Review article

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THE EFFECT OF NO/EDRF AND MONOCYTES/MACROPHAGES ON LDL-OXIDATION

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Beside prostaglandin (PG) I2 and tissue plasminogen activator (tPA), nitric oxide (NO) is a key repellant substance contributing to haemostatic balancing. The role of low-density lipoproteins (LDL) in the pathogenesis of atherosclerosis has been gaining increasing importance. It is well accepted that LDL in their modified (i.e. oxidized) form are no longer recognized by the LDL-receptor, but are taken up by cells of the arterial wall, especially macrophages, in a non-regulated manner through the so called scavenger-receptor pathway. This process leads to the formation of foam cells, the hallmark of the atherosclerotic lesion. NO is also produced in relevant amounts by macrophages. The interaction of NO and LDL with macrophages is thus of key importance in the onset of aery lesions. While oxidized LDL (oxLDL) are resulting in a decreased NO availability, NO seems to prevent LDL-oxidation. In contrast, however, in the presence of superoxides oxidation may result. All these potential actions have to be discussed in view of the extremely short half-life of NO indicating that these actions are restricted most likely to the local site of biosynthesis being dependent on the actual concentration, the duration of availability and the presence of transition metals. These findings indicate that NO may play a dual pro- and antiatherosclerotic role being dependent on local factors only.

Key words: LDL-oxidation, endothelium-derived relaxing factor (EDRF), nitric oxide (NO), monocytes/macrophages, atherosclerosis.

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INTRODUCTION

Many recent studies have demonstrated that LDL, the major cholesterol carrier in human plasma, can be modified by enzymatic as well as by non-enzymatic processes (1, 2). The modifications of LDL might occur in vivo as a consequence of the interactions of the lipoprotein with cells of the arterial wall (3–5), blood cells (6–9), immune complexes (10) and also components of the arterial wall matrix (11). The properties of enzymatically modified LDL, involving lipoprotein lipase, hepatic lipase, cholesterol esterase, cholesterol oxidase, phospholipases and lipoxygenases have been reviewed by Aviram (12).

Various forms of non-enzymatically modified LDL, such as acetyl LDL (1), acetoacetyl LDL (13) and malondialdehyde-conjugated LDL (14), have been described but the in vivo relevance of most these forms of LDL is not clear yet. However, acetyl LDL cannot be formed under in vivo conditions (1, 15). Glycosylation of the LDL apolipoprotein B-100 may also play an important role in cellular lipid accumulation, especially in hyperglycemic patients (16).

However, cell-induced oxidation of LDL has been demonstrated to occur in vivo and although the mechanism is not completely clear yet, the properties of this form have been extensively reviewed (1, 12, 17–25). OxLDL are recognized by the scavenger receptor of macrophages, currently considered as the main step in inducing foam cell formation. Modification of LDL results in an enhanced uptake by macrophages via the scavenger receptor which in contrast to the classical LDL receptor shows no regulation (1, 15, 26). The accumulation of LDL in the arterial intima is considered to be an important step in the formation of fatty streaks. Recently, it has been demonstrated that a specific binding of LDL to its receptor on macrophages is required in order to permit subsequent oxidation of the lipoprotein (27). However, oxLDL also bind to the scavenger receptor expressed by rabbit arterial smooth muscle cells (SMC) and macrophages. It seems that the receptor expression in SMCs is regulated as demonstrated by treatment with phorbol ester and platelet secretory products (28, 29).

The intact arterial wall reacts upon a great variety of stimuli with the release of a number of vasoactive mediators (30). Under physiological conditions, the inhibitory repellant substances such as endothelium-derived relaxing factor (EDRF/NO), PGI₂ and tPA among others are dominating to synergistically protect endothelial integrity (30–33). EDRF/NO and PGI₂ are potent inhibitors of smooth muscle contraction and proliferation, and they also inhibit platelet aggregation, stimulate platelet disaggregation, and inhibit platelet and monocyte adhesion to the endothelial surface (31, 32, 34). Several papers have shown that experimental atherosclerosis induced by
Fig. 1. Interactions between monocyte/macrophage (МΦ), oxLDL, EDRF/NO and PGI₂. Abbreviations: cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; i(c)COX: inducible (constitutive) cyclooxygenase; i(c)NOS: inducible (constitutive) nitric oxide synthase; LO: lipoxygenase; ROS: reactive oxygen species; ↓: inhibition

hypercholesterolemia is associated with increased vascular tone (35—39), mostly due to diminished release of EDRF/NO (40). There is evidence that the function of EDRF/NO is greatly impaired by LDL, most likely in its oxidized form. On the other hand, NO seems to influence the oxidation of LDL. Physiologically, the production of NO might act to scavenge superoxide anions released from monocytes/macrophages. As the oxidation of LDL is a process consuming time and on the other hand the life-span of NO is extremely short (41) the potential effects certainly are restricted to the site of production being dependent on presence of transition metals (42). In this review we are going to summarize some of the recent research emphasizing the influence of EDRF/NO, native and oxidized LDL and monocytes/macrophages on the pathogenesis of atherosclerosis (Fig. 1).
1. Influence of NO/EDRF on LDL-oxidation

