Lysophosphatidylcholine Regulates Synthesis of Biglycan and the Proteoglycan Form of Macrophage Colony Stimulating Factor

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Objective—We have shown that copper-oxidized LDL (Ox-LDL) regulates proteoglycan synthesis by arterial smooth muscle cells. Ox-LDL specifically upregulates biglycan expression while causing elongation of glycosaminoglycan chains on all of the major secreted proteoglycans (biglycan, decorin, and versican), resulting in enhanced lipoprotein-binding interactions. It is not known which component of Ox-LDL is responsible for these effects. This study investigated the ability of several bioactive components of Ox-LDL to regulate proteoglycan synthesis.

Methods and Results—Those tested included 2 oxysterols (7-ketocholesterol and 7β-hydroxycholesterol) and 2 lysolipids (lysophosphatidylcholine and lysophosphatidic acid) formed during LDL oxidation. 7-ketocholesterol, lysophosphatidylcholine, and lysophosphatidic acid all increased proteoglycan MWapp, which is correlated with chain elongation and enhanced lipoprotein-binding properties in vitro. Lysophosphatidylcholine mimics the ability of Ox-LDL to stimulate biglycan expression and also causes a marked induction of the core protein for the proteoglycan form of macrophage colony stimulating factor.

Conclusions—Multiple oxidized lipid molecules can modulate proteoglycan synthesis and may have important consequences to atherogenesis via processes that involve enhanced lipoprotein retention as well as the promotion of macrophage survival and differentiation. (Arterioscler Thromb Vasc Biol. 2003;23:1111-1119.)

Key Words: lysophosphatidylcholine n oxidized LDL n proteoglycan n biglycan n proteoglycan form of macrophage colony stimulating factor

The interactions of proteoglycans with LDL are hypothesized to be the major cause of extracellular lipid accumulation within atherosclerotic lesions.1–3 Such proteoglycan-mediated lipid accumulation can be attributable to either direct interaction between these molecules or indirect interaction via bridging molecules.4,5 Several consequences result that potentially contribute to atherogenesis, including increased susceptibility of lipoproteins to oxidation6 or aggregation7 and rapid internalization of lipoprotein-proteoglycan complexes by both macrophages and smooth muscle cells.6

Much of the understanding of arterial proteoglycan structure and function has been derived from studies of smooth muscle cell proteoglycans. These are a heterogeneous group of complex macromolecules that have the common structure of a core protein, to which one or more glycosaminoglycan chains are covalently attached. An individual proteoglycan is defined by both its core protein and the disaccharide content of its glycosaminoglycan chains. Thus, the major interstitial proteoglycans in blood vessels are versican, decorin, biglycan, and perlecan.8 Each of these can bind to lipoproteins in vitro.5,9 However, biglycan shows the strongest colocalization with epitopes for apolipoprotein (apo) B and E in human atherosclerotic plaques.10 Thus, biglycan may be particularly important in lipoprotein retention within the arterial wall.

The human monocyte-derived macrophage, another important cell type in the pathogenesis of atherosclerosis,11 secretes a single species of proteoglycan identified as the proteoglycan form of the cytokine macrophage colony stimulating factor (PG-MCSF).12 PG-MCSF can bind LDL in vitro12,13 and could potentially participate in lipoprotein retention, although this has not been demonstrated. It is thought that the glycosaminoglycan chains serve to anchor the cytokine to lipoproteins or other matrix molecules, where it participates in the survival and differentiation of mononuclear phagocytic cells.12,13

