The Oxidation of Lipoproteins by Monocytes-Macrophages

BIOCHEMICAL AND BIOLOGICAL MECHANISMS*

Guy M. Chisolm III‡, Stanley L. Hazen, Paul L. Fox, and Martha K. Catheart

From the Department of Cell Biology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio 44195

The oxidation of lipoproteins has been proposed as a biological process that initiates and accelerates arterial lesion development (1–5). Oxidized lipoproteins accumulate in lesions (6) and may form at other inflammatory sites. Whether the oxidized lipoprotein is an initiator or accelerator of disease is the subject of speculation, debate, and intensive study. Various cellular and biochemical mediators of lipoprotein oxidation in vivo have been proposed, but none has yet been proven to be responsible.

Two decades ago we demonstrated that low density lipoprotein (LDL), the plasma level of which correlates with the risk of atherosclerosis, could injure endothelial cells (ECs) in culture (7). The capacity of LDL to injure cells was directly related to the level of LDL oxidation, and we speculated on a possible role for oxidized LDL-mediated endothelial injury in atherogenesis (8, 9). Contemporaneously, Dr. Daniel Steinberg’s group (10, 11) demonstrated that LDL exposed to cultured ECs was altered such that it became a ligand for scavenger receptors. In 1984, both Steinberg’s group and ours (12, 13) demonstrated that the “EC-modified LDL” they had characterized and the “oxidized LDL” we had described were the same entity. Their report highlighted the macrophage recognition of the EC-oxidized lipoprotein, and our capacity of EC or smooth muscle cell (SMC)-oxidized LDL to injure cells. These papers introduced the concept that reactive oxygen species from vascular cells could transform LDL, causing it to exhibit dramatically altered composition and atherogenic properties. The first demonstration that certain leukocyte populations could oxidize LDL employed human neutrophils and activated populations of adherent human monocytes, cells well known to generate abundant reactive oxygen species in vitro and in vivo (14).

The identity of the cells responsible for the oxidation of LDL that accumulates in lesions is uncertain. Although it is well known that free ferrous or cupric ions catalyze lipid peroxidation reactions in vitro, the presence of free metal ions in vivo is doubted (15). Multiple mechanisms exist in vitro for binding free transition metal ions, rendering them redox-inactive (15–17). In this minireview, we take the position that monocyte-derived macrophages are likely candidates to mediate the in vivo oxidation of lipoproteins, because they are prominent in arterial lesions, known to generate activation-dependent reactive oxygen species, and, unlike EC and SMC (12, 18), capable in vitro of LDL oxidation in media free of metal ion additives. In vitro LDL appears to be oxidized extracellularly without interaction with the LDL receptor (19–21). There are multiple potential pathways through which monocyte-macrophages may promote extracellular LDL oxidation. In this review we evaluate cellular mechanisms (both enzymatic and non-enzymatic) for LDL oxidation. We use the term “monocyte-macrophage” as a shorthand reference to in vitro studies performed on isolated monocytes, macrophages, and monocyte-like cell lines.

The Role of Ceruloplasmin

The presence of free copper or iron ions in vivo is unlikely, but an interesting concept in redox-metal-dependent oxidation of LDL by phagocytes was recently introduced. The idea followed the surprising observation that the copper-containing acute phase plasma protein, ceruloplasmin (Cp), studied for years as an antioxidant, could act instead as a potent oxidant of LDL (22). The oxidant capacity of Cp may have been overlooked previously because of the difficulty in preserving Cp in an undegraded state during its purification (23). Cp is a 132-kDa glycoprotein that contains seven copper atoms per molecule and carries most of the copper in blood. We found that LDL exposed to Cp exhibited many characteristics of LDL oxidized in the presence of free cupric ion (22). This oxidant activity of Cp in vitro has been verified by other laboratories (24–26).

