Antioxidants Inhibit the Ability of Lysophosphatidylcholine to Regulate Proteoglycan Synthesis

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Objective—We previously have shown that lysophosphatidylcholine (lysoPC) regulates proteoglycan synthesis by vascular smooth muscle cells (SMCs). Given the accumulating evidence for reactive oxygen species (ROS) as mediators of a variety of effects of lysoPC, the present study evaluates the potential role of ROS as intermediate molecules in the regulation of proteoglycan synthesis by lysoPC.

Methods and Results—LysoPC (10 μmol/L) was found to stimulate rapid and sustained generation of ROS by SMC, as indicated using a fluorescent probe for measuring intracellular oxidants and fluorescence-activated cell sorting. This was not associated with cytotoxicity, as evaluated by fluorescence microscopy using MitoTracker Red or propidium iodide, cell number, cell protein, or lactate dehydrogenase release. Pretreatment with catalase or superoxide dismutase, specific scavengers of hydrogen peroxide and superoxide, respectively, blocked the ability of lysoPC to stimulate both accumulation of ROS and proteoglycan synthesis. Most importantly, these enzymatic antioxidants prevented lysoPC from stimulating the synthesis of proteoglycans with enhanced lipoprotein-binding properties, as quantified by a gel shift binding assay.

Conclusions—These findings strongly suggest that ROS are key mediators in the ability of lysoPC to regulate proteoglycan synthesis and that these effects can be inhibited by antioxidants. (Arterioscler Thromb Vasc Biol. 2006;26:494-500.)

Key Words: antioxidants • catalase • lysophosphatidylcholine • PG-MCSF • proteoglycans • reactive oxygen species • superoxide dismutase

Many lines of evidence implicate low-density lipoprotein (LDL) oxidation in atherogenesis.1-3 We have shown that oxidized LDL (ox-LDL) regulates several aspects of proteoglycan synthesis by arterial smooth muscle cells (SMCs).4 Of a number of bioactive components of ox-LDL tested, only lysoPC had similar effects, including the ability to influence both glycosaminoglycan chain elongation and proteoglycan core protein synthesis.5

LysoPC is formed during metal ion-mediated oxidation of LDL.6 It also can be generated by the enzymatic action of secretory phospholipase A2 (sPLA2) on the phosphatidylcholine component of LDL.6 Both pathways may contribute to the production of lysoPC in human plasma7 and atherosclerotic lesions.8 Whereas lysoPC elicits a variety of responses that contribute to atherogenesis, the complexity of its modes of action has made it difficult to understand how these responses occur. It also is not known how lysoPC regulates proteoglycan synthesis. Core protein synthesis occurs in the rough endoplasmic reticulum and glycosaminoglycan chain formation occurs in the Golgi,9 so lysoPC regulation of these 2 aspects of proteoglycan synthesis is a complex response that occurs in different intracellular sites via different pathways.5

The ability of lysoPC to cause glycosaminoglycan chain elongation on proteoglycans is functionally significant because longer chains facilitate greater interaction with LDLs.4,10,11 This interaction between circulating LDL and arterial proteoglycans is a process central to atherogenesis.1

The ability of lysoPC to influence core protein synthesis of biglycan and the proteoglycan form of the cytokine macrophage colony-stimulating factor (CSF) (PG-MCSF), also is functionally significant. Of the major interstitial proteoglycans in blood vessels that are known to bind to lipoproteins in vitro,12-14 biglycan shows the strongest colocalization with epitopes for apolipoprotein (apo) B and apoE in human atherosclerotic plaques.13 Thus, biglycan may be particularly important in lipoprotein retention within the arterial wall. PG-MCSF is a proteoglycan that we have described to be secreted by both human monocyte-derived macrophages15 and stimulated SMCs.5 PG-MCSF can bind LDL in vitro15,16 and could potentially participate in lipoprotein retention. It is thought that the glycosaminoglycans of biglycan serve to anchor the cytokine to lipoproteins or other matrix molecules, where it participates in the survival and differentiation of mononuclear phagocytic cells.15,16

Given the accumulating evidence that reactive oxygen species (ROS) such as hydrogen peroxide and superoxide play a role in atherosclerosis, and that oxidants are major signaling molecules involved in gene regulation,17 the present study evaluates the potential role of ROS as mediators of the...
lysoPC effects. LysoPC is shown to stimulate ROS production in this system that is not associated with cytotoxicity. Antioxidant pretreatment can: (1) block lysoPC-induced accumulation of ROS; (2) block the ability of lysoPC to regulate both glycosaminoglycan chain elongation and core protein synthesis of proteoglycans; and, most importantly, (3) block the ability of lysoPC to stimulate synthesis of proteoglycans with enhanced lipoprotein-binding properties.

