Class A Scavenger Receptor Up-regulation in Smooth Muscle Cells by Oxidized Low Density Lipoprotein*

ENHANCEMENT BY CALCIUM FLUX AND CONCURRENT CYCLOOXYGENASE-2 UP-REGULATION*

Received for publication, September 23, 1999, and in revised form, February 7, 2000

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Oxidative stress caused by phorbol esters or reactive oxygen up-regulates the class A scavenger receptor (SR-A) in human smooth muscle cells (SMC), which normally do not express this receptor. The increase in SR-A expression correlates with activation of the redox-sensitive transcription factors activating protein-1 c-Jun and CCAAT enhancer-binding protein β. Here we show that coincubation of SMC with macrophages or oxidized low density lipoproteins (LDL) from macrophage-conditioned medium activates these same regulatory pathways and stimulates SR-A expression. The increased SR-A gene transcription induced by cell-oxidized LDL up-regulated SR-A mRNA and increased by 30-fold the uptake of acetyl LDL, a ligand for the SR-A. Copper-oxidized LDL also increased SR-A receptor expression. Oxidized LDL with a lipid peroxide level of 80–100 nmol/mg of LDL protein and an electrophoretic mobility ~1.5 times that of native LDL exhibited the greatest bioactivity. Inhibition of calcium flux suppressed SR-A induction by oxidized LDL. Conversely, calcium ionophore greatly enhanced SR-A up-regulation by oxidized LDL or other treatments that promote intracellular oxidative stress. This enhancement was dependent upon concurrent up-regulation of SMC cyclooxygenase-2 expression and activity and was blocked by the cyclooxygenase-2 inhibitors NS-398 and Resveratrol. In THP-1 cells, oxidized LDL induced monocyte-to-macrophage differentiation and increased SR-A expression. These findings support a role for mildly oxidized LDL in the redox regulation of macrophage differentiation and SR-A expression and suggest that increased vascular oxidative stress may contribute to the formation of both SMC and macrophage foam cells.

The traditional risk factors for atherosclerosis, which include hypercholesterolemia, hypertension, cigarette smoking, diabetes, and high fat diet, have all been associated with endothelial dysfunction (1). Under these conditions, circulating monocytes adhere to the arterial endothelium, migrate to the subendothelial space, and differentiate into resident macrophages within the subendothelial cell matrix. The differentiated cells express scavenger receptors that take up modified lipoproteins, leading to a massive accumulation of cholesterol esters and the appearance of foam cells. These macrophage-derived foam cells make up the fatty streak lesions that precede more advanced atherosclerotic lesions, which can ultimately cause thrombosis and myocardial infarction (2).

Advanced atherosclerotic lesions also contain smooth muscle cells (SMC), which migrate from the media of the blood vessels to the neointima, where they proliferate. Intimal SMC can also accumulate large amounts of cholesterol esters and become foam cells. Although the cholesterol that accumulates in foam cells is derived from lipoproteins, the mechanisms by which this occurs are not fully understood. Plasma lipoproteins are oxidatively modified in the subendothelial matrix, where they cause the release of cytokines that attract monocytes to the subendothelial space (3). With further oxidation, these low density lipoproteins (LDL) become ligands for several scavenger receptors on macrophages. Since they are not down-regulated as the intracellular content of cholesterol increases, these receptors are thought to contribute to the excessive uptake of modified lipoproteins and to the lipid engorgement characteristic of macrophage-derived foam cells (4). The first of these scavenger receptors to be cloned was the class A scavenger receptor (SR-A) (5). Knockout mice lacking this receptor were resistant to the development of atherosclerosis, suggesting that the SR-A contributes to the uptake of modified lipoproteins and to cholesterol ester accumulation in macrophages in vivo (6).