To determine whether Cp could substitute as a “physiological” source of redox-active metal, the equivalent of normal (unevoked) plasma levels of Cp was added to cultures in which ECs or SMCs were exposed to LDL in RPMI 1640, a cell culture medium without transition metal ion additives. Cp markedly enhanced LDL oxidation by both cell types (27). Proteolytic cleavage of Cp or removal of one of its seven copper atoms that is near His426 prevented its oxidative action (27–29). Cell-derived superoxide anion (O2−) was essential for the enhancement of LDL oxidation by Cp under these conditions.

Because monocyte-macrophages express Cp mRNA and protein (30, 31), the hypothesis that cell-derived Cp contributes to LDL oxidation by these cells was tested. A model system similar to that of Cathcart et al. (20, 32) was adopted, which used the human myelomonocytic cell line, U937, activated by zymosan in RPMI 1640 medium. Exogenous Cp added to unactivated U937 cells caused LDL oxidation to an extent similar to that by zymosan in the absence of exogenous Cp (33). Zymosan induced Cp mRNA and (after a 5–6-h lag following activation) Cp protein synthesis (33). This lag coincided with the lag prior to measurable LDL oxidation previously reported (34, 35). A neutralizing antibody to Cp blocked LDL oxidation by activated U937 cells, as did antisense oligodeoxyribonucleotides (ODN) targeted against segments of the Cp mRNA (35). These studies suggested a possible in vivo role for Cp in monocyte-macrophage-mediated LDL oxidation, but they also revealed that cell-derived factors in addition to Cp are required for optimal oxidation. Among the candidates are, for example, O2− and lipoxigenase, both of which are increased by activation of monocyte-macrophage-related cells. The role of O2− may be to reduce the Cp-bound “oxidant” copper (28).

Factors that stimulate monocyte-macrophage Cp production and could contribute to Cp accumulation in vivo include endotoxin (31) and interferon (IFN)-γ (36). The observation that a bacterial product is an agonist may be significant given the possible link between bacterial infection and atherosclerosis (37). Because T-cells, a major source of IFN, populate arterial lesion sites, Cp accumulation could be enhanced by a local inflammatory response. However, IFN-γ also stimulates antioxidant production by monocytes-macrophages (38) and has been shown under certain conditions to inhibit LDL oxidation (39, 40).

The studies of Cp in cell-mediated LDL oxidation suggest other protein-bound redox-active transition metals might also participate in extracellular oxidation events. Under physiological conditions...
tions globin degradation products such as hemin (41) and holo forms of iron binding proteins such as transferrin (25) and ferritin (42) may catalyze oxidation reactions. The reducing agents required for activity and the physiological significance of these protein-bound redox metals remain uncertain.

The Role of O2

The role of O2 in monocyte-macrophage LDL oxidation is as debated as that of metal ion, and the two controversies are interrelated. The dependence on O2 is less in cell systems in which the culture medium contains free redox metal ions. Using metal ion-containing (Ham's F12) medium, Garner et al. (47) reported O2-independent LDL oxidation by human monocytes-macrophages. Our early studies with monocytes-macrophages were conducted in RPMI 1640 in an effort to mimic the predicted absence of free metal ions in vivo. Our studies showed that LDL oxidation required activation of the cells by certain activators, including opsonized zymosan (Zop) or LPS; other activators were not effective (14, 32).

(Addition of free metal ions to monocytes incubated in RPMI 1640 resulted in activation-independent LDL oxidation (20)). The activation dependence in RPMI 1640, the successful inhibition by numerous antioxidants (14, 32), and the well described enhancement of O2 production by monocytes following activation were consistent with a requirement for O2. Weiss and others have found cell-derived, extracellular O2 to be required, but not sufficient, for LDL oxidation by monocytes-macrophages (34, 35). O2-generating systems alone do not mediate LDL oxidation (43–45). Others have speculated that superoxide dismutase could be inhibitory by acting as a metal chelator, rather than an O2 scavenger, giving the false impression of O2 dependence (46). However, further evidence for the requirement for O2 in this system is derived from recent studies using in vitro knockout of p47phox. O2 and LDL oxidation were concomitantly ablated when this requisite component of the NADPH oxidase complex was inhibited by antisense ODN.