**Methods**

**Cell Culture**

Monkey (Macaca nemestrina) arterial SMCs were maintained as described, grown to confluence, and made quiescent in DMEM/0.1% calf serum. Cells were treated with lysoPC containing palmitic and stearic acids (Sigma) (dissolved in phosphate-buffered saline [PBS]) or 5-cholesten-3β-ol-7-one (7-KC, Steraloids) (dissolved in ethanol) in fresh DMEM/0.1% calf serum. PBS or ethanol, alone, was used as controls. For experiments in which the effects of antioxidants were studied, quiescent cells were pretreated with catalase from bovine liver (Sigma), superoxide dismutase from bovine erythrocytes (Sigma), EDTA, γ-tocopherol, or α-tocopherol (Sigma) for up to 24 hours before the treatment period. For some experiments, the antioxidant-containing media was removed and fresh antioxidant-free DMEM/0.1% calf serum was added before lysoPC treatment. At the indicated times, cells were harvested by trypsin to remove surface-associated proteoglycans; and, most importantly, (3) block the ability of lysoPC to stimulate synthesis of proteoglycans with enhanced lipoprotein-binding properties.

**Proteoglycan Isolation and Characterization**

Media were harvested with protease inhibitors (0.1 mol/L 6-aminohexanoic acid, 5 mmol/L benzamidine HCl, and 0.1 mmol/L phenylmethylsulfonyl fluoride) and purified on DEAE-Sephacel. Apparent molecular size of intact [[35S]-labeled proteoglycans was evaluated by SDS-PAGE with Cyclone phosphor screen imaging and quantification using OptiQuant software (Packard). Molecular sizes of [[S-Met]-core proteins from chondroitin ABC lyase (CABC lyase) digested samples were evaluated by SDS-PAGE with Cyclone phosphor screen imaging and quantification using OptiQuant software (Packard). Relative core protein levels were quantified by densitometric scanning of PhosphorImager analyses using OptiQuant software (Packard).

**Quantification of ROS**

ROS generation was assessed using CM-H$_2$DCFDA (Molecular Probes), a membrane-permeable dye that can be oxidized by intracellular ROS to the fluorescent product CM-DCF, which can be monitored by fluorescence-activated cell sorting (FACS) and fluorescence microscopy. This dye is most useful for the direct measurement of H$_2$O$_2$ and the indirect measurement of ·OH. 5 μmol/L CM-H$_2$DCFDA was added to cells 15 minutes before lysoPC treatment. At the indicated times, cells were harvested by trypsin and washed with PBS. CM-H$_2$DCF fluorescence was evaluated immediately without fixation of cells. Experiments which included antioxidant pretreatment, as described earlier, were used to verify that CM-H$_2$DCF fluorescence is a valid indicator of ROS production.

**Detection of Intracellular Catalase Accumulation**

Two methods were used to demonstrate that pretreatment of quiescent SMCs with catalase for 24 hours before treatment with lysoPC resulted in intracellular catalase accumulation. First, quiescent SMCs were treated with increasing concentrations (0 to 0.4 mg/mL) of $^{125}$I-catalase for 24 hours, after which cells were washed with PBS, treated with trypsin to remove surface-associated $^{125}$I-catalase, washed again with PBS, and sonicated in 50 mmol/L potassium phosphate with 0.1% Triton X-100 at pH 7.8. Cellular $^{125}$I-catalase uptake was determined on a Packard gamma counter. Second, quiescent SMCs were treated with 100 or 250 U/mL (corresponding to 0.05 or 0.125 mg/mL) catalase for 24 hours, after which cells were washed with PBS, treated with trypsin to remove surface-associated catalase, then washed again with PBS containing protease inhibitors (0.1 mol/L 6-aminohexanoic acid, 5 mmol/L benzamidine HCl, and 0.1 mmol/L phenylmethylsulfonyl fluoride). Cells were lysed by the addition of 0.2% Tween-20. Cellular catalase accumulation was evaluated by enzyme-linked immunosorbent assay (ELISA) using a rabbit anti-catalase horseradish peroxidase conjugate ( Fitzgerald Industries International).