NO has been demonstrated to exert a dual effect on LDL oxidation. In the presence of superoxide \( \text{O}_2^- \) NO is prooxidant. Like the reaction product peroxynitrite (OONO) \( (43,44) \) it has been shown to cause oxidation in pure lipid systems \( (44, 45) \) and in LDL \( (46) \) probably through the formation of hydroxyl radicals \( (43, 44, 46–48) \). In addition there is evidence that the superoxide radical is a poor initiator of lipid peroxidation unless in its protonated form, the perhydroxyl radical \( (45, 49) \). However, the efficiency of the decomposition reaction of peroxynitrite to form the hydroxyl radical under physiological conditions is a matter of debate but is possibly in the range of about 5—10\% \( (48) \).

NO alone seems to inhibit LDL oxidation \( (50) \). NO generation has been shown to exert a protective effect on macrophage-dependent \( (51, 52) \) and on endothelial cell-mediated oxidation of LDL \( (53) \). Using murine macrophages Jessup and Dean demonstrated that cell-mediated oxidation can be inhibited by stimulating nitric oxide synthesis in these cells \( (54) \). Darley-U斯mar et al. incubated LDL with NO-liberating substances like sodium nitroprusside (SNP), S-nitroso-n-acetylpenicillamine (SNAP) or a syndnonimine (SIN-1). The nitrovasodilators SNP and SNAP which produce NO but no superoxide had little or no ability to oxidize LDL while SIN-1 which liberates both NO and superoxide during autooxidation was capable of initiating lipid peroxidation in LDL. Superoxide dismutase (SOD) was shown to inhibit lipid peroxidation in these experiments, indicating that superoxide plays a key role in this process. Catalase on the other hand had no effect on the SIN-1-induced peroxidation of LDL, suggesting that hydrogen peroxide does not play a prominent role. The peroxyl radical scavenger BHT (butylated hydroxy toluene) but not the hydroxyl radical scavengers mannitol and ethanol were able to prevent the formation of lipid peroxides in LDL, suggesting that the peroxidation of LDL initiated by SIN-1 is propagated by the peroxyl radical \( (46) \).

For \textit{in vitro} studies LDL can be oxidized by incubation with cultured endothelial cells \( (55) \), macrophages \( (5) \) or copper-ions \( (56) \). The influence of NO/EDRF on LDL oxidation in such experiments seems to be dependent on the susceptibility to oxidation of the LDL from different donors which is expressed in the length of the lag phase during which the endogenous antioxidants are consumed and which can be shortened by the addition of higher concentrations of metal ions. The reason why in the study of Darley-U斯mar et al. only a minor influence of NO on copper-induced LDL oxidation could be observed, probably was that the copper concentration they used for oxidizing LDL was very high \( (200 \mu\text{M}) \). They also measured the degree of oxidation after 18 hours, at a time point when the oxidation status of the different LDL samples was already equalized \( (46) \). The influence of NO/EDRF and NO-donors such as nitrovasodilators on copper-mediated
oxidation of LDL has also been investigated by Hogg and colleagues (Tab. 1). They suggested that NO generated from the decomposition of SNAP inhibits LDL-oxidation by scavenging lipid peroxyl radicals (LOO-) and lipid radicals (L-) formed during lipid peroxidation rather than by chelation of copper ions or removal of lipid hydroperoxides (LOOH) within the LDL particle (50).