Xing et al. (48) provided evidence that systems using zymosan as an activator may in fact be metal ion-dependent because the yeast cell walls, like transferrin and ferritin, can carry iron in an oxidized (Fe3+) state. Their results are consistent with Zop-bound Fe3+ being reduced to Fe2+ by cell-derived O2, which then catalyzes LDL oxidation, thus putting the Zop-activated cell system into a category parallel with the concept presented above: that activated monocytes-macrophages oxidize LDL by a pathway requiring O2 to reduce transition metal (e.g. Cp-borne cupric or ferritin-, transferrin- or hemin-borne ferric).

The formation of O2 and/or its dismutation product, H2O2, by monocytes-macrophages appears to be essential for LDL oxidation in vitro, whether the process is Cp-, myeloperoxidase- (MPO) (see below), or Zop-dependent. Accordingly, considerable effort has been devoted to elucidate signaling pathways regulating the generation of O2. Multiple approaches have demonstrated that the pathway for optimal oxidation of LDL by Zop-activated monocytes-macrophages involved calcium via both influx and release from intracellular stores (50). Using PKC inhibitors, down-regulation of PKC expression by PMA, and antisense ODN, the requirement for activation of the calcium-dependent ePKC isoenzyme, PKCo, was convincingly shown (51, 52). Selective inhibition of cytosolic phospholipase A2 (cPLA2) using antisense ODN and pharmacological inhibitors, revealed cPLA2 activity to be another essential step in the signaling sequence for O2 production and LDL oxidation (53). Addition of arachidonic acid, the product of cPLA2, restored both the production of O2 and LDL oxidation (53). Recent data, mentioned above, using p47phox antisense ODN suggest that the enzymatic source of O2 is NADPH oxidase. PKC activation ODN.2 may be involved in phosphorylation of various protein components of this enzyme complex.

The Role of 15-Lipoxigenase

Are there other cell-derived factors required for optimal monocyte-macrophage oxidation of LDL? Several have been studied; these include not only Cp but also LPS. MPO, LOs are iron-containing enzymes found in various cells, including reticuloocytes, monocytes-macrophages, and certain endothelial cells. They catalyze the direct insertion of molecular oxygen into polyenoic fatty acids, forming hydroperoxides. There are a number of ways in which LOs could participate in LDL oxidation. LO could oxidize cellular fatty acid, cholesterol ester, or phospholipid substrates, and the hydroperoxide products could transfer to LDL, making LDL more susceptible to oxidation (54). If LO could come in contact with LDL, it could use phospholipid or cholesterol ester as substrate (55), leading to lipid peroxidation. LO products could also participate in signal transduction pathways regulating other monocyte-macrophage functions involved in oxidation.

In monocyte-macrophage systems, 5-LO has been ruled out as a participant in LDL oxidation (58, 59). Sparrow et al. (56) demonstrated that incubation of LDL with 15-LO plus phospholipase A2 led to LDL oxidation in a cell-free system; however, 15-LO alone was also shown to oxidize LDL significantly in the absence of cells (57). 15-LO inhibitors are able to block cell-mediated oxidation of LDL, but many of these inhibitors are nonspecific antioxidants, making the assertion of a role for 15-LO more difficult to demonstrate (60–62). One study reported a lack of LO involvement in LDL oxidation by monocytes-macrophages by pointing out discrepancies between the concentrations of inhibitors required for LO inhibition and those required to inhibit LDL oxidation (61).