How SMC in the artery wall accumulate lipid is less clear, since SMC were initially thought to be devoid of scavenger receptors. We have shown, however, that SMC express SR-A and that receptor expression is regulated over a wide range (7–10). SR-A activity is induced in SMC by treatments that increase intracellular oxidative stress, such as phorbol esters and the combination of H2O2 and vanadate (11). Certain growth factors that increase SMC SR-A activity (10) have also been associated with increased oxidative stress, including interleukin-1, tumor necrosis factor α (12), epidermal growth factor (13), platelet-derived growth factor (10, 14), and transforming growth factor β (10, 15). The latter two factors are

* This work was funded in part by National Institutes of Health Program Project Grant HL-47660. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: SMC, smooth muscle cells(s); AA, arachidonic acid; LDL, low density lipoproteins(s); AcLDL, acetylated LDL; AP-1, activating protein 1; bp, base pair(s); BAPTA, 1,2-bis(O-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid; C/EBP, CAAT/enhancer-binding protein; COX, cyclooxygenases; DI, 1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; FBS, fetal bovine serum; FPLC, fast performance liquid chromatography; H/V, combination of hydrogen peroxide (100 μM) and sodium orthovanadate (10 μM); JNK, c-Jun amino-terminal activating kinase; PGE2, prostaglandin E2; PLA, phospholipase A; sPLA, cytoplasmic phospholipase A; tPLA, extracellular group II PLA; ROS, reactive oxygen species; SR-A, class A scavenger receptor; PCR, polymerase chain reaction.
Scavenger Receptor Regulation in SMC

Regulation of Human SMC SR-A by Macrophages—Platelet secretory products and ROS, as well as certain cytokines and growth factors alone and in combination, synergistically increase SR-A expression in SMC (7, 10, 22, 23, 31). Since activated macrophages in atherosclerotic lesions secrete several of these factors and are in intimate contact with SMC, we hypoth-

**EXPERIMENTAL PROCEDURES**

**Materials and Cells**—Fetal bovine serum (FBS), Dulbecco’s phosphate-buffered saline, and the fluorescent probe 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) were obtained as described previously (7). Phorbol 12-myristate 13-acetate, sodium orthovanadate (vanadate), H2O2, Resveratrol, EGTA, BAPTA, diltiazem, and verapamil were from Sigma. Mibefradil was obtained from New Zealand White rabbits by peritoneal lavage 3 days prior to a C/EBP site in the SR-A promoter 21 (bp) relative to the transcriptional start site was also necessary for full up-regulation of SR-A expression in SMC (22).

SMC SR-A activity is up-regulated in vivo by an atherogenic diet in rabbits (23), suggesting that hyperlipidemia also contributes to intracellular oxidative stress, thereby altering SMC gene expression either directly or through paracrine factors secreted by local inflammatory cells. Cytokines known to be secreted by activated leukocytes up-regulated SR-A expression in rabbit SMC in vitro (23). In developing atherosclerotic lesions, intimal SMC are closely associated with macrophages that secrete numerous growth factors, cytokines, and ROS (24), suggesting that factors secreted by macrophages might interact with SMC to increase SR-A expression. In this study, we tested the hypothesis that coculture of SMC with macrophages would increase the SMC SR-A activity. Here we report that coculture with macrophages increased SR-A expression in SMC; however, the enhanced SR-A activity was caused by cell-oxidized LDL and not by growth factors or cytokines secreted by the macrophages. Further characterization of the mechanism of receptor up-regulation revealed a role for calcium flux and cyclooxygenase (COX)-2.

**Prostaglandin E2 Measurement**—Prostaglandin E2 (PGE2), a specific product of COX-2-catalyzed arachidonic acid (AA) metabolism, was measured with a monoclonal enzymatic immunoassay (Cayman Chemical Co., Ann Arbor, MI).

