tissues during atherogenesis *in vivo* and overexpression in endothelium but not macrophages leads to accelerated lesion formation, one is led to hypothesize that the endothelial expression of this activity plays a role in the initiation of the disease. These transgenic models need further evaluation to clarify the many questions that remain.

WHAT DO WE LEARN FROM ANALYSIS OF THE STEREOCHEMISTRY OF LESIONAL LIPIDS?

The analysis of the oxidized lipid products extracted from lesions is not obviously consistent with either a purely enzymatic or nonenzymatic process and may result from some combination of the two. Although it is clear from *in vitro* studies that 15-lipoxygenase can initiate the production of lipid hydroperoxides in LDL, the mechanism for this effect is unclear. Since this enzyme generates hydroperoxides with the (S) configuration, a direct effect of the enzyme on LDL lipids would be expected to generate products with this stereochemistry. If, on the other hand, the lipoxygenase functioned primarily to seed a nonenzymatic chain reaction, the products should be racemic. For example, the linoleate product isolated from LDL incubated with purified 15-lipoxygenase *in vitro* is essentially pure 13(S)-HODE [13]. Although there are no data concerning the stereochemistry of LDL hydroperoxides *in vivo*, several attempts have been made to determine the composition of the bulk lipids isolated from atherosclerotic lesions. These studies have generally found that lesions contain a racemic mixture of 13-HODE usually esterified to cholesterol [35], suggesting that the bulk of the oxidized lipid is generated by a nonenzymatic mechanism. This, of course, does not rule out a critical role for the lipoxygenase as an initiating factor in the oxidation process since minimal oxidation of lipoprotein makes the particle more susceptible to further oxidation, perhaps by nonstereospecific mechanisms. It is interesting to note that, in some experiments, a slight enantiomeric excess of the "enzymatic" stereoisomer has been detected [35, 36]. This has been demonstrated most convincingly at early time points in the development of lesions. These findings have led to the proposal that the lipoxygenase is the major contributor to oxidation at some critical time during but not throughout atherogenesis.

15-Lipoxygenase initially converts linoleate to 13(S)-HPODE with high specificity. *In vitro* studies using biological membranes as substrate for purified 15-lipoxygenase have found that, as the concentration of membrane increases, this stereospecificity disappears, and products with only 10–40% enantiomeric excess were observed. In addition to the loss of stereospecificity, secondary reactions leading to further oxidation of both lipids and proteins were readily detected [12]. Although it is certainly dependent upon the source of membranes, it is interesting to note that oxidation products of both linoleate and arachidonate were detected under conditions that generate secondary reaction products. This is in contrast to the apparent lack of oxidized arachidonic acid products in the lipids obtained from atherosclerotic lesions [35, 36]. In addition, no oxidation of membrane cholesterol was noted. However, in incubations of cholesterol with the soybean lipoxygenase and linoleate, it was shown that a carbon-based radical can initiate the oxidation of cholesterol [37].

There has been a clear bias in the literature that in order

for 15-lipoxygenase to play a role in atherogenesis, stereochemically pure product ought to be readily detectable in the bulk phase of vascular or lesioned-area lipids, and this does not appear to be the case. If this evidence supports a nonenzymatic mechanism for lipid oxidation, it remains to be explained why the lipid products are predominantly 13-HODE, that is, why larger amounts of the positional isomer 9-HODE are not found. A second problem is the apparent lack of arachidonic acid oxidation products that ought to be generated under conditions that oxidize linoleate. One possible explanation for this is that the nonenzymatic oxidations may be limited to the lipoprotein core or foam cell lipid droplets, which are composed primarily of cholesteryl linoleate, although why cholesterol oxidation does not occur under these conditions is unclear.

It has also been noted that racemic 13-HODE is detectable in lipids extracted from vessels of cholesterol-fed rabbits in the absence of gross lesions or macrophage accumulation (as in PD146176-treated animals). This suggests that the presence of these linoleate products is not sufficient to initiate atherogenesis. In fact, the presence of these oxidized lipids in the absence of atherosclerotic lesions raises several questions. For example, if the 15-lipoxygenase is inhibited, what is the mechanism by which the racemic 13-HODE found in the artery is generated? It remains to be determined what role, if any, the presence of esterified forms of oxidized linoleate in the vascular cell membranes plays in the pathophysiology of the developing atherosclerotic lesion. In fact, the apparent transient nature of the generation of 15-lipoxygenase products in the vessel wall that occurs early in atherogenesis [36] suggests the possibility that the products of this enzyme may initiate cellular events through changes in gene expression that occur or are maintained far beyond the time period of their generation.

WHAT OTHER EFFECTS OF 15-LIPOXYGENASE INDEPENDENT OF ENHANCED LDL UPTAKE COULD BE CRITICAL FOR ATHEROGENESIS?

The ability of lipoxygenase to catalyze the modification of LDL and render it able to bind to scavenger receptors may not be the only role for this pathway in atherogenesis. In fact, lipoxygenases are well known to generate cascades of biologically active metabolites that play important roles in processes as diverse as inflammation, asthma, and modulation of neuronal function [38–40]. How might some of the linoleate products of 15-lipoxygenase participate in atherogenesis at this level?