Despite caveats, substantial indirect evidence links 15-LO to LDL oxidation. In cell-free systems LO was inhibited by O2 (57). O2 scavengers in a cell-free soybean LO system and an intracellular O2 scavenger, hemin, in an activated monocyte-macrophage system both enhanced LDL oxidation (57, 60). Fibroblasts overexpressing 15-LO oxidized LDL moderately more than control transfected cells (63). Cytokines that modulate 15-LO activity in Zop-activated monocytes-macrophages (enhancement by interleukin-4 and interleukin-13; inhibition by IFNγ) modulated in parallel LDL oxidation by these cells (64). Angiotensin II enhanced both LO activity and LDL oxidation in mouse peritoneal macrophages and J774 cells (49). Peritoneal macrophages from animals without 12/15-LO demonstrated impaired LDL oxidation (119). These in vitro studies are consistent with a contributory role for LO.

The Role of Myeloperoxidase

Another cell-derived factor that may participate in phagocyte-dependent oxidation of LDL is MPO. MPO is an abundant heme protein released by activated neutrophils and monocytes and present in some tissue macrophages such as those in vascular lesions (65). MPO may play a role in monocyte-macrophage oxidation of LDL. A variety of distinct pathways (66–72). MPO can act to amplify the oxidizing potential of H2O2, the dismutation product of O2, by using it as a co-substrate to generate a variety of oxidants, including diffusible radical species (69, 73), reactive halogens (66, 68, 74), aldehydes (70, 75), and nitrating agents (72, 76–78). The heme group of MPO is buried deep within a hydrophobic binding pocket and catalyzes the oxidation of a variety of small substrates that can then diffuse away from the enzyme and damage cellular targets. Thus, MPO-mediated oxidation reactions occur in the absence of free transition metal ions and are resistant to inhibition by chelators. Because of its high concentration in biological matrices, chloride is regarded as a major substrate for MPO (79, 80). MPO catalyzes the two-electron oxidation of chloride forming the powerful oxidant, hypochlorous acid (HOCl). Exposure of LDL to reagent HOCl results in chlorination and oxidation of protein and lipid constituents of LDL, induces LDL aggregation, and converts the lipoprotein into a high uptake form for macrophages (66, 67, 81, 82). MPO-generated HOCl also oxidizes free α-amino acids, abundant nutrients in plasma and extracellular fluids, converting them into aldehydes (70, 75, 83, 84). MPO-generated aldehydes can then modify nucleophilic targets on LDL protein and lipids (85, 86). In addition, MPO can catalyze the one-electron oxidation of t-tyrosine, generating the tyrosyl radical. PMA-activated phagocytes can produce tyrosyl radical and initiate LDL lipid peroxidation and dityrosine cross-linking of proteins (69, 73).

Recent studies have identified another potential MPO-dependent pathway that monocytes-macrophages may employ for LDL oxidation that involves formation of reactive nitrogen species. MPO can use H2O2 and nitrite (NO2−), a major end product of nitric oxide (nitrogen monoxide, 'NO) metabolism, to generate a reactive inter-
mediate capable of nitrating aromatic compounds and tyrosine residues (77, 78, 87). In addition, HOCl can react with NO2 to form a nitrating and chlorinating intermediate (76). Recent studies demonstrate that exposure of LDL to NO2 and either elutriated human monocytes or isolated MPO and an H2O2 source results in LDL lipid peroxidation and protein nitration (72). Moreover, LDL modified by MPO-generated nitrating intermediates is rendered a ligand for high affinity binding and uptake by macrophages, resulting in cholesteryl ester accumulation and foam cell formation (72). The biological consequences of LDL modification by MPO-generated chlorinating intermediates, reactive aldehydes, nitrating intermediates, and diffusible radical species are areas for future study.