**Table 1:** Effect of NO-liberating substances on copper-induced LDL oxidation

<table>
<thead>
<tr>
<th>Oxidant</th>
<th>Inhibitor</th>
<th>LDL-oxidation</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIN-1 (1 mM)</td>
<td>—</td>
<td>no</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>yes</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>SOD (20 U/ml)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>boiled SOD (activity = 0,6/ml)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Catalase (500 U/ml)</td>
<td>—</td>
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<tr>
<td></td>
<td>Mannitol (100 mM)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>EtOH (174 mM)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>BHT (50 μM)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>SNP (2 mM)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>SNAP (1 mM)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>—</td>
<td>SNP (2 mM)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>—</td>
<td>SNAP (1 mM)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cu ++ (200 μM)</td>
<td>—</td>
<td>no</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>SNP (2 mM)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>SNP (1 mM)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cu ++ (10 μM)</td>
<td>SNP (100 μM) dark</td>
<td>slightly increased</td>
<td>46</td>
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<tr>
<td></td>
<td>SNP (100 μM) light</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>SNAP (0,1—2,5 mM)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>ABAP (1 mM)</td>
<td>SNAP (1 mM)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>SNP (100 μM)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>SNP (1—3 mM)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cu ++ (5 μM)</td>
<td>SNP (1—3 mM)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cu ++ 5 μM)+SIN-1 (1 mM)</td>
<td>—</td>
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</tr>
</tbody>
</table>

Abbreviations: ABAP (2,2-azo-bis-amidinopropane)

Incubating native LDL (nLDL) with increasing concentrations of SNP we could not observe formation of thiobarbituric acid reactive substances (TBARS). When we incubated nLDL with 5 μM copper, however, the formation of TBARS seemed to be attenuated by increasing concentrations of SNP (1—3 mM, Fig. 3). However, the maximal amount of TBARS tended to increase after the addition of SNP. The same effect was observed when using increasing concentrations of copper. In agreement with the results of Darley-Usmar et al. (46), SIN-1 was able to oxidize LDL. Coincubation of SIN-1 was able to oxidize LDL. Coincubation of SIN-1 and copper even further enhanced this effect (Fig. 2).
Fig. 2. Influence of SIN-1 on LDL-oxidation. LDL (0.2 μg/μl) were incubated with either different concentrations of SIN-1 alone or together with 5 μM copper at 37°C for indicated periods. Oxidation was stopped by adding 10 μM of BHT dissolved in EtOH. Evaluation of formation of thiobarbituric reactive substances (TBARS) was performed as described by Wallin et al. (144). Interpretation in the text. Data are presented as mean ±SD. Statistical analysis was performed using Students t-test. A p < 0.05 was considered as significant. * p < 0.05, ** p < 0.005, *** p < 0.0005, compared with the control.

Chang et al. demonstrated that the influence of NO on LDL-oxidation in the absence of superoxide is pH-dependent (57). Lowering the pH they observed an increased ability of NO and nitrite to oxidize LDL in aerobic solutions. SOD (10 to 30 U/ml) was ineffective in inhibiting LDL oxidation. Copper-dependent oxidation was shown to be accelerated by NO even at pH 7.4. Low pH-levels have been measured in the thickened intima (58). Under such conditions NO may act in the absence of superoxide to cause LDL oxidation. NO also seems to modulate oxLDL metabolism in macrophages. Using SNP and SIN-1 Wybranska et al. demonstrated that these compounds (30—300 μM and 300 μM, respectively) stimulated the accumulation and degradation of oxLDL by peritoneal rat macrophages (59). However, inhibition of endogenous NO formation by L-NMMA had the same effect. These authors conclude that NO at low physiological concentrations downregulates scavenger receptor expression of macrophages and therefore
Influence of SNP on LDL-oxidation

![Graph showing the influence of SNP on LDL-oxidation.](image)

Fig. 3. Influence of SNP on copper-induced LDL-oxidation. LDL (0.2 μg/μl) were incubated with indicated concentrations of SNP together with 5 μM copper at 37°C for indicated periods. Procedure as for figure 2. Interpretation in the text. Data are presented as mean ± SD. Statistical analysis was performed using Students t-test. A p < 0.05 was considered as significant. * p < 0.05, ** p < 0.005, compared with the control.

inhibits oxLDL uptake by these cells. Higher NO concentrations may lead to toxic effects as expressed by an increased uptake of oxLDL by the cells.