**Evaluation of Cytotoxicity**

LysoPC effects on cell viability were evaluated by fluorescence-activated cell sorter (FACS) and fluorescence microscopy using 2 selective probes. MitoTracker Red CM-H$_2$X-ROS (Molecular Probes) is a reduced dye that does not fluoresce until it enters an actively respiring cell, where it is oxidized to a fluorescent mitochondrion-selective probe and retained. Cells without functioning mitochondria (ie, apoptotic cells) do not accumulate this probe. Propidium iodide is a DNA-selective probe that is membrane-impermeable for cells with intact plasma membranes, but easily passes through the compromised membranes of necrotic cells. In addition, effects on cell number, cell protein, and lactate dehydrogenase release were measured, as previously described.

**Lipoprotein Preparation**

LDLs (d=1.019 to 1.063 g/mL) were isolated from plasma of normal human volunteers, dialyzed against 150 mmol/L NaCl with 1 mmol/L EDTA, stored under N$_2$ at 4°C, and used within 3 weeks.

**Analysis of Lipoprotein–Proteoglycan Binding**

Binding of LDL to proteoglycans was evaluated by a mobility shift assay. Briefly, [[35S]-labeled proteoglycans and LDL were dialyzed against a physiological MOPS buffer. [[35S]-labeled proteoglycans then were incubated with native LDL (0 to 0.5 mg/mL) for 1 hour at 37°C and electrophoresed in 0.7% agarose gels. In this system, proteoglycans bound to lipoproteins remain near the gel origin and free proteoglycans migrate to the gel front. Gels were quantified by phosphorimagery analysis. Binding of LDL to proteoglycans also was evaluated by a plate assay. Briefly, increasing concentrations of LDL (0 to 1 mg/mL) were coated on a 96-well flexible assay plate (Falcon) overnight at 4°C. An equal amount of [[35S]-labeled proteoglycans was added to each well and binding was allowed to occur for 2 hours at room temperature. Wells were thoroughly washed and dried before counting in a Beckman liquid scintillation counter. Binding parameters were determined with SAAM II software.

**Statistical Analyses**

The significance of differences in mean values was determined by the 2-sample t test assuming unequal variances.

**Results**

LysoPC Induces ROS Generation

The focus of the present study is to identify the potential role of ROS as mediators of the effects of lysoPC on proteoglycan synthesis by SMCs. To determine whether ROS were generated in response to lysoPC, a fluorescent probe (CM-H$_2$DCFDA) for monitoring intracellular oxidants was used. ROS generation was detected by 1 minute and was maximal by 10 to 15 minutes after lysoPC treatment (Figure 1A). ROS generation also was dependent on the concentration of lysoPC. The minimum concentration of lysoPC at which ROS could be detected was 10 μmol/L (Figure 1B), the concentration chosen for the remainder of this study. ROS remained elevated for up to 36 hours after treatment with 10 μmol/L.
lysoPC (Figure 1C). Thus, lysoPC induced both immediate and sustained ROS generation in SMCs.

The ability of lysoPC to induce ROS formation was contrasted with those of 7-ketocholesterol, another known bioactive component of oxidized LDL. We have previously shown that 7-ketocholesterol (up to 20 μmol/L) affects only glycosaminoglycan chain formation but not core protein synthesis of proteoglycans. 7-Ketocholesterol did not induce an immediate ROS response as measured for up to 30 minutes but did induce a delayed ROS response that could be detected after 24 hours (Figure 1D). These findings demonstrate that lysoPC and 7-ketocholesterol, 2 of the major bioactive components of oxidized LDL, have differential abilities to induce ROS formation by SMCs that are associated with differential abilities to regulate proteoglycan synthesis.