**RESULTS**

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**RESULTS**
esized that macrophage products might up-regulate SMC SR-A activity. To test this hypothesis, we cocultured SMC and rabbit peritoneal macrophages for 24 h and used fluorescence microscopy to assess the uptake of DiI-labeled AcLDL. Coincubated SMC internalized labeled AcLDL, indicating increased SR-A activity, whereas the control cells did not (Fig. 1). Conditioned medium alone also increased SR-A activity, indicating that cell-cell contact was not required (Fig. 2). However, the increased activity did require lipoproteins. Conditioned medium containing LDL (100 μg/ml) or 10% FBS increased SMC SR-A activity 25–30-fold, whereas lipoprotein- or serum-free conditioned medium was inactive (Fig. 2).

Next, we determined whether the bioactivity resided within the LDL or was mediated by factors secreted by the macrophages in response to incubation with LDL. LDL were isolated from conditioned medium by FPLC and tested for their ability to up-regulate SMC SR-A activity. The column fractions that contained the LDL, as determined by cholesterol content, coincided with the fractions that up-regulated SMC SR-A activity (Fig. 3). To confirm that the LDL fraction contained the active component, LDL (d = 1.063 g/ml) were reisolated from conditioned medium by ultracentrifugation and tested for bioactivity. The uptake of DiI-labeled AcLDL increased markedly in SMC incubated with the “top” lipoprotein-containing fraction (21.8 ± 0.9-fold over control cells); the “bottom” nonlipoprotein fraction had minimal activity (2.7 ± 0.3-fold increase over control).

To rule out the possibility that cytokines or growth factors secreted by the macrophages contributed to the increased SMC SR-A activity, we performed antibody blocking experiments. Under conditions that prevent platelet secretory products from increasing SR-A activity in SMC (10), antibodies to interleukin-1, interleukin-6, platelet-derived growth factor, fibroblast growth factor, and tumor-like growth factor, and transforming growth factor β, alone and in combination, did not block the bioactivity in the conditioned medium (data not shown).

Chemically Oxidized LDL Up-regulate SMC SR-A Activity—Because LDL incubated with cells undergo oxidative modification, we hypothesized that oxidatively modified LDL were the active species in the conditioned medium. We tested this hypothesis in three ways. First, the active preparations of LDL from cell-conditioned medium were assayed for lipid peroxide level and electrophoretic mobility. The maximal ability to increase SR-A activity correlated with mild lipoprotein oxidation, as indicated by a peroxide level of 80–100 nmol/mg LDL protein and a relative electrophoretic mobility ~1.5 times greater than that of native LDL (data not shown). The thiobarbituric acid-reactive substance content of the active LDL preparations was consistently <10 nmol/mg LDL protein. More extensively oxidized LDL were toxic to the SMC (data not shown).

Second, LDL were incubated for 6 h with THP-1 cells in the presence of EDTA (200 μM) and butylated hydroxytoluene (20 μM) to prevent oxidation. Under these conditions, no lipid peroxides formed, the electrophoretic mobility of the LDL was unchanged, and the conditioned medium did not up-regulate SR-A (data not shown).

Third, we evaluated the ability of mildly chemically oxidized...
LDL to activate SMC SR-A expression. Chemically modified LDL with lipid hydroperoxide levels and electrophoretic mobilities similar to those of cell-modified LDL up-regulated SMC SR-A activity 10-fold more than native LDL; however, the level of SR-A activation was notably less than when cell-modified LDL were used (Fig. 4). This difference in bioactivity might have resulted from differences in the specific oxidized lipids formed by the two oxidation methods or from effects of additional bioactive factors released by activated macrophages into the medium.

AP-1/c-Jun Is Induced and Activated in SMC by Cell-modified LDL—Up-regulation of SR-A activity in SMC treated with phorbol ester or H/V is dependent upon increased binding of the AP-1/c-Jun transcription factor to SR-A promoter elements (22). We therefore hypothesized that incubation of SMC with cell-modified LDL would increase the level of this transcription factor. Electrophoretic mobility shift assays showed a time-dependent increase in AP-1 binding in nuclear extracts from human SMC incubated with cell-modified LDL; supershift analyses identified c-Jun and c-Fos in the binding complex, and competition studies confirmed the specificity of binding (Fig. 5A). Because AP-1/c-Jun must be phosphorylated to be active, we examined the activity of the specific c-Jun-activating kinase, JNK. Increased JNK activity was present within the SMC lysates after incubation with conditioned medium (Fig. 5B).