Haem-containing proteins like myoglobin and haemoglobin are known to exist in several oxidation states and in the presence of hydrogen peroxide (H₂O₂) can develop into radicals which are able to oxidize cell membranes (60). It is well known that NO reacts very strongly with haem-containing proteins e.g. with the haem moiety of guanylate cyclase (61). Oxidation of erythrocyte membranes by myoglobin radicals was shown to be inhibited by the addition of NO after mixing metmyoglobin and H₂O₂ with the membranes (62). NO also inhibited the oxidation of LDL in the presence of myoglobin and H₂O₂. However, in the presence of excess hydrogen peroxide, the action of NO is fundamentally different and appears to enhance the oxidation of LDL. The mechanism for this latter effect is uncertain and requires further studies (63).
2. Influence of nLDL and oxLDL on NO/EDRF

There is a constant basal release of NO/EDRF from the endothelium under the stimulus of pulsatile flow. Impairment of endothelium-dependent relaxation occurs in experimental atherosclerosis (64) even if the endothelium is still in place. In human coronary arteries impairment of relaxation occurs in areas of endothelial dysfunction or atherosclerosis (65). This could also be related to increased plasma cholesterol concentrations (66). An increasing body of evidence has been accumulated suggesting a role for LDL in mediating at least some of the altered vasomotor properties known to accompany atherosclerotic vascular disease. A characteristic property for the atherosclerotic coronary artery appears to be a reduced responsiveness to NO/EDRF-mediated vasodilatation (35).

Impairment of NO/EDRF release occurs extremely early during atherogenesis. Verbeuren et al. showed that the release of NO/EDRF via the constitutive enzyme was decreased during fatty streak formation (64). However, cholesterol enrichment of arterial smooth muscle cells upregulates cytokine-induced nitric oxide synthesis (67). Native LDL has been claimed to inhibit endothelium dependent relaxation by a rapid and reversible mechanism that may result from a direct interaction between LDL and NO/EDRF (68, 69). Using a bioassay system, Galle et al. demonstrated that LDL directly inactivates NO/EDRF (70). These authors proposed that NO/EDRF may be sequestered and inactivated within the hydrophobic core of the lipoprotein molecule. Conversely, several studies have failed to demonstrate any effect of native LDL on endothelium-derived relaxations (41, 71, 72). Native LDL has also been reported to cause irreversible inhibition of endothelium-dependent relaxation (68, 71), but this may result from the oxidation of the LDL which is likely to occur in the highly oxygenated environment used in these studies (68).

Most studies have demonstrated that oxLDL, but not native LDL, is able to inhibit NO/EDRF (41, 68, 71—73). Copper-oxidized LDL, for example, inhibits endothelium-dependent relaxations mediated by nitric oxide in the rabbit aorta (68) and in pig coronary arteries (41). The underlying mechanisms for the inhibition of NO/EDRF-mediated vasodilatation by oxDL remain controversial. However, the inhibitory effect of oxLDL on endothelium-dependent relaxation is associated with the lipid fraction of the molecule. Lysolecithin (lysophosphatidylcholine), which is converted from lecithin (phosphatidylcholine) during the oxidative modification of LDL, was shown to be active compound, whereas oxidized free fatty acids, sphingomyelin, and other phospholipids had no effect (71, 72). The effects of oxLDL could even be mimicked by synthetic lysolecithin (palmitoyl). Evidence has been presented that this effect is due to lysolecithin-mediated alteration of endothelial cell membranes resulting in disruption of receptors specific for vasodilatory agonists (71). Others have suggested other mechanisms, such as
direct inactivation of ON/EDRF after its release from the endothelial cells (70) or a direct action on the vascular smooth muscle cells themselves, maybe elicited by a decrease in cyclic nucleotides (74, 71), or an increase in intracellular free calcium concentrations (75).

It has been shown that at low concentrations (< 50 µg/ml) oxLDL inhibited endothelium-dependent relaxation evoked by receptor-dependent (acetylcholine) but not receptor-independent (A23187) stimuli (71). At higher concentrations (> 50 µg/ml), however, oxLDL caused non-specific inhibition of endothelium-dependent relaxation. However, Plane et al. demonstrated a different inhibitory potency on endothelium-dependent relaxation of preparations of oxLDL with comparable contents of lysophosphatidylcholine (LPC) from different donors, suggesting that LPC is not the only active compound in oxidized LDL (76). The suggested mechanism for the inhibitory effect is an interaction of oxLDL with endothelial cells to reduce NO/EDRF (33, 71). There is also evidence that oxLDL could inhibit endothelial relaxation by inhibiting the pertussis toxin-sensitive Gi protein pathway for cellular signalling (77, 78).

Jorens et al. demonstrated a diminished inducible NO synthase activity by loasing J774-macrophages with oxidatively modified LDL but not with acetylated LDL (79). Their results show that lipid loaded J774-macrophages were still capable of expressing the inducible NO synthase, but that oxLDL significantly decreased the output of NO metabolites. Both acetylated and oxidized LDL led to a similar accumulation of cholesteryl esters, and citrulline biosynthesis decreased in a comparable way, thus supporting the assumption that NO synthase activity was inhibited in cells loaded with oxLDL.

