Oxidized Low Density Lipoproteins Stimulate Phosphoinositide Turnover in Cultured Vascular Smooth Muscle Cells

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Atherogenesis is associated with alterations in the properties of different cell types, including monocytes/macrophages (foam cell formation), platelets (increased aggregation), endothelial cells (injury), and smooth muscle cells (SMCs) (lipid accumulation or foam cell formation). Oxidized low density lipoproteins (ox-LDL) play a key role in this vascular pathology. This study investigated the ability of ox-LDL to elicit chemical signaling events in cultured human vascular smooth muscle cells (VSMCs). Ox-LDL was found to stimulate phospholipase C-mediated phosphoinositide turnover in human VSMCs. This response occurred rapidly (within 1 minute) and at low concentrations of ox-LDL (half-maximal effective concentration, \(\approx 5 \mu g/ml\)). Ox-LDL-stimulated inositol phosphate accumulation in human VSMCs was inhibited by pretreatment of cells with phorbol 12-myristate 13-acetate and with compounds that elevate cyclic AMP or cyclic GMP. Ca\(^{2+}\) antagonists also blocked the effects of ox-LDL on phosphoinositide turnover. Inhibitors of receptor-endocytotic processes (including receptor clustering, cross-linking, and cytoskeleton-dependent internalization) effectively prevented ox-LDL-induced inositol phosphate generation. The data suggest that ox-LDL promotes phospholipase C-mediated phosphoinositide turnover in a manner analogous to that for other Ca\(^{2+}\)-mobilizing hormones. The results also support an association between phosphoinositide turnover and receptor-mediated endocytosis. Prevention of the direct effects of ox-LDL on SMCs could prove an interesting therapeutic avenue for the prevention of atherosclerosis. (Arteriosclerosis and Thrombosis 1992;12:278-285)

KEY WORDS • second messengers • atherosclerosis • inhibitors • phospholipase C • receptor endocytosis

Low density lipoproteins (LDLs) are a well-known atherogenic risk factor, and they accumulate in the vessel wall during hypercholesterolemia.\(^1\)\(^-\)\(^3\) Oxidation of LDL (ox-LDL) enhances its atherogenicity, and inhibition of such oxidation decreases the rate of progression of atherosclerotic lesions.\(^1\)\(^-\)\(^4\)\(^-\)\(^6\) LDL is oxidatively modified when incubated in vitro with three major cellular constituents of the vascular wall: endothelial cells, vascular smooth muscle cells (VSMCs), and macrophages.\(^7\)\(^-\)\(^9\) Oxidation of LDL also occurs in vivo,\(^10\) and ox-LDL has been detected in atherosclerotic lesions in humans and rabbits.\(^11\) The uptake of modified lipoproteins such as ox-LDL occurs via the “scavenger receptor” pathway, and expression of scavenger receptors has been demonstrated on macrophages,\(^9\) endothelial cells,\(^12\) fibroblasts,\(^13\) and smooth muscle cells (SMCs).\(^13\) Unlike the LDL receptor, expression of the scavenger receptor is not downregulated by an increase in intracellular cholesterol.\(^14\) Therefore, uptake of modified lipoproteins including ox-LDL contributes to the accumulation of cholesteryl esters in foam cells of atherosclerotic lesions.\(^12\)\(^-\)\(^15\) Both SMCs and macrophages are major precursors of the arterial foam cells found in the early stages of the development of atherosclerotic lesions.\(^14\)\(^-\)\(^16\)\(^-\)\(^17\)

Recently, increasing attention has been focused on the contribution of ox-LDL to abnormal vascular reactivity during hypercholesterolemia and atherosclerosis. Endothelium-dependent vasoconstrictive effects of ox-LDL have been demonstrated in pig coronary\(^18\) and rabbit femoral\(^19\) arteries. Ox-LDL has also been shown to inhibit endothelium-derived relaxations to serotonin\(^18\) and acetylcholine,\(^20\) an effect partially attributed to suppressed release of endothelium-derived relaxing factor.\(^18\) However, there is also evidence for direct effects of ox-LDL on vascular smooth muscle, independent of its effects on the endothelium. In rabbit femoral artery segments, ox-LDL potentiates vasoconstrictions to threshold concentrations of various contractile agonists, and the degree of potentiation is significantly greater in endothelium-denuded segments than in endothelium-intact segments.\(^19\) Inhibition of such ox-LDL-elicited vasoconstrictions by Ca\(^{2+}\) antagonists suggests that ox-LDL induces increased transmembrane Ca\(^{2+}\) influx.\(^19\) Additionally, ox-LDL has been demonstrated to inhibit endothelium-independent relaxations to nitric oxide and nitroglycerin.\(^21\) This direct effect of ox-LDL on vascular smooth muscle may involve a direct...
interaction of ox-LDL with soluble guanylate cyclase, which, at least in guanylate cyclase preparations from platelets, inhibits activation of the enzyme by nitric oxide–containing compounds.22