Linoleate, arachidonate, and the lipoxygenase products of these lipids have been suggested to modulate mitogenesis and cellular adhesion in a variety of systems. For example, 13-HPODE at nanomolar levels dramatically potentiates epidermal growth factor-dependent mitogenesis in cultured fibroblasts [41]. 13-HODE was found to be nearly as active as its precursor hydroperoxy acid. Further, the effect was found to be exclusively associated with the (S)-isomer, suggesting that enzymatic production is necessary [42]. This chiral requirement for activity is of special importance for two reasons. First, it is clear, as discussed above, that substantial amounts of racemic 13-HODE can be found in atherosclerotic tissue. Second, evidence from other systems suggests that some *but not all* of the direct effects of hydroperoxy lipids discriminate between the stereoisomers of these metabolites [43].

More recent studies have demonstrated similar effects of oxidized lipids on mitogenesis in vascular smooth muscle cells. Linoleate (20 μM) increased expression of c-*myc*, c-*fos*, and c-*jun* from 3- to 40-fold in this cell type, and the effect could be blocked by the antioxidant lipoxygenase inhibitor NDGA [44, 45]. Furthermore, equal levels of expression of these transcription regulators could be achieved with a twenty times lower dose of 13-HPODE, suggesting that metabolism by the 15-lipoxygenase was critical in regulating this gene expression. In addition, oxidant stress has been noted to induce the production of arachidonate hydroperoxides, which have been shown to induce the expression of c-Fos and c-Jun protein along with transcription factor activating protein (AP-1) activity [46]. The importance of these effects on mitogenesis in the area of carcinogenesis has been reviewed [47], but it has equally important implications in the pathogenesis of atherosclerosis.

In addition to modulating gene expression, linoleate metabolites have been observed to have potentially important effects on cellular kinases. For example, linoleate and 13-HPODE are potent activators of MAPK in vascular smooth muscle cells [44]. On the other hand, calcium/calmodulin-dependent protein kinase IIα, which may activate MAPK in vascular smooth muscle under some conditions [48], has been found to be inhibited by arachidonate hydroperoxides (although the effects of linoleate metabolites have not been tested) [49]. Interestingly, 13-HODE has been observed to induce transient increases in intracellular calcium levels, although the precise consequences of this effect are not clear [50]. In addition, some isoforms of PKC (particularly PKCβ) may be inhibited by 13-HODE-containing DAG. 13-HODE-DAG displaces diolein, believed to mimic an endogenous activator of PKC, and blocks activation of the kinase [51]. Thus, these lipids may play multiple and complex roles as regulators of kinases that are central to the process of cellular proliferation.

The most likely source for the linoleate that is the precursor of these biologically active 15-lipoxygenase products is the LDL core, which is rich in cholesteryl linoleate, and not the cell membrane phospholipids, which are relatively rich in arachidonate. Therefore, minimal modification of LDL by lipoxygenase-mediated processes may generate locally active modulators of cell function without enhancing the scavenger receptor-mediated uptake of the particle. This has been seen in a second key area: modulation of monocyte adhesion to endothelium. MM-LDL has been shown to induce generation of both soluble and membrane-bound monocyte chemotactic factors as well as

expression of a variety of cellular adhesion molecules. For example, MM-LDL activates the production of colony-stimulating factors [52] and monocyte chemotactic protein 1 (MCP-1) in endothelial cells [53] and induces the expression of a member of the GRO family of peptides [54].

MM-LDL activates monocyte adhesion to microvascular endothelium in an *in vivo* model [55] and has been found to induce intracellular accumulation and histamine-induced expression of P-selectin [56]. Unfortunately, the factor(s) associated with this mildly oxidized form of LDL that is responsible for these effects has not been clearly identified and, thus, the potential role of lipoxygenase, although clearly present, is not yet determined. Importantly, Sigari *et al.* [57] have shown that fibroblasts that overexpress 15-lipoxygenase convert LDL into a particle that behaves exactly as MM-LDL. This further supports a role for this enzyme as an activator of atherogenesis.

A more convincing role for this pathway has been demonstrated in the ability of oxidized LDL to augment TNFα-induced expression of both VCAM-1 and ICAM-1 on cultured human aortic endothelial cells [58]. In this case, the activity of oxidized LDL could be mimicked by the addition of 13-HPODE to the cultures prior to activation with the cytokine. A similar effect could be induced in cultured bovine aortic endothelial cells that overexpressed human 15-lipoxygenase. TNF induced VCAM expression, and this expression was augmented in 15-lipoxygenase overexpressing cells [59].

CONCLUDING REMARKS

Data suggesting that lipoxygenase plays an active role in the development of atherosclerosis have continued to accumulate. The earliest pharmacological results suffered for the lack of specific agents and had little biochemical evidence to support their conclusions. These findings have been largely corroborated by newer research with better drugs and by the application of molecular biological techniques. Unfortunately, the role of the lipoxygenase and/or its products in atherosclerosis remains unclear, in spite of recent data that strengthen the connection. It appears that active lipoxygenase alone is not sufficient to induce the disease, but that a transient expression of the 15-lipoxygenase products may play a critical role in the development of vascular lesions. This effect appears to require a concomitant elevation of plasma cholesterol or of LDL. Biochemical studies that attempt to discover the nature of this critical but ephemeral role by quantifying bulk lesional or vascular wall lipids appear to be unlikely to shed much light on the pathogenesis of atherosclerosis. However, the development of novel specific lipoxygenase inhibitors and the advent of transgenic murine models prone to atherosclerosis, as well as the development of a lipoxygenase-knockout mouse, present an interesting opportunity to determine at least the necessity and cellular localization of the lipoxygenase pathway in the genesis of atherosclerosis.

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