A decreased availability of L-arginine or enzymatic cofactors has also been proposed to explain the inhibitory influence of lipoproteins on endothelium-dependent relaxation (77). The inactivation of NO by native and oxLDL (70) might account for the antagonism for the stimulatory effects of NO on purified soluble guanylate cyclase by oxLDL (80). While oxLDL might interfere with the inducible NO synthase activity via the suppression of TNF-alpha mRNA (81) the activity of the constitutive enzyme seems to be unaffected (70, 80). Flavahan suggested that oxLDL may even activate the inducible NO synthase that could convert arginine to NO (33). However, Yang et al. demonstrated that lipids extracted from oxLDL but not from native LDL inhibited the inducible nitric oxide synthase in macrophages activated by lipopolysaccharide or gamma interferon (82).

3. Oxidation of LDL by Monocytes/Macrophages

Using cultured cell systems, it has been shown that endothelial cells (55), monocytes (7, 83, 84), macrophages (5, 85), lymphocytes (6), platelets (8, 9) and
smooth muscle cells (4) are capable of modifying LDL into their oxidized form. In this review we will focus on the action of monocytes/macrophages on LDL and the resulting increased uptake of oxLDL by these cells.

The precise mechanisms of cell-mediated oxidation of LDL are still subject to speculation. It is possible that the removal of endogenous lipoprotein antioxidants, the introduction of proxides into LDL by direct action of enzymes or by transfer of peroxidized lipids from cell membranes or the direct action of cell-derived oxidants on the lipoprotein contribute to the oxidative modification of LDL. Trace amounts of redox-active metals are essential and metal catalysed oxidation may also play an important role (55). Macrophages accumulate in the atherosclerotic region and are known to release both superoxide anions and NO when activated. NO may limit the effects of macrophage-induced oxidation of LDL (54, 63). The macrophage may contribute to the development of the lesion, but would also seem to provide an essential protective function in scavenging oxLDL which is known to be toxic to other cells in the arterial wall (86). It has been shown in mouse peritoneal macrophages that oxLDL is capable of inducing apoptosis (87), which might explain the source of the necrotic base of advanced atherosclerotic plaques. The most cytotoxic compounds in oxLDL when tested on porcine aortic smooth muscle cells were found in the lipid fraction and analysed as 7-ketocholesterol and 7-hydroxycholesterol (88). However, there is evidence for a protective mechanism in these cells. Human monocytes and macrophages show elevated glutathione levels after exposure to oxLDL. Gotoh et al. demonstrated that this is due to oxLDL-stimulated de-novo synthesis of glutathione in these cells. Inhibition of glutathione synthesis renders oxLDL cytotoxic at concentrations well tolerated by untreated cells (89). These results suggest that the glutathione status of macrophages in the arterial wall could be important for the detoxification of oxLDL and the development of atherosclerotic lesions.

Lesion derived LDL and oxLDL share a lability for aggregation leading to enhanced macrophage degradation (90). Monoclonal antibodies against LDL further enhance macrophage uptake of LDL aggregates (91). Since oxLDL is highly immunogenic, it could form immune complexes being subsequently taken up by macrophages through the Fc-receptor (1, 92). After the recruitment of monocytes oxLDL could stimulate the release of M-CSF (93) thus affecting growth and maturation of macrophages and subsequent release of other cytokines and growth factors. At least in fibroblasts components of oxidized lipid can also stimulate collagen gene expression (94). It has been demonstrated in isolated peritoneal macrophages that oxLDL are able to repress the LPS-stimulated expression of mRNA for TNF-α and IL-1 (81). However, in human peripheral blood mononuclear cells oxLDL seem to induce the release of interleukin-1-beta. Constituents of oxLDL responsible for this effect were shown to be 9-hydroxyoctadecadienoic acid (9-HODE) and alkenals
(2,4-decadienal, 2-octenal) (95). These results suggest that the biochemical response of the macrophage to oxLDL may be important in determining the role of the foam cell in the progression of the lesion (96). Moreover, oxLDL have been shown to be responsible for the induction of a variety of gene products. Among others, oxLDL significantly enhanced tissue factor expression in adherent monocytes inducted by bacterial lipopolysaccharide (LPS). In contrast, they did not alter LPS-mediated production of interleukin-8 (IL-8) and inhibited LPS-induced secretion of tumor necrosis factor-alpha (TNF-α) (97). IL-1 alpha, IL-1 beta and IL-6 were shown to be decreased by oxLDL in LPS-stimulated mouse macrophages (98). However, IL-1 beta release from human monocytes seems to be promoted by oxLDL (99, 100). A regulation of tissue factor expression by minimally modified LDL (MM-LDL) and LPS has also been reported in human endothelial cells further confirming the influence of oxLDL at the level of gene transcription (101, 102).