Lipoproteins are normally transported through the intact endothelium of blood vessels by the process of diacytosis and thereby come into direct contact with VSMCs present in the media.22 Furthermore, in atherosclerotic lesions medial SMCs that have migrated through the internal elastic lamina and have undergone proliferation to form a neointima are in direct contact with plasma lipoproteins.24,25 Given the possibility for direct exposure of VSMCs to lipoproteins under both physiological and pathophysiological conditions, together with the existence of scavenger receptors on these cells and the effects of ox-LDL on vascular smooth muscle contraction and lipid metabolism, a study of the molecular mechanisms of action of ox-LDL in VSMCs seems pertinent. The present study demonstrates that ox-LDL stimulates phosphoinositide turnover in cultured human VSMCs. This effect of ox-LDL could be blocked by a variety of compounds, including Ca2+ antagonists, inhibitors of endocytosis, and some second messengers or their analogues. These findings provide important insights for the possible pharmacological regulation of ox-LDL action in the vessel wall.

Methods

Cell Culture and Measurement of Phosphoinositide Catabolism

The isolation, phenotypic characterization, and propagation of VSMCs were performed as described previously;26 the tissue of origin consisted of microarterioles associated with omental fat. Human VSMCs were immunocytochemically characterized in second passage by checking for the presence of α-smooth muscle actin (isolates were >95% positive) and the absence of factor VIII antigen (undetectable in human VSMC isolates but present in bovine endothelial cell culture controls). For the experiments described herein, human VSMC isolates between passages 7 and 11 were used. Human VSMCs were seeded into 24-well Nalgene multiwell plates (1.2×10^4 cells/cm^2) and grown to confluence (2 days, 2×10^5 cells/cm^2). Thereafter, human VSMCs were rendered quiescent by serum deprivation and maintenance in serum-free medium containing 0.1% (wt/vol) bovine serum albumin for 48 hours and with inclusion of 2.5 μCi/ml of [2-3H]inositol to prelabel the inositol phospholipids.26 Before experimentation, the radioactive-containing medium was aspirated; cells were washed with two 1.0-m1 aliquots of phosphate-buffered saline and 0.5 ml minimal essential medium containing 0.1% (wt/vol) bovine serum albumin, 25 mM N-Tris(hydroxy-methyl)methyl-2-aminophenol, and 25 mM N-2-hydroxyethylperazine-N’-2-ethanesulfonic acid (both at pH 7.3), and 15 mM LiCl was added to each well. Unless otherwise stated, dishes were incubated for 30 minutes at room temperature and then transferred to a water bath at 37°C. Experimental protocols were initiated after 5 minutes. Human VSMCs were exposed either to vehicle (minimal essential medium containing 0.1% [wt/vol] bovine serum albumin) or to ox-LDL at selected concentrations and for time intervals indicated in the figure legends. Incubations were terminated by addition of 1.0 ml 5 mM EDTA/1% sodium dodecyl sulfate.27 Lysates were applied to columns containing 0.5 g Dowex-AG 1-X8 (formate form), and inositol phosphates were resolved as described previously.27 Elution buffers (in 7-m1 aliquots) for inositol mono-, bis-, and trisphosphates were, respectively, 0.2 M ammonium formate/0.1 M formic acid, 0.4 M ammonium formate/0.1 M formic acid, and 1.0 M ammonium formate/0.1 M formic acid.27 Radioactivity of inositol phosphates in eluates was quantified by liquid scintillation spectrophotometry. All experimental procedures were performed in triplicate within a single experiment and on at least three separate occasions. Unless otherwise stated, data in the text and figures are given as mean±SD. Where appropriate, Student’s t test was applied for significance determinations.