LysoPC Induces ROS Generation Without Causing Cytotoxicity

To establish whether the ability of lysoPC to regulate proteoglycan synthesis was associated with cell death, several different indices of cytotoxicity were monitored. MitoTracker Red CM-H2-X-ROS is retained in the mitochondria of actively respiring cells, but not in cells without functioning mitochondria (ie, apoptotic cells). Although there was a difference in cell morphology, there was no difference in MitoTracker uptake between control cells and those treated with 10 μmol/L lysoPC (Figure 1, available online at http://atvb.ahajournals.org). Propidium iodide is membrane-impermeable for viable cells with intact plasma membranes but easily passes through the compromised membranes of necrotic cells. Neither control nor lysoPC-treated cells had significant propidium iodide uptake even 24 hours after treatment (data not shown). Therefore, the effects of lysoPC on proteoglycan synthesis are not associated with cytotoxicity.

Enzymatic Scavengers of ROS Inhibit LysoPC Effects

To confirm the role of ROS in the regulation of proteoglycan synthesis, several compounds were tested for their ability to modulate the lysoPC response. First, the ability of enzymatic scavengers to block lysoPC-induced ROS accumulation was evaluated by DCF fluorescence. The mean fluorescence intensity (MFI) under control conditions was 74±1.1 (n=7). This increased to 170±9.8 with lysoPC treatment (n=7). Pretreatments of SMCs with 100 U/mL catalase (MFI 69±0.0, n=5), 100 U/mL superoxide dismutase (MFI 79±2.8, n=5), or 0.05 mol/L EDTA (MFI 67±6.0, n=3) were effective at blocking both the immediate and sustained accumulation of ROS induced by lysoPC (Figure 2). Because these enzymes specifically scavenge hydrogen peroxide and...
superoxide, respectively, these observations provide strong evidence that these reactive oxygen species play a role in the generation of DCF fluorescence. EDTA was also inhibitory, suggesting that redox-active metal ions might be involved in the production of ROS. A number of other antioxidant compounds were ineffective at blocking lysoPC-induced ROS accumulation. These include γ-tocopherol (100 μmol/L) and α-tocopherol (100 μmol/L), the thiol-reducing agent 2-mercaptopropionylglycine (300 μmol/L), and the mitochondrial oxidase inhibitors rotenone (1 μmol/L) and thenoyltrifluoroacetone (10 μmol/L). Of the antioxidant compounds tested, only pretreatment with catalase also blocked the sustained accumulation of ROS induced by 7-ketocholesterol (Figure 2).

Second, the ability of these antioxidants to block lysoPC-induced glycosaminoglycan chain elongation and, thus, overall proteoglycan size was evaluated. As previously described, proteoglycans from control SMCs are resolved into 4 major broad bands on SDS-PAGE (Figure 3A, lane 1). Band 1 is the large chondroitin sulfate proteoglycan versican that does not enter the resolving gel. Band 2 (MW_{app} > 300 kDa) is a mixture of heparan and chondroitin sulfate proteoglycans, which are not fully characterized. Band 3 (MW_{app} ~ 200 kDa) contains biglycan under control conditions and PG-MCSF on stimulation with lysoPC. Band 4 (MW_{app} ~ 100 kDa) contains decorin. Biglycan, PG-MCSF, and decorin are all small dermatan sulfate proteoglycans. Note that this broad banding pattern is typical for proteoglycans, which do not migrate uniformly on SDS-PAGE because of their size heterogeneity.

LysoPC induced a large increase in overall size of proteoglycans in bands 3 and 4, as compared with the control (Figure 3A, lane 2 versus lane 1). Pretreatments with the enzymatic scavengers catalase and superoxide dismutase (Figure 3A, lanes 3 and 4), as well as EDTA (data not shown) were effective at blocking this effect of lysoPC. Interestingly, both γ-tocopherol and α-tocopherol also blocked the lysoPC-induced increase in proteoglycan size (data not shown), although they had no effect on lysoPC-induced ROS generation. 2-Mercaptopropionylglycine and rotenone/TTFA were ineffective at blocking lysoPC-induced increase in proteoglycan size (data not shown).