Polymorphonuclear leucocytes (PMN) and monocytes have been identified in the early lesions of atherosclerosis in animals and in man (103). LDL have been described as activators of monocyte oxidative metabolism and lysosomal enzyme release (104, 105). PMNs from hyperlipoproteinemic subjects show increased oxidative metabolism (106). LDL per se stimulated PMN O₂ production and decreased PMN chemotaxis (107).

3a. Role of Lipoxygenases

Lipoxygenases are cellular enzymes that introduce peroxide groups into unsaturated fatty acids and have been considered as potential agents for direct or indirect introduction of lipid peroxides into LDL (108, 109). 5-lipoxygenase catalyzes the oxygenation of free arachidonic acid, the first step in the biosynthesis of leukotrienes, known as potent inflammatory mediators. 12- and 15-lipoxygenases are involved in the biosynthesis of other bioactive metabolites but their physiological roles are less clear. The presence of 15-lipoxygenase mRNA and protein in macrophage-rich areas of atherosclerotic lesions in rabbits (110) and man (111) has been reported. It has furthermore been demonstrated that it colocalized to areas staining positively for oxLDL epitopes. O'Leary et al. demonstrated that 5- and 15-lipoxygenase-derived peroxides may promote the peroxidation of LDL in the presence of a transition metal catalyst (e.g. copper) (112).

It has been shown that 15-lipoxygenase is able to oxidize LDL sufficiently to cause high cellular uptake of LDL in vitro (108, 109, 113—116). However, it is highly unlikely that cellular lipoxygenases, which are cytosolic enzymes, do have access to LDL, either in the extracellular space or in an endocytotic
vesicle, under normal circumstances in vivo (117). An alternative possibility suggested is that fatty acid peroxides generated via the action of lipoxygenases on cellular lipids are released from the cells, either as peroxyl radicals or as hydroperoxides, and relocate in an adjacent LDL particle where they can act as substrates for redox-active metal-catalysed decomposition in the propagation phase of LDL oxidation (114, 118). It seems very likely that 15-lipoxygenase does not contribute to the oxidative modification of LDL by acting directly on LDL but via the production of intracellular hydroperoxy lipids. However, whether these products are able to initiate lipid peroxidation in LDL or if they only contribute to the propagation of this process is still matter of debate.

On the other hand it has been reported that lipoxygenases are not involved in cell-mediated oxidation of LDL (119—121). However, there are problems in determining the role of lipoxygenases in cellular systems using inhibitors. For example, many lipoxygenase inhibitors are antioxidants as well, and, when added to culture media together with LDL, can protect the lipoprotein against both cell-mediated and cell-free oxidation (122).

3b. Role of free radicals

Reactive oxygen species (ROS) produced by monocytes/macrophages are likely to be involved in LDL modification (7, 84, 123). Even monocytes obtained from hypercholesterolemic patients with no clinical signs of vascular disease are showing an increased capacity of ROS generation if compared to cells obtained from normolipemic individuals (124). It has also been shown by different groups that endothelial (125—127) and arterial smooth muscle cells (4) release superoxide anions, which may initiate or accelerate the oxidative modification of LDL. Under physiological conditions, the level of superoxide may be low due to its interaction with NO/EDRF (128, 129). However, it was suggested by Flavahan that inactivation of NO/EDRF by high concentrations of LDL will decrease the superoxide scavenging effect of NO/EDRF. This in turn might enable superoxide to initiate oxidation of LDL (33).