Isolation and Modification of Low Density Lipoprotein

LDL was isolated from human plasma collected in EDTA (1 mM) and butylated hydroxytoluene (BHT, 10 μM), using sequential ultracentrifugation with density adjustments by addition of potassium bromide.28 The LDL fraction (density, 1.019–1.063 g/ml) was dialyzed against isotonic phosphate buffer (pH 7.4) containing 1 mM EDTA and 1 μM BHT and then sterilized by filtration (0.45-μm pore size). LDL samples were stored at 4°C in the dark and used within 2 weeks. Before oxidation, LDL was dialyzed against isotonic phosphate buffer (pH 7.4) to remove EDTA and BHT. LDL (200 μg/ml) was oxidized by exposure to 5 μM CuCl₂ for 24 hours at 37°C.8 The copper-oxidized LDL, which has previously been shown to be indistinguishable from LDL that was biologically oxidized by endothelial cells in culture,8 was dialyzed for 24 hours against three changes of phosphate-buffered saline. The extent of lipid peroxidation was estimated as thiobarbituric acid-reactive substances.8,29 Tetramethoxypropane was used as a standard, and results are expressed as nanomoles of malondialdehyde equivalents per milliliter of LDL suspension. In the present series of experiments, the degree of oxidation corresponded to 9.0±1.9 nmol malondialdehyde equivalents/ml (n=5). Methylated LDL (methyl-LDL) was prepared using the protocol described by Basu et al.30 Ox-LDL and methyl-LDL preparations were sterilized by filtration (0.45-μm pore size), stored at 4°C in the dark, and used within 2 weeks. Protein content was measured by the method of Lowry et al.31 using bovine serum albumin as a standard. Lipid extractions of LDL and ox-LDL were performed according to the chloroform/methanol extraction method of Folch et al.32 The chloroform phase was evaporated under nitrogen, and the lipids were dissolved in ethanol. Appropriate amounts were added to minimal essential medium containing 0.1% (wt/vol) bovine serum albumin to correspond to the original concentrations of intact LDL and ox-LDL.

Materials and Drugs

All materials and media for tissue culture were obtained from Gibco AG (Basel, Switzerland) except for fetal calf serum, which was purchased from Fakola...
**Results**

**Stimulation of Phosphoinositide Turnover by Oxidized Low Density Lipoprotein**

Exposure of confluent, quiescent human VSMCs to ox-LDL resulted in a time-dependent (Figure 1A) and dose-dependent (Figure 1B) accumulation of inositol phosphates. Increases in inositol mono-, bis-, and trisphosphates were rapid ($p<0.01$ above control values within 1 minute). Prolonged exposure (10–30 minutes) resulted in a gradual decline of stimulated increases in inositol bis- and trisphosphates, whereas the increase of $^3$H content in inositol monophosphate (in the presence of 15 mM LiCl) was sustained (data not shown). This effect of ox-LDL was elicited at low concentrations of the lipoprotein (Figure 1B), and the half-maximal concentration of ox-LDL for generation of $[^3H]$inositol phosphates was calculated to be $4.8\pm2.2$ $\mu$g/ml. The present study used five preparations of ox-LDL, each of which was derived from a different batch of LDL. These ox-LDL preparations were essentially identical with respect to efficacy (individual dose profiles not shown). Methyl-LDL (5–100 $\mu$g/ml), which does not bind either to LDL receptors or to scavenger receptors, was found incapable of stimulating phosphoinositide catabolism in either human VSMC preparation (data not shown).