C/EBPβ Is Induced and Processed in SMC Treated with Cell-modified LDL—The SR-A promoter also contains a C/EBP binding site, and full SR-A transcriptional activity in SMC requires C/EBPβ as well as AP-1/c-Jun binding (22). Electrophoretic mobility shift assays showed greater C/EBP binding to nuclear extracts from SMC treated with cell-modified LDL than to nuclear extracts from untreated control SMC (Fig. 6A). The binding activity was supershifted with C/EBPβ antibody but not with antibody to the related transcription factor GADD 153 (Fig. 6B). The specificity of C/EBPβ binding was further demonstrated by complete competition with unlabeled oligonucleotide corresponding to the consensus C/EBP wild-type sequence but not with a mutant sequence containing an 8-bp substitution in the binding motif. The central band in the tripartite binding complex was consistently more prominent at the later time points (Fig. 6B). Variable band intensities in the complex C/EBPβ binding pattern are due to the differential translational and posttranslational processing characteristic of the C/EBPβ trans-activator protein (32).

Cell-modified LDL and Calcium Ionophore Increase SR-A Activity—Treatment with H/V increases intracellular oxidative stress and activates redox-sensitive SR-A gene expression in SMC (22). Because oxidants stimulate calcium signaling (17, 18), we examined the effect of the calcium ionophore A23187 on the ability of H/V or cell-modified LDL to regulate receptor activity (Fig. 7A). Incubation of SMC with A23187 and either H/V or cell-modified LDL significantly increased SR-A activity. The up-regulation was 3.4-fold greater than was obtained with H/V alone and 2.2-fold greater than with conditioned medium alone. Cotreatment with nickel chloride, a calcium channel blocker that also exerts intracellular effects on calcium, completely blocked the ionophore-induced increase in SR-A activity.
Three specific calcium channel blockers, diltiazam, verapamil, and mibefradil, did not suppress SR-A up-regulation by either H/V or cell-modified LDL (data not shown). However, chelation of either extracellular or intracellular calcium with EGTA or BAPTA, respectively, partially blocked the up-regulation of SR-A activity by H/V plus A23,187 (Fig. 7B). Together, they almost completely inhibited SMC SR-A up-regulation by H/V plus A23,187. SR-A up-regulation by cell-modified LDL was also partially suppressed by both EGTA and BAPTA, but their effects on the up-regulation of receptor activity by cell-modified LDL were not additive.

The enhancement of SMC SR-A expression by calcium ionophore appeared to be mediated by effects at both the transcriptional and posttranscriptional levels. Although ionophore did not augment AP-1 binding on electrophoretic mobility shift assays (data not shown), it did increase phosphorylation of c-Jun (Fig. 8), indicating more sustained activation of JNK, consistent with the promotion of AP-1/c-Jun-dependent transcription. Interestingly, the ionophore may promote alternative splicing or RNA editing that favors the SR-AII mRNA transcript. Reverse transcription-PCR of mRNA isolated after treatment with H/V or cell-modified LDL showed a synergistic increase in SR-AII mRNA with the addition of ionophore (Fig. 9). A slight but reproducible increase in SR-AI mRNA was observed under the same conditions that strongly up-regulated SR-AII mRNA; however, ionophore did not further increase SR-AI mRNA (Fig. 9).

**COX-2 Up-regulation Correlates with Enhanced SR-A Expression in the Presence of Calcium Ionophore**—Because the COX-2 and SR-A gene promoters have similar transcription factor binding sites (22, 33), we hypothesized that COX-2 expression would be regulated by factors that increased SR-A expression in SMC. Indeed, both H/V and cell-modified LDL increased COX-2 mRNA levels, and the increase after either treatment was further enhanced by calcium ionophore (Fig. 9).