Wilkins and Leake tested the influence of free-radical scavengers and inhibitors of free-radical-generating enzymes on LDL oxidation by mouse resident peritoneal macrophages in culture. The modification of LDL was quantified by degradation by a second set of macrophages for 24 hours (121, 130). When they tested esculetin, a lipoxygenase/cyclooxygenase inhibitor, there was no significant inhibitory effect at concentrations up to 10 μM. At 100 μM esculetin completely inhibited macrophage modification of LDL, but was obviously toxic to the cells. In addition, the general antioxidant activity of
this compound probably was at least in part responsible for the inhibitory effect. Eicosatetraynoic acid (ETYA) exerted no effect at a concentration of 2 $\mu$M, but inhibited LDL-modification at concentrations of 20 and 200 $\mu$M. However, ETYA is toxic to cells in these concentrations. Arachidic acid, the saturated analogue of ETYA, which is not toxic to cells did not show any inhibitory effect on LDL modification at comparable concentrations. Oxypurinol (up to 450 $\mu$M), a xanthine oxidase inhibitor only caused little inhibition and the NADPH-oxidase inhibitors wortmannin and thiopropionic acid dilauryl ester (TPDE) had no effect on macrophage modification of LDL over a concentration range of 1–1000 nM and 0,1–100 $\mu$M, respectively. Diphenylene iodonium and diphenyl iodonium, both of which are potent NADPH-oxidase inhibitors, showed a dose-dependent inhibitory effect on LDL-oxidation by macrophages at concentrations of 0,1 $\mu$M and 1–100 $\mu$M, respectively. Both compounds did not significantly affect copper-induced LDL-oxidation, demonstrating that possible general antioxidant properties of these compounds were not responsible for the inhibitory effect. However, the iodoniums also appeared to be toxic to the cells. The results of this study are summarized in Table 2.

Table 2. Influence of free-radical scavengers and inhibitors of free-radical-generating enzymes on LDL-oxidation

<table>
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<th>Tested compound</th>
<th>Concentration</th>
<th>Inhibition</th>
<th>Remarks</th>
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<tr>
<td>Free radical scavengers:</td>
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<td></td>
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<tr>
<td>Superoxide dismutase (SOD)</td>
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<td>no</td>
<td>no consistent inhibition</td>
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<td>Catalase</td>
<td>25–100 $\mu$g/ml</td>
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<td>hydrogen- peroxide scavenger</td>
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<td>1–100 mM</td>
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<td>hydroxyl- radical scavenger</td>
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<td>Diazabicyclooctane</td>
<td>10 mM</td>
<td>no</td>
<td>singlet oxygen scavenger</td>
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<tr>
<td>Lipoxygenase inhibitors:</td>
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</tr>
<tr>
<td>5,8,11,14-eicosatetraynoic acid (ETYA)</td>
<td>1 $\mu$M</td>
<td>no</td>
<td>compound is toxic to macrophages</td>
</tr>
<tr>
<td></td>
<td>20 $\mu$M</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>yes (100%)</td>
<td></td>
</tr>
<tr>
<td>Esculetin</td>
<td>10 $\mu$M</td>
<td>no</td>
<td>in non-toxic concentrations</td>
</tr>
<tr>
<td></td>
<td>100 $\mu$M</td>
<td>yes</td>
<td>compound is toxic; general antioxidant activity</td>
</tr>
<tr>
<td>Xanthine oxidase inhibitor:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxypurinol</td>
<td>40–450 $\mu$M</td>
<td>no</td>
<td></td>
</tr>
<tr>
<td>NADPH-oxidase inhibitors:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wortmannin</td>
<td>1–1000 nM</td>
<td>no</td>
<td>toxic in higher concentrations</td>
</tr>
<tr>
<td>TPDE</td>
<td>0,1–100 $\mu$M</td>
<td>no</td>
<td>toxic in higher concentrations</td>
</tr>
<tr>
<td>Diphenyl iodonium</td>
<td>1–100 $\mu$M</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>Diphenylene iodonium</td>
<td>0,1–10 $\mu$M</td>
<td>yes</td>
<td></td>
</tr>
</tbody>
</table>
NADPH-oxidase-derived superoxide anions have been shown to play a role in LDL-modification by activated monocytes (84, 123). A role for superoxide anions generated by NADPH-oxidase in LDL oxidation by macrophages was suggested by Wilkins and Leake (121). Why superoxide dismutase (SOD) does not inhibit LDL oxidation by macrophages in this system, however, remains unclear. It is possible that superoxide anions exert their influence on LDL oxidation in such a manner that SOD does not have access to them, suggesting a reaction with different molecules, maybe with nitric oxide to form the hydroxyl radical. However, there are conflicting results concerning the role of SOD as an inhibitor of cell-derived superoxide in the oxidative modification of LDL. Various groups were measuring different degrees of inhibition (84, 109, 127) or no effect (3, 121) of SOD. Jessup et al. claim that SOD should not be used when metal-dependent oxidation is involved because of its metal chelating properties (131). Since both cell-mediated and cell-free systems used for LDL oxidation are metal-dependent, any activity of SOD in such systems may be attributed to a metal chelating effect. In the same study Jessup et al. stimulated NADPH-oxidase in macrophages by phorbol myristate acetate (PMA) and opsonized zymosan to increase extracellular superoxide generation and incubated LDL with these stimulated cells to generate high-uptake LDL. However, neither PMA nor zymosan caused any detectable increase in the rate at which high-uptake LDL was formed. This implies that extracellular superoxide radical may not be required during macrophage mediated oxidation of LDL. Although they point out that the cells used in this study did not express active nitric oxide synthase (51), stimulation of NO synthesis could occur after incubation with oxLDL (33). Thereby, the possibility that superoxide was consumed by the reaction with NO can not be excluded.