The Role of NO and NO-derived Oxidants

The role of NO and reactive nitrogen species derived from NO in LDL oxidation is an area of intense research. NO is a long lived free radical formed by multiple inflammatory and vascular wall cells (88). Both pro- and antioxidant effects of NO have been proposed. NO is relatively unreactive toward most biomolecules. In cell culture systems, enhanced production of NO by monocytes-macrophages exerted an antioxidant effect, attenuating the extent of lipid peroxidation (89). However, a variety of reactive nitrogen species derived from NO are capable of oxidizing biological targets. The cellular and biochemical consequences of NO and NO-derived reactive nitrogen species formation on biological targets are thus complex; they have recently been comprehensively reviewed (90–92).

Methodological Considerations Affecting Mechanisms of Oxidation

The mechanisms of LDL oxidation by monocytes-macrophages in vitro are strongly influenced by methodological considerations. These include the presence of metal ion additives, the protection of LDL during preparation, and the source and treatment of the cells. There are multiple ways that transition metal ions have been shown to participate in lipid peroxidation. The hydroxyl radical (·OH) can be formed by metal ion-dependent processes and can initiate lipid peroxidation (93, 94); however, transition metals can also catalyze ·OH-independent lipid peroxidation. O2·− and H2O2 support metal-catalyzed lipid peroxidation through their participation in redox reactions, e.g. as a reductant for Fe3+ and as an oxidant for Fe2+, respectively (93, 95). Fe2+ can also react with preferred lipid hydroperoxides to generate lipid alkoxyl radicals and facilitate lipid peroxidation (96). Thus LDL that carries even minor amounts of lipid hydroperoxides will be more susceptible to metal ion-mediated oxidation (97, 98). Trace levels of hydroperoxides may be formed in vivo and circulate on lipoproteins, or they may arise from spurious oxidation during isolation and storage. LDL will also readily bind LPS, altering (sometimes dramatically) the outcomes of LDL-cell interactions (99). The cell isolation method also influences the pathways involved in monocyte-macrophage-dependent oxidation of LDL. For example, protocols that employ adhesion of monocytes in vitro, both to simulate adherence in vivo and to facilitate removal of other mononuclear cells, can lead to release of granule contents, including MPO.1 Human monocyte-derived macrophages generated in culture also lose MPO during differentiation unless grown in the presence of specific growth factors and cytokines (100, 101). Thus, the role of MPO-dependent oxidation events can best be studied in fresh monocytes isolated by methods that do not require adhesion, such as buoyant density centrifugation or elutriation.

Perspectives

Multiple, distinct pathways exist through which monocytes-macrophages can promote LDL oxidation. Are there clues from in vivo studies that would aid in discriminating which among the different mechanisms pertains in vivo? Cp, LO, and MPO are all abundantly present in lesions (65, 102, 103), as perhaps would be expected considering all have been shown to be enhanced in monocyte-macrophage systems activated in vitro, and lesions can be a rich source of macrophages. There is also evidence of LO and MPO activities in lesions. Non-equilibrium distributions of chiral fatty acid oxidation products, expected after LO-dependent oxidation of esterified linoleic acid, have been observed in early lesions (and less so in later stage lesions) in rabbits (104), as well as humans (105, 106). Multiple distinct, stable end products of MPO are enriched both in human vascular lesions and LDL recovered from human atherosclerotic aorticx, including proteins damaged by HOCl (107), chlorotryptosine (66), dityrosine, and nitrotirosine (108–110), among others (71).

Does oxidation involving a metal carrier, as modeled in vitro using Cp, ferritin, or Zop, take place in vivo? Because one well characterized pathway for metal-dependent lipid peroxidation is mediated by ·OH, the absence from early human atherosclerotic lesions of significant increases in ·OH-generated protein oxidation products (109) has led to the suggestion that metal-mediated LDL oxidation may not take place in vivo (5, 109). However, such a suggestion is tempered because of known pathways, mentioned above, for ·OH-independent, metal ion-mediated lipid peroxidation (94, 96). Significant amounts of ·OH-dependent protein oxidation products were observed in advanced lesions (111).