The direct interactions between proteoglycans and lipoproteins can be modulated by changes to either component of the interaction. Thus, changes in proteoglycan amount or structure could affect their interactions with lipoproteins.14–16 We previously have shown that changes in proteoglycan synthesis also result from stimulation with oxidized LDL (Ox-LDL),17 a factor important to atherogenesis.1 Lysophosphatidylcholine preferentially stimulates biglycan core protein synthesis and causes nonspecific glycosaminoglycan chain elongation on versican, biglycan, and decorin that is associated with enhanced proteoglycan-lipoprotein interactions.
The present study additionally examines these effects by investigating the ability of known bioactive components of Ox-LDL to regulate proteoglycan synthesis. The primary compounds of interest are 7-ketocholesterol (7-KC) and lysophosphatidylcholine (lysoPC), 2 of the major products formed during LDL oxidation.18,19 To assess the specificity of the effects of these compounds, another oxysterol, 7β-hydroxycholesterol (7β-OH), and another lysolipid, lyso-phosphatidic acid (lysoPA), also were examined. These compounds also are formed during the oxidation of LDL but are present in lower abundance than 7-KC and lysoPC, respectively. 7-KC, lysoPC, and lysoPA caused nonspecific chain elongation on each of the major secreted proteoglycan species, resulting in enhanced LDL-binding by these modified proteoglycans. In contrast, lysoPC alone specifically stimulated expression of the biglycan core protein. Of great interest is the finding that lysoPC dramatically induces the expression of PG-MCSF, a proteoglycan not previously known to be synthesized by smooth muscle cells.

**Methods**

**Cell Culture**

Monkey (Macaca nemestrina) arterial smooth muscle cells (SMCs) were maintained as described.4 Cells were grown to confluence, made quiescent in DMEM/0.1% calf serum, and incubated with Ox-LDL, LysoPC containing palmitic and stearic acids, palmitic acid alone, or stearic acid alone (Sigma), LysoPA containing stearic acid or Ox-LDL, LysoPC containing palmitic and stearic acids, palmitic acid alone, or stearic acid alone (Sigma), LysoPA containing stearic acid (lysoPA, Avanti), 5-cholesten-3β-ol-7-one (7-KC, Steraloids), 5-cholesten-3β,7β-diol (7-OH, Steraloids), or PBS (control) in fresh DMEM/0.1% calf serum. SMCs were metabolically labeled with [35S]methionine (50 μCi/mL) for the last 24 hours of the treatment period.

**Lipoprotein Preparation**

LDLs (1.019 to 1.063 g/mL) were isolated from plasma of normal human volunteers,20 dialyzed against 150 mmol/L NaCl with 1 mmol/L EDTA, stored under N2 at 4°C, and used within 3 weeks. For preparation of Ox-LDL, native LDL (300 μg protein/mL) was dialyzed against PBS and incubated with 5 μmol/L copper sulfate for 18 hours at 37°C in air.21

**Proteoglycan Isolation and Characterization**

Media were harvested with protease inhibitors, purified on DEAE-Sephacel, and applied to preparative Sepharose CL-2B. Proteoglycans eluting at Kav ≤0.3 were pooled for peak 1; those eluting at 0.55 ≤Kav ≤0.8 were pooled for peak 2. Treated cell media were harvested with protease inhibitors, purified on DEAE-Sephacel, and applied to preparative Sepharose CL-2B. Proteoglycans eluting at Kav ≤0.3 were pooled for peak 1; those eluting at 0.55 ≤Kav ≤0.8 were pooled for peak 2. 24 Hour treatment with 50 μmol/L lysoPC caused elution of small proteoglycans into peak 3, with Kav of 0.8. Three peaks of glycosaminoglycan containing proteoglycans were identified by Western analysis using antisera specific for human biglycan (1:1000)22 or human osteosarcoma PG-100/PG-MCSF (1:1000).23 Total RNA was isolated using the RNAqueous system (Ambion) for Northern analysis. [32P]-labeled cDNA probes for biglycan and decorin (CABC lyase)-digested samples were identified by Western analysis using antisera specific for human biglycan (1:1000)22 or human osteosarcoma PG-100/PG-MCSF (1:1000).23

**Analysis of Lipoprotein-Proteoglycan Binding**

Binding of LDL to proteoglycans was evaluated by a mobility shift assay.25 Briefly, [35S]proteoglycans were incubated with native LDL (0 to 0.5 mg/mL) in a physiological HEPES buffer 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 PhosphorImager analysis. 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**