In a separate series of experiments, human VSMCs were exposed in parallel to ox-LDL and the parent LDL fraction. We consistently observed that the $[^3H]$inositol monophosphate production response of human VSMCs to ox-LDL was significantly greater than that in LDL-treated human VSMCs (Figure 2). However, the half-maximal concentrations of LDL (4.4±2.4 $\mu$g/ml) and ox-LDL (4.6±2.1 $\mu$g/ml) for generation of $[^3H]$inositol monophosphate were not significantly different (Figure 2). Similar findings were also made with respect to ox-LDL- and LDL-stimulated generation of $[^3H]$inositol bis- and trisphosphates (profiles not shown). In
were found to have no significant effect on basal levels with chloroform/methanol and added back in ethanol as we investigated the effects of lipid extracts (extracted in either human VSMC preparation (data not shown). The blocking effects of both PMA and forskolin occurred rapidly (>50% inhibition within 10 minutes of preincubation) for various times before addition of vehicle or ox-LDL (50 μg/ml) and further incubation for 10 minutes. After cell lysis and application/elution on ion-exchange columns, radioactivity in the fraction containing inositol monophosphate was determined. Results (mean±SD, n=3) express the increases in [%] inositol trisphosphate relative to those levels (100%) in samples from human VSMCs with or without (○) prior exposure to PMA or forskolin. In panel B, prelabeled human VSMCs were exposed to ox-LDL (25 μg/ml for 5 minutes) after prior incubation without or with PMA (10^{-7} M for 30 minutes); forskolin (10^{-8} M for 30 minutes); NaF (10^{-2} M for 30 minutes); 8-Br-cGMP (10^{-4} M for 60 minutes); ANP (10^{-7} M for 60 minutes); or verapamil, diltiazem, or nifedipine (10^{-3} M each for 10 minutes). [%] content in inositol trisphosphate was determined, and results (mean±SD, n=3) express the change in radioactivity as a percentage of [%] inositol trisphosphate in respective samples (100%) not exposed to ox-LDL. Experimental procedures are given in “Methods.” PMA, phorbol 12-myristate 13-acetate; 8-Br-cGMP, 8-bromo-cyclic GMP; ANP, atrial natriuretic peptide.

From such control experiments, we can exclude the possibility that the stimulatory effects of ox-LDL on [%]inositol phosphate production (Figure 1) are not due to the oxidized lipid components per se.

Lipid A, the active core of lipopolysaccharide from gram-negative bacterial cell walls, has been shown to stimulate diacylglycerol synthesis in a variety of cell types, including mesangial cells. This effect of lipid A does not involve the phosphoinositide-specific phospholipase C cycle. Nevertheless, as lipopolysaccharide is a common contaminant in LDL preparations, we considered it pertinent to exclude the possibility that the observed effects of LDL on phosphoinositide metabolism in human VSMCs might be due to some effect of lipopolysaccharide. Exposure of rat aortic VSMCs and mesangial cells (at quiescence and mono-[2-^[3]H]inositol-prelabeled) to 100 ng/ml lipopolysaccharide from Escherichia coli had no effect on phosphoinositide turnover, and levels of inositol mono-, bis-, and trisphosphates after either a 5- or a 20-minute exposure did not differ significantly from basal values (data not shown).

**Inhibition of Effects of Oxidized Low Density Lipoprotein**

The ability of ox-LDL to stimulate inositol monophosphate accumulation was inhibited in human VSMCs preincubated with PMA (an activator of protein kinase C) or forskolin (which elevates cyclic AMP) (Figure 3A). The blocking effects of both PMA and forskolin occurred rapidly (>50% inhibition within 10 minutes of preincubation of human VSMCs), and complete inhibition of the ability of ox-LDL to generate inositol monophosphate was possible (after ~30–60 minutes of preincubation). For reasons pertaining to the sensitivity of quantification, only changes in inositol monophosphate were determined in

The process of atherogenesis has been attributed in part to peroxidized lipid components of oxidatively modified LDL. The major cholesterol oxidation products formed from LDL via transient metal (Cu^{2+}) catalysis or in the presence of endothelial cells have been identified as cholest-3,5-dien-7-one and cholest-3,5-dien-7-one, respectively. In a study of the effects of these products on cultured fibroblasts, cholest-3,5-dien-7-one was found to be a potent stimulator of cholesterol esterification, whereas cholest-3,5-dien-7-one was only mildly effective in this regard. Extracted lipids from modified LDL (either endothelial cell-modified or Cu^{2+}-oxidized) have been shown to be strongly chemotactic for human monocytes, whereas extracted lipids from native LDL exhibited no chemotactic activity. To exclude the possibility that our observations of phosphoinositide catabolism in human VSMCs exposed to ox-LDL were due to oxidized lipid components per se, we investigated the effects of lipid extracts (extracted with chloroform/methanol and added back in ethanol as described in “Methods”) on VSMC phosphoinositide metabolism. Lipid extracts from LDL and ox-LDL both were found to have no significant effect on basal levels of [%]inositol mono-, bis-, or trisphosphate production in either VSMC preparation (data not shown).
Accumulation to Ca\(^{2+}\) Antagonists and which can be internalized after ligand binding. 12-14-3445