Third, the ability of enzymatic scavengers of ROS to inhibit lysoPC-induced core protein regulation was evaluated. As previously described, lysoPC causes a marked, 5.3±1.8-fold relative to control (mean±SEM, n=3), induction of PG-MCSF core, a 100-kDa protein that is not abundant in monkey SMCs under control conditions5 (Figure 3B). Pretreatment with catalase was effective at inhibiting the lysoPC-mediated induction of PG-MCSF core protein (2.0±1.2-fold relative to control, n=3), as was pretreatment with superoxide dismutase (2.8±1.0-fold relative to control, n=3). The antioxidant pretreatments had no effect on biglycan (data not shown) or versican (Figure 3B) core proteins.

Thus, the ability of catalase and superoxide dismutase to block the accumulation of ROS, glycosaminoglycan chain...
elongation, and induction of PG-MCSF core protein in response to lysoPC treatment support the hypothesis that ROS are involved in the regulation of proteoglycan synthesis by lysoPC. It should be noted that the ability of catalase and superoxide dismutase to prevent these effects of lysoPC required a 24-hour pretreatment with these antioxidants. Short pretreatment periods (up to 2 hours) were not effective for blocking any of the lysoPC effects (data not shown). Intracellular accumulation of increasing concentrations of catalase was demonstrated after 24 hours of incubation with 125I-catalase (Figure II, available online at http://atvb.ahajournals.org) and verified by ELISA using an anti-catalase antibody (data not shown). In addition, after the 24-hour pretreatment period, catalase and superoxide dismutase could be removed from the media without affecting their ability to block the ability of lysoPC to influence glycosaminoglycan chain elongation or core protein synthesis (data not shown).

Antioxidants Inhibit the Ability of lysoPC to Stimulate Synthesis of Proteoglycans With Enhanced LDL-Binding Properties

Proteoglycans synthesized in the presence of lysoPC exhibit enhanced LDL-binding properties as compared with those synthesized by control untreated cells. Therefore, the ability of antioxidants to prevent this effect of lysoPC was evaluated (Figure 4). Antioxidant pr-treatment had no effect on the LDL-binding affinity of proteoglycans synthesized by lysoPC-treated cells or control cells. However, both catalase and superoxide dismutase prevented the ability of lysoPC to induce synthesis of proteoglycans with greater total binding capacity for native LDL. This key result establishes a role for ROS as mediators in the ability of lysoPC to influence proteoglycan synthesis and lipoprotein retention by arterial wall proteoglycans.

Discussion

We previously have shown that lysoPC regulates multiple aspects of proteoglycan synthesis that impact on lipoprotein retention within the arterial wall. The rationale for the present study is based on the accumulating evidence that ROS play a role in atherosclerosis and that ROS function as mediators of a variety of the effects of lysoPC. Therefore, the aim of this study was to evaluate the potential role of ROS as mediators in the regulation of proteoglycan synthesis by lysoPC. The major findings are 4-fold. First, lysoPC stimulates rapid and sustained ROS production in this system. Second, this accumulation of ROS can be blocked by pretreatment with the enzymatic scavenger catalase or superoxide dismutase. Third, these enzymes also block the ability of lysoPC to regulate proteoglycan synthesis. Fourth, catalase and superoxide dismutase prevent the ability of lysoPC to stimulate synthesis of proteoglycans with enhanced lipoprotein-binding properties.

Results of this study strongly suggest that ROS are key mediators in the ability of lysoPC to regulate proteoglycan synthesis and to influence lipoprotein retention in the arterial wall.
The finding that lysoPC stimulates ROS production has been reported previously in neutrophils,27 endothelial cells,28 and SMCs.29 Others also have reported that ROS can regulate synthesis of collagen,30 another extracellular matrix molecule. However, it has not previously been reported that ROS can regulate synthesis of proteoglycans. On the contrary, ROS have been implicated in the degradation of matrix molecules.6,7 Thus, one novel aspect of this study is that lysoPC-mediated generation of ROS promotes proteoglycan synthesis. Furthermore, lysoPC-mediated generation of ROS promotes both glycosaminoglycan chain formation and core protein synthesis, 2 aspects of proteoglycan synthesis that occur in different intracellular sites and likely involve different pathways of regulation.