**Lipid Components of Ox-LDL Increase Proteoglycan MW<sub>app</sub>**

We have shown that 72 hours of treatment with Ox-LDL (5 μg protein/mL) increases proteoglycan size.17 As described, proteoglycans from control SMCs are resolved into 4 major bands on SDS-PAGE (Figure 1). Band 1 is the large chondroitin sulfate proteoglycan versican that does not enter the resolving gel. Band 2 (MW<sub>app</sub> >300 kDa) is a mixture of heparan and chondroitin sulfate proteoglycans, which are not fully characterized. Bands 3 (MW<sub>app</sub> ~200 kDa) and 4 (MW<sub>app</sub> ~100 kDa) contain biglycan and decorin, respectively, 2 small dermatan sulfate proteoglycans.26

The effects of several bioactive lipid components of Ox-LDL were investigated. The ability of 7-KC to affect proteoglycan size could be detected at 48 hours (Figure 1A). Band 4 MW<sub>app</sub> increased with up to 50 μmol/L 7-KC. In contrast, treatment with 7β-OH for 48 hours (Figure 1B) had no effect. The response to lysoPC was evident by 24 hours (Figure 1C). Band 4 MW<sub>app</sub> increased with up to 50 μmol/L lysoPC, and band 3 also increased in MW<sub>app</sub>. LysoPA increased band 4 MW<sub>app</sub> at 24 hours (Figure 1D) with no effect on band 3. Because band 1 does not enter and band 2 only just enters the resolving gel, these proteoglycans could not be evaluated. Thus, the MW<sub>app</sub> of proteoglycans in bands 3 and 4 are increased in response to 7-KC, lysoPC, and lysoPA, each of which exerted more rapid effects than Ox-LDL itself.

As a point of reference, the content of 7-KC in Ox-LDL (5 μg/mL) has been determined to be ~0.7 μmol/L.27 That of lysoPC to be ~20 μmol/L.28 7-KC at 1 μmol/L increased the MW<sub>app</sub> of band 4 slightly, but it was difficult to assess effects on band 3 because of its low abundance (data not shown). LysoPC at 20 μmol/L increased the MW<sub>app</sub> of bands 3 and 4 and also increased the amount of band 3 relative to band 4. The increase in MW<sub>app</sub> was comparable to that found with Ox-LDL.27 Thus, at concentrations relevant to those found in Ox-LDL, 7-KC and lysoPC each mimic some of the effects of Ox-LDL on proteoglycan synthesis.

**LysoPC Alters the Ratio of Small Proteoglycans**

The effect of lysoPC on band 3 is striking. This band is comparable to that found with Ox-LDL.27 As described, treatment with 50 μmol/L lysoPA increased the MW<sub>app</sub> of band 4 slightly, but it was difficult to assess effects on band 3 because of its low abundance (data not shown). LysoPC at 20 μmol/L increased the MW<sub>app</sub> of bands 3 and 4 and also increased the amount of band 3 relative to band 4. The increase in MW<sub>app</sub> was comparable to that found with Ox-LDL.27 Thus, at concentrations relevant to those found in Ox-LDL, 7-KC and lysoPC each mimicked some of the effects of Ox-LDL on proteoglycan synthesis.
SDS-PAGE. Thus, whereas multiple components of oxidized LDL modulate proteoglycan size, only lysoPC alters the amounts of individual proteoglycan species.

**Specificity of Response**
The specificity of the lysoPC response was evaluated by several means. First, the effects of lysophospholipids with different head group or fatty acid components were compared with those of lysoPC containing a mixture of palmitic and stearic acids. LysoPC containing either palmitic or stearic acid alone both increased proteoglycan size and enriched for band 3. LysoPA increased size with no enrichment for band 3. Lysophosphatidylethanolamine had no effect on size or amount. These results suggest that head group structure determines the ability of lysophospholipids containing long chain saturated fatty acids to alter proteoglycan synthesis (data not shown). Second, lysoPC exists primarily in protein-bound forms in plasma, raising the question of whether