Does the incidence of atherosclerosis offer hints indicating a particular oxidative mechanism? Serum Cp was reported to be an independent risk factor for coronary heart disease (112), and increases in ultrasound-detectable lesion size correlated with LDL cholesterol only in subjects with high serum Cp copper levels (113). Furthering the notion that LO may promote atherosclerosis are one report of a putatively specific 15-LO inhibitor significantly blunting diet-induced atherosclerosis in the rabbit (114) and another showing that disruption of the 12/15-LO gene in apolipoprotein E-deficient mice dramatically reduced atherosclerosis (115). In surprising contrast, transgenic rabbits overexpressing macrophage-specific 15-LO were partially protected from atherosclerosis (116). Despite remaining controversies, cell culture models of monocyte-macrophage-mediated oxidation of LDL have facilitated the formulation of multiple feasible mechanisms of LDL oxidation and have generated ideas for intervention in vivo. It is possible that more than one of the mechanisms discussed above pertains in vivo. Delineation among them awaits more studies of selective inhibition, activation, or overexpression of key proteins such as MPO, LO, Cp, those required for ·O2·− generation, or perhaps extracellularly active forms of superoxide dismutase or catalase.

REFERENCES

1. Chisolm, G. M., and Penn, M. S. (1996) in Oxidized Lipoproteins and Atherosclerosis (Fuster, V., Ross, R., and Topol, E. J., eds) pp. 129–149. Raven Press, New York
2. Steinberg, D. (1997) J. Biol. Chem. 272, 29963–29966
3. Hessler, J. R., Robertson, A. L., Jr., and Chisolm, G. M. (1979) Arteriosclerosis 9, 225–233
4. Witzum, J. L., and Horkko, S. (1997) Ann. N. Y. Acad. Sci. 811, 88–99
5. Heinecke, J. W. (1998) Atherosclerosis 141, 1–15
6. Yla-Herttuala, S. (1998) Curr. Opin. Lipidol. 9, 337–344
7. Hessler, J. R., Robertson, A. L., Jr., and Chisolm, G. M. (1979) Atherosclerosis 32, 213–229
8. Hessler, J. R., Morel, D. W., Lewis, L. J., and Chisolm, G. M. (1983) Arteriosclerosis 3, 215–222
9. Morel, D. W., Hessler, J. R., and Chisolm, G. M. (1983) J. Lipid Res. 24, 1070–1076
10. Henriksen, T., Mahoney, E. M., and Steinberg, D. (1983) Arteriosclerosis 3, 195–204
11. Henriksen, T., Mahoney, E. M., and Steinberg, D. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 6499–6503
12. Steinbrecher, U. P., Parthasarathy, S., Leake, D. S., Witzum, J. L., and Steinberg, D. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 3883–3887
13. Morel, D. W., DiCorleto, P. E., and Chisolm, G. M. (1984) Atherosclerosis 4, 357–364
14. Cathcart, M. K., Morel, D. W., and Chisolm, G. M. (1985) J. Leukocyte Biol. 34, 341–350
15. Dabbagh, A. J., and Frei, B. (1995) J. Clin. Invest. 96, 1588–1596
16. Aasa, R., Malmstrom, B. G., Saltman, P., and Vangard, T. (1963) Biochim. Biophys. Acta 74, 203–222
17. Thomas, C. E. (1992) Biochim. Biophys. Acta 1128, 50–57
18. Heinecke, J. W., Rosen, H., and Chait, A. (1984) J. Clin. Invest. 74, 1390–1394
19. Tangirala, R. K., Mol, M. J., and Steinberg, D. (1996) J. Lipid Res. 37, 835–843
20. Cathcart, M. K., Li, Q., and Chisolm, G. M. (1995) J. Lipid Res. 36, 1857–1865

S. L. Hazen and M. K. Cathcart, unpublished observations.
E. Ehrenwald and P. L. Fox, unpublished observations.