The process of internalization is a composite of events, which include receptor clustering and receptor cross-linking by transglutaminase on the plasma membrane as well as cytoskeleton-dependent translocation into the cytoplasm.\(^{46,47}\) Therefore, we studied the effects of compounds known to inhibit either plasma membrane or translocative processes of receptor internalization.

Ox-LDL-stimulated inositol phosphate accumulation was inhibited by the covalent sulphydryl modifying agent phenylarsine oxide (Figure 4B), which inhibits receptor clustering.\(^{48}\) The transglutaminase inhibitors bacitracin and dansylcadaverine\(^{49}\) both prevented the stimulation of phosphoinositide turnover by ox-LDL (Figure 4B). Cytochalasin B, which promotes actin-filament depolymerization,\(^{50}\) and colchicine, which interacts with tubulin to promote microtubule depolymerization,\(^{50}\) also blocked the effects of ox-LDL on phosphoinositide metabolism in human VSMCs (Figure 4C). The effects of all the aforementioned endocytic inhibitors were dose dependent (Figures 4B and 4C) and required preincubation of human VSMCs for 30–60 minutes (time dependencies not shown).

### Discussion

The generation of inositol trisphosphate in human VSMCs exposed to ox-LDL provides evidence for the ability of this modified lipoprotein to activate phospholipase C–mediated phosphoinositide turnover. This signal transduction pathway plays a pivotal role in the regulation of smooth muscle contraction and SMC growth.\(^{51-55}\) Inositol trisphosphate produced via this pathway is the second messenger for Ca\(^{2+}\) mobilization from intracellular stores\(^{51}\) and may also play a role in promoting Ca\(^{2+}\) influx.\(^{55}\) Another second-messenger product of phospholipase C–mediated phosphoinositide hydrolysis is diacylglycerol, which activates protein kinase C.\(^{39,51,53}\) Protein kinase C promotes Ca\(^{2+}\) influx in SMCs\(^{51,53}\) and phosphorylates S6-kinase, an enzyme involved in the reinitiation of protein synthesis in quiescent cells.\(^{36,52}\) Additionally, protein kinase C induces expression of nuclear proto-oncogenes, which increase transcription and DNA synthesis, events that ultimately
lead to cell growth. Furthermore, both Ca\(^{2+}\) (via the intracellular Ca\(^{2+}\) receptor calmodulin) and protein kinase C are important regulators of several metabolic enzymes involved in cellular processes such as glycolysis, lipolysis, and secretion.

Ox-LDL is believed to play an important role in the development of atherosclerotic lesions, within which the constituent SMCs exhibit increased proliferation, increased lipid accumulation, and increased secretion of extracellular matrix components. The stimulation of phosphoinositide turnover by ox-LDL has been reported to occur in macrophage-derived macrophages, which can be identified in atheromatous lesions and are considered to play a key role in the pathogenesis of atherosclerosis. Stimulation of phosphoinositide turnover in human VSMCs by ox-LDL, a finding also made for cultured rat aortic SMCs (V. Tkachuk and T.J. Resink, unpublished results), may be an additional mechanism of action of this modified lipoprotein on the development of such vascular lesions.

Many of the described effects of ox-LDL on the vessel wall have been attributed to the interaction of ox-LDL with endothelial cells, with resultant alterations in the morphological and functional properties of these cells. However, ox-LDL has been reported to alter smooth muscle contractile function even in endothelium-denuded vessels, and additionally, this modified lipoprotein can be located within the intima of the vessel wall. Therefore, ox-LDL could plausibly interact with SMCs in both intact and injured vessels. Such a direct interaction of ox-LDL with SMCs is supported by its ability to stimulate phosphoinositide turnover in cultured human VSMCs, and this may indeed represent an endothelium-independent mechanism of action for modified LDL on vessels. Furthermore, we have demonstrated a sensitivity of the effects of ox-LDL on phosphoinositide turnover to the Ca\(^{2+}\) antagonists verapamil, diltiazem, and nifedipine (Figures 3 and 4). These results are in accord with observations of inhibition of ox-LDL–induced contraction by these Ca\(^{2+}\)– influx inhibitors and further invoke the notion that stimulation of phosphoinositide turnover in VSMCs contributes to the action of ox-LDL on smooth muscle contractile and metabolic processes. Stimulation of phosphoinositide catabolism in VSMCs by ox-LDL occurred at concentrations that have been estimated to exist in the intima of the vessel wall and to elicit and/or modify the contractile responses of isolated blood vessel segments.