**Figure 1.** Lipid components of Ox-LDL affect proteoglycan synthesis in a concentration-dependent manner. SDS-PAGE of [35S]proteoglycans secreted by SMCs treated as indicated. A total of 40,000 dpm were loaded per lane.
lysoPC that is protein-bound would have similar effects as
when in low serum conditions. No difference was found in
the ability of lysoPC to modulate proteoglycan synthesis
when cells were treated in the presence of 0.1%, 1%, or 5%
serum (data not shown). Third, these effects are not attribut-
able to PAF contamination of the lysoPC preparation,29
because pretreatment with PAF-acetyl hydrolase to degrade
possible contaminants did not alter the ability of lysoPC to
modulate proteoglycan synthesis (data not shown). Fourth,
these effects are not attributable to cytotoxicity. The concen-
trations of lysoPC and 7-KC used did not cause a decrease in
cell number or protein or an increase in lactate dehydrogenase
(data not shown).

LysoPC and 7-KC Increase Proteoglycan
Hydrodynamic Size
Proteoglycan size also was evaluated by size exclusion
chromatography (Figure 2). This procedure establishes hy-
drodynamic size and resolves the proteoglycan mixture into 2
peaks, with versican eluting in peak 1 (\(K_{av} \approx 0.37\)) and
biglycan and decorin coeluting in peak 2 (\(K_{av} \approx 0.63\)) under
control conditions.14 Both peaks from cells stimulated with
7-KC shifted to a lower \(K_{av}\) (peak 1 \(K_{av} \approx 0.35\); peak 2 \(K_{av} \approx 0.59\)), indicating increased hydrodynamic size with treatment. Peaks 1 and 2 from cells stimulated with lysoPC shifted
even lower (peak 1 \(K_{av} \approx 0.23\); peak 2 \(K_{av} \approx 0.59\)). The shifts
toward a smaller \(K_{av}\) for peak 1 indicate an increase in size for
versican that could not be detected by SDS-PAGE. This
method does not resolve the smaller proteoglycans, but the
shifts to smaller \(K_{av}\) values for peak 2 are in agreement with
the increase in size of bands 3 and 4 observed by SDS-PAGE.
Thus, multiple components of Ox-LDL modulate proteogly-
can size. These components do not affect the size of selected
proteoglycans but rather nonspecifically increase overall size
of each of the proteoglycan species secreted by SMCs.

Figure 2. 7-KC and lysoPC increase proteoglycan hydrodynamic size. Sepharose CL-2B chromatography of \([^{35}S]O_4\)-proteoglycans
secreted by SMCs treated for 48 hours, as indicated. Profiles shown are representative of \(n=3\). Dashed lines indicate the \(K_{av}\) for peaks
1 and 2.

7-KC and lysoPC Cause Chain Elongation That Is Correlated With Enhanced Lipoprotein Binding
To evaluate whether the increase in proteoglycan size is
attributable to chain elongation, chains from peaks 1 and 2
were liberated for additional analysis (Figure 3). Under
control conditions, versican chains (peak 1) eluted with \(K_{av} \approx 0.38\), corresponding to MW \(\approx 35\) kDa.30 Versican chains
shifted to lower \(K_{av}\) values of \(\approx 0.32\) for 20 \(\mu\)mol/L 7-KC and
\(\approx 0.28\) for 20 \(\mu\)mol/L lysoPC, indicating larger molecular
weights of \(\approx 55\) and 68 kDa10 for chains synthesized in the
presence of 7-KC and lysoPC, respectively. Identical results
were obtained for peak 2 proteoglycans. Thus, the ability of
7-KC and lysoPC to increase proteoglycan size is attributable,
at least in part, to their ability to cause chain elongation.
These compounds exerted similar effects on chain elongation
for all of the proteoglycans and were not selective for specific
molecules.

Increases in chain length are correlated with increased
LDL binding.25,31 Therefore, the LDL-binding properties of
proteoglycans synthesized in the presence of lysoPC were
evaluated (Figure 4). Because of the finding that the ratio of small proteoglycans is altered by lysoPC, we chose to focus on peak 2 in this assay. Peak 2 from control cells bound LDL with $K_a = 1.78 \times 10^{-7}$ mol/L. Peak 2 from cells treated with 20 $\mu$mol/L lysoPC bound LDL with $K_a = 7.76 \times 10^{-8}$ mol/L, indicating higher affinity binding of these proteoglycans compared with proteoglycans from control cells. Thus, proteoglycans with increased glycosaminoglycan chain length have enhanced lipoprotein-binding properties.