We have previously described the ability of oxidized LDL and lysoPC to influence proteoglycan size.4,5 This effect was found to be caused, at least in part, by glycosaminoglycan chain elongation with ≈25% to 50% increase in chain length.4 We and others10,32 also have found that proteoglycans with such increases in chain length have increased LDL-binding properties. The ability of catalase and superoxide dismutase to inhibit both lysoPC-induced ROS and synthesis of proteoglycans with increased molecular size accurately predicts the resulting inhibition in the ability of lysoPC to stimulate synthesis of proteoglycans with enhanced lipoprotein-binding properties. It is interesting that superoxide dismutase pretreatment resulted in the synthesis of proteoglycans with even lower overall molecular size and lower total binding capacity for native LDL than proteoglycans synthesized under control conditions. This suggests that superoxide dismutase has additional effects on glycosaminoglycan chain formation and proteoglycan synthesis that are separate from its ability to block the accumulation of ROS.

It should be pointed out that the concentration of lysoPC (≈20 μmol/L) used in this study was below its critical micellar concentration (50 μmol/L), a concentration above which lysoPC is cytotoxic.33 Moreover, this concentration is physiologically relevant, because lysoPC is present in albumin- and lipoprotein-bound forms in plasma at even higher levels (>200 μmol/L).34 It also should be emphasized that both catalase and superoxide dismutase were effective at inhibiting lysoPC effects only when preloaded into cells. It is commonly believed that these enzymes are not internalized and are effective only at neutralizing extracellular ROS. However, preincubation has been shown to be effective at increasing intracellular enzyme activity,35,36 presumably by endocytotic bulk phase transport. This was verified here by monitoring intracellular 125I-catalase uptake as well as by ELISA. In addition, it was established that a 24-hour preincubation was required for these enzymes to inhibit lysoPC effects (data not shown). If these enzymes were active only in the extracellular milieu, then such a preincubation would not be necessary. Furthermore, after the preincubation period, catalase and superoxide dismutase could be removed from the media without affecting their ability to block the effects of lysoPC on proteoglycan synthesis. Thus, these enzymes most likely did enter the cells to inhibit the intracellular accumulation of ROS.

It is interesting that γ-tocopherol and α-tocopherol both blocked the lysoPC-induced increase in proteoglycan size though they had no effect on lysoPC-induced DCF fluorescence. This finding reflects on the limitations of specificity of the fluorescent probe, CM-H2DCFDA, as an indicator of ROS, and gives some indication as to the nature of the free radicals and ROS being generated in this system. The tocopherols are scavengers of fatty acid peroxyl and alkoxyl radicals that result from lipid peroxidation reactions. Most likely, these highly reactive free radical species are produced in response to lysoPC treatment but are not being detected with CM-H2DCFDA. This reagent is most useful as a direct measure of peroxynitrite and hydroxyl radicals, and only indirectly as a measure of hydrogen peroxide hydrogen peroxide in combination with cellular peroxidases.23,24 These results indicate, not surprisingly, that multiple free radicals and reactive oxygen species are produced as a result of lysoPC treatment and have an impact on proteoglycan synthesis. However, the increase in DCF fluorescence being measured in this system most likely represents an increase in hydroxyl radical formation.

Findings from our studies, taken together with reports in the literature, suggest the following scenario (Figure 5). The initiating event of atherogenesis is subendothelial LDL retention via interaction with extracellular matrix proteoglycans.1 Retained LDL is then modified by oxidation or sPLA2 action,1 two processes that lead to lysoPC generation. LysoPC regulates synthesis of biglycan and PG-MCSF that can bind LDL and promote macrophage migration, proliferation, and differentiation. The most important aspect of this scenario is that enzymatic scavengers can inhibit the ability of lysoPC to influence proteoglycan synthesis and lipoprotein retention. It is of interest that M-CSF upregulates synthesis of cytoplasmic PLA2 by macrophages, leading to activation of phosphatidylcholine hydrolysis and increased production of lysoPC.37 In summary, a positive feedback mechanism is suggested in which the initial retention of LDL leads to generation of lysoPC and ROS that results in upregulation of proteoglycan synthesis with significant bearing on further lipoprotein retention and macrophage survival in atherogenesis.
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