The physiological response of smooth muscle to vasoactive hormones is sensitive not only to Ca\(^{2+}\) antagonists but also to modifications by cyclic AMP, cyclic GMP, and phorbol ester. Hormone-stimulated phosphoinositide turnover is also sensitive to these second messengers or their analogues. A surprising observation in the present study was that ox-LDL–stimulated phosphoinositide turnover in human VSMCs could be inhibited by the same spectrum of compounds (Figure 3) that block the effects of Ca\(^{2+}\)– mobilizing hormones on phosphoinositide turnover in SMCs. The mechanism of action of these hormones on phosphoinositide turnover involves the coupling of phospholipase C to receptors via G-proteins. Receptors that interact with G-proteins are structurally homologous and quite different from the receptors for high and low density lipoproteins as well as the scavenger receptors for modified lipoproteins. Therefore, the mechanism of phospholipase C stimulation by ox-LDL might be expected to differ from that of G-protein–dependent hormones and could even be indirect. However, the kinetics for ox-LDL–stimulated phosphoinositide turnover (Figure 1) were akin to those established for hormone action on this signal transduction system. Kinetic similarities include both a rapid (within 1 minute) generation of inositol tris-, bis-, and monophosphates and a saturability of the generation response. Importantly, stimulation of phosphoinositide turnover in human VSMCs by ox-LDL occurred at concentrations comparable to the affinity of the scavenger receptor for modified lipoprotein. Taken together, such findings allow us to suggest that ox-LDL–stimulated phosphoinositide turnover in human VSMCs is receptor mediated. Although the phosphoinositide turnover responses to equivalent concentrations of ox-LDL and LDL differ quantitatively (ox-LDL > LDL; this study and Reference 35), these data per se are insufficient to unequivocally conclude that the signaling responses of VSMCs to native and oxidatively modified LDL are mediated via different receptors. Nevertheless, scavenger receptors have recently been demonstrated to exist on VSMCs, and it is tempting to speculate that the presently observed biochemical responses of VSMCs to ox-LDL are indeed mediated via these receptors.

Further evidence for a receptor-mediated mechanism of action of ox-LDL on phosphoinositide turnover in human VSMCs was obtained from observations that inhibitors of receptor endocytotic processes prevented stimulation of phosphoinositide turnover by ox-LDL. These inhibitors included compounds that variously block receptor clustering, receptor cross-linking, or cytoskeleton-dependent receptor internalization. Such compounds are well recognized to inhibit endocytosis of both hormonal and lipoprotein receptors. Furthermore, our results suggest a close association between phosphoinositide turnover and receptor-mediated endocytosis. Such an association has previously been made in studies that investigated the relation between receptor internalization and phosphoinositide catabolism in response to vasoactive hormones angiotensin II and endothelin.

If ox-LDL–stimulated phosphoinositide turnover in smooth muscle cells is indeed part of the mechanism of action of this lipoprotein on contractile or metabolic processes of the vasculature, the diversity of compounds found to be effective inhibitors of ox-LDL–induced phosphoinositide turnover in human VSMCs indicates a broad base of pharmacological possibilities for regulating the action of ox-LDL on the vasculature. Such pharmacological intervention could include compounds that elevate cyclic nucleotides, prevent Ca\(^{2+}\) influx, or interfere with endocytotic processes. Present avenues for preventing or slowing the development of atherosclerosis concern the use of cholesterol-lowering agents and antioxidants that interfere with LDL modification. Use of pharmacological compounds that prevent a direct cellular action of ox-LDL might increase the therapeutic regimen for prevention of atherosclerosis.
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