**LysoPC Stimulates Core Protein Synthesis**

The ability of lysoPC to regulate band 3 was examined additionally by preparation of core proteins. The SDS-PAGE profile of [35 S]-methionine intact proteoglycans and core proteins synthesized under control conditions is shown (Figure 5, 1st and 2nd lanes). The doublet with $M_W_{app} \sim 40$ to 45 kDa represents the biglycan and decorin core proteins from intact bands 3 and 4, respectively. Treatment with lysoPC increased biglycan core protein levels 1.8-fold ($P < 0.05$) and also caused a marked 2.9-fold ($P < 0.05$) induction of a 100-kDa core protein that is not abundant under control conditions. 7-KC had no effect on either the biglycan or 100-kDa core proteins. Note that the 100-kDa band is indeed a core protein derived from an intact proteoglycan, because it appears only after CABC lyase digestion. No significant changes in other core protein bands were observed. Also note that metabolic labeling with [35 S]-methionine (Figure 5) and [35 S]-SO$_4$ (Figure 1) similarly show increased intensity of intact band 3 relative to band 4 with lysoPC treatment, indicating increased abundance of proteoglycans in band 3 evaluated (Figure 4).

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**Figure 3.** 7-KC and lysoPC cause glycosaminoglycan chain elongation. Sepharose CL-6B chromatography of chains from [35SO$_4$]-proteoglycans secreted by SMCs treated for 48 hours, as indicated. Profiles shown are representative of n=3. Dashed lines indicate the $K_w$ for peaks 1 and 2 under control conditions.

**Figure 4.** Proteoglycans synthesized in the presence of 20 $\mu$mol/L lysoPC have enhanced lipoprotein-binding properties. Peak 2 proteoglycans from SMCs treated for 48 hours were evaluated for their lipoprotein-binding capacity in a gel mobility shift assay.
rather than simply increased incorporation of $^{35}$S-SO$_4$ into glycosaminoglycan chains. Thus, lysoPC specifically increases the expression of 2 core protein molecules, 1 of which is known to be biglycan.

### 100-kDa Protein Is the Core Protein of PG-MCSF

Western analyses were performed to identify the 100-kDa protein. An antibody specific for biglycan recognizes the 45-kDa core protein but not the 100-kDa protein (Figure 6A). It also shows that lysoPC, but not 7-KC, increases biglycan expression, confirming the metabolic labeling experiments (Figure 5). In contrast, an antibody specific for PG-MCSF recognizes the 100-kDa core protein but not the 45-kDa protein. The 100-kDa protein is markedly upregulated by lysoPC (Figure 6B). Northern analyses confirm that both biglycan and MCSF mRNA levels are increased with lysoPC, although with different time courses of response (Figure 6C). MCSF mRNA is markedly increased by 2.5 hours and remains elevated up to 48 hours after exposure to lysoPC. Biglycan mRNA also increases but not until 48 hours after exposure to lysoPC. These findings indicate that the 100-kDa protein is derived from PG-MCSF, a molecule not previously identified in these cells.

### Discussion

We previously have shown that copper-oxidized LDL regulates multiple aspects of proteoglycan synthesis. These effects could be mimicked by the lipid fraction of Ox-LDL. The aim of the present study was to identify the active lipid components responsible for these effects. The major findings are 3-fold. First, 7-KC and lysoPC each are able to increase the size of all secreted proteoglycans attributable, at least in part, to chain elongation. Importantly, this is correlated with enhanced lipoprotein-binding properties of these proteoglycans. Second, lysoPC, but not 7-KC, increases synthesis of biglycan. Third, lysoPC markedly induces synthesis of PG-MCSF, a proteoglycan not previously identified in arterial SMC.