4. Monocyte-endothelium interactions

OxLDL, but not native LDL, are potent chemoattractants for circulating monocytes, but not for neutrophils (132). OxLDL is at the same time a potent inhibitor of the motility of the resident macrophage (133). Both activities were shown to reside in the lipid component, especially in lyssolecithin. Even minimally modified LDL (MM-LDL), which are not recognized by the scavenger receptor, were shown to enhance monocyte endothelial interactions (134). MM-LDL were also demonstrated to induce monocyte chemoattractic protein 1 (MCP-1) in human endothelial cells and smooth muscle cells (135). On the other hand, NO and PGI2 are able to inhibit monocyte (and platelet) adhesion to the endothelial surface (32, 136). LDL was also shown to induce rapid activation of monocyte adhesiveness to endothelial cells. This effect
appeared to be mediated by interaction of LDL with its receptor rather than LDL-receptor complex internalization or integrin membrane mobilization from intracellular pools. The integrin system nevertheless seems to be involved (137). It has also been reported that oxLDL can stimulate platelet aggregation (138) and promote procoagulant activity on the surface of human monocyte/macrophages by an increase in tissue thromboplastin activity (139) or by stimulating the expression and secretion of tissue factor by monocytes or aortic endothelial cells (97, 101, 102). The recent observation that antioxidants like alpha-tocopherol and probucol could inhibit agonist (IL-1, LPS, thrombin, PMA)-induced monocyte adhesion to cultured human endothelial cells (140) points to a novel alternative antiatherosclerotic mechanism of these compounds. The inhibition seems to correlate with a decrease of E-selectin mRNA levels. When monocyte adhesion to endothelial cells was induced by treatment with MM-LDL, it could be blocked by inhibitors of glycoprotein synthesis (cycloheximide, tunicamycin) and low levels of trypsin. However, no increase in E-selectin levels, vascular cell adhesion molecule-1 (VCAM-1) or intercellular adhesion molecule-1 (ICAM-1) could be detected after MM-LDL treatment. There is also evidence that MCP-1 and fibronectin were not responsible for monocyte binding. However, several sugars (lactose-1-phosphate, maltose-1-phosphate, N-acetylglucosamine) were able to inhibit monocyte binding to endothelial cells, indicating that carbohydrate chains are involved (141).

DISCUSSION

Mounting evidence suggests that the oxidative modification of LDL within the arterial wall is influenced among many other factors by EDRF/NO. However, the first studies about the influence of EDRF/NO on LDL-oxidation showed contradictory results. Indeed, it seems very likely that NO can exert a dual effect on LDL-oxidation, dependent on the microbiological environment. NO alone seems to inhibit oxidation of LDL, but in the presence of superoxide anion it shows strong prooxidant activity.

A paradoxical effect of NO has been shown on the oxidation of LDL but also on the nervous system. In the nervous system NO seems to have both neurotoxic and neuroprotective effects. Lipton et al. showed that the effect was dependent on the redox state of NO. Neurotoxicity occurs when NO is present in the reduced form (as NO⁻), the effect being mediated, at least in part, by peroxynitrite (ONOO⁻), formed by reaction with superoxide anion (O₂⁻). Neuroprotection occurred when NO in the oxidised form (NO⁺, or nitrosonium ion) reacted with the thiol group of the N-methyl-D-aspartate (NMDA) receptor to down-regulate neurotransmission. Drugs that manipulate the
The mechanisms by which LDL are oxidized by cells in vitro may vary in different cell types. The generation of superoxide anion appears to be the predominant pathway by which LDL is modified in vitro by smooth muscle cells, whereas 15-lipoxygenase appears to be involved in LDL modification by endothelial cells and macrophages. It is noteworthy that human monocytes and human tissue macrophages do not express 15-lipoxygenase, implying a lesion specific expression of this enzyme in foam cells (Fig. 4).
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