Oxidation of LDL leads to the formation of a complex array of products, of which 7-KC and lysoPC are particularly pluripotent. 7-KC is formed by cholesterol autoxidation. It is the major oxysterol in copper-oxidized LDL and the second-most abundant oxysterol in atherosclerotic lesions (after 27-OH, which is not abundant in copper-oxidized LDL). LysoPC is formed by oxidation of phosphatidylcholine in native LDL. It is abundantly present in copper-oxidized LDL and in high concentrations both in human plasma and in atherosclerotic lesions.

7-KC and lysoPC regulate chain elongation on all of the major secreted proteoglycan species. This may be functionally significant, because longer chains are correlated with increased LDL binding, and these compounds did indeed stimulate synthesis of proteoglycans with enhanced lipoprotein-binding properties. The ability of both compounds to cause chain elongation suggests nonspecific regulation, perhaps attributable to increases in the amount/activity of the enzymes responsible for chain synthesis. These enzymes are membrane-associated and may be sensitive to alterations in membrane lipid composition. This suggests a plausible, but untested, hypothesis whereby oxidized lipids perturb membrane composition, affecting function of glycosaminoglycan synthesizing enzymes. This would explain both the relatively nonspecific nature of the stimulus (multiple compounds cause chain elongation) as well as the nonspecific nature of the result (all proteoglycans are affected).

In contrast, the regulation of core protein synthesis is fairly specific. LysoPC alone regulates expression of core proteins for biglycan and PG-MCSF but not versican or decorin. Such differential regulation of proteoglycan genes has been shown for other molecular mediators of atherogenesis. Ox-LDL also selectively enhances biglycan expression; we now show that this ability is mediated by lysoPC. It is not clear how lysoPC affects core protein synthesis. Other molecules...
may play an intermediary role in these effects of Ox-LDL and lysoPC.\textsuperscript{14,15} It should be pointed out that the concentrations of lysoPC used in this study were at or below its critical micellar concentration (50 $\mu$mol/L), a concentration above which lysoPC is cytotoxic,\textsuperscript{37} and are physiologically relevant, because lysoPC is present in albumin- and lipoprotein-bound forms in plasma at even higher levels ($>200\;\mu$mol/L).\textsuperscript{38} In contrast, the concentrations of 7-KC used here exceed physiological relevance.\textsuperscript{32} Although 1 $\mu$mol/L 7-KC does increase proteoglycan size, these effects are subtle and not as dramatic as those observed with greater concentrations. However, examining the abilities of several compounds to cause chain elongation contributes to the understanding of the mechanisms by which this occurs.

The specific functions of biglycan and PG-MCSF are not known. However, there are 2 intriguingly similar potential roles for these molecules. First, the colocalization of biglycan with apo B and apo E in human coronary arteries suggests that biglycan may be important in lipoprotein retention.\textsuperscript{10} Likewise, PG-MCSF can bind LDL in vitro,\textsuperscript{12,13} suggesting that it could contribute to lipoprotein retention. Second, biglycan is reported to stimulate growth and differentiation of monocytic cells, suggesting a hemopoietic role for this proteoglycan.\textsuperscript{39} PG-MCSF is a proteoglycan form of a known
factor that promotes growth and differentiation of mononuclear phagocytic cells. It will be of interest to compare the lipoprotein-binding and hemopoietic properties of these 2 proteoglycans whose synthesis is responsive to lysoPC.

Not surprisingly, multiple oxidized lipid molecules impact proteoglycan synthesis. However, we believe the effects of lysoPC to be most relevant, in agreement with other studies. Two processes that are believed to be central to atherogenesis are the initial retention of lipoproteins by proteoglycans and the subsequent modification of retained lipoproteins by processes such as oxidation or digestion by phospholipase A2, both of which generate lysoPC. Our results suggest that lysoPC aggravates atherogenesis in several ways. Its abilities to regulate biglycan synthesis and also to cause chain elongation on all proteoglycans could dramatically enhance lipoprotein retention with additional consequences. Perhaps most interestingly, lysoPC induces PG-MCSF, a molecule not known to be synthesized by SMC. Thus, the generation of lysoPC may have important consequences via processes that promote lipoprotein retention as well as macrophage survival and differentiation.

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References


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