Next, to determine whether the increase in COX-2 activity is important for the up-regulation of SMC SR-A activity, we evaluated the effects of two COX-2 inhibitors on the uptake of DiI-labeled AcLDL by SMC treated with H/V plus ionophore (Fig. 10A). With H/V plus A23,187 alone, the mean fluorescence intensity of the cells was 292, indicative of a high level of uptake of DiI-labeled AcLDL. Both NS-398, a specific inhibitor of COX-2 activity, and Resveratrol, a phenolic antioxidant that inhibits COX-2 at the transcriptional and posttranscriptional
levels (33), essentially blocked the ionophore-induced increase in SR-A activity, shifting the mean relative fluorescence intensity to 16 and 19, respectively. The mean basal fluorescence was 17. Both inhibitors also substantially inhibited the ionophore-induced increase in SR-A activity in SMC treated with cell-modified LDL (Fig. 10B), reducing the mean fluorescence intensity from 123 for the cells treated with cell-modified LDL plus A23,187 to 31 (NS-398) and 53 (Resveratrol).

Although treatment with ionophore alone significantly increased COX-2 mRNA levels (Fig. 9), treatment of SMC with ionophore alone did not increase SR-A mRNA or activity. These data suggest either that increased COX-2 activity is not sufficient for up-regulation of SMC SR-A or that increased COX-2 mRNA does not necessarily correlate with COX activity. We
ionophore-stimulated production of PGE2. Therefore, under
increase. Resveratrol and NS-398 also blocked the calcium
I). Coincubation with Resveratrol or NS-398 abolished this
expression of ionophore alone and ionophore with H/V (Fig. 9), PGE2
levels. Although COX-2 mRNA levels were similar in the pres-
duction of bioactive COX-2 metabolites (Table I). PGE2 produc-
tion did not always correlate with increased COX-2 mRNA
increased COX-2 mRNA levels were associated with the pro-
product of COX-mediated AA metabolism, to determine if the
inhibition of SMC COX-2 activity and PGE2 production
ucts of AA metabolism contribute to increased SR-A expres-
sion. Inhibition of SMC COX-2 activity as measured by PGE2 production correlated
with increased SR-A expression. In SMC treated with H/V and
ionophore, both NS-398 and Resveratrol significantly inhibited
the ionophore-induced increases in PGE2 levels and, as
described above, the enhancement of SR-A up-regulation.

Similar results were obtained with cell-modified LDL (Table
I). The conditioned medium containing cell-modified LDL had a
PGE2 level of 2560 pg/ml, reflecting secretion into the medium
by the THP-1 cells. However, when SMC were incubated in this
conditioned medium, the PGE2 level increased 3.5-fold (Table
I). Coincubation with Resveratrol or NS-398 abolished this
increase. Resveratrol and NS-398 also blocked the calcium
ionophore-stimulated production of PGE2. Therefore, under
conditions in which ionophore increases SR-A activity, both
COX-2 mRNA and PGE2 levels in the medium were considerably
increased. This finding suggests that the metabolic prod-
ucts of AA metabolism contribute to increased SR-A expres-
sion. Inhibition of SMC COX-2 activity and PGE2 production correlated with decreased SR-A activity.

While PGE2 is a readily identifiable marker for COX-2 ac-
tivity, it is not likely to be the sole eicosanoid responsible for
increased COX-2-dependent SR-A expression. Purified PGE2
added directly to SMC did not induce SR-A activity (data not
shown). Because PGE2 is only one of a large family of eico-
sanoids downstream of COX-catalyzed AA metabolism, its
identification in the medium implies the presence of a full
complement of prostaglandins. These findings suggest that H/V
treatment triggers enzymatic AA metabolism in SMC and that
ionophore significantly enhances this process. Much higher
levels of AA metabolites generated by COX-2-mediated path-
ways can be identified after SMC treatment with cell-modified
LDL. The addition of ionophore minimally increased the levels
of AA metabolites beyond those achieved with cell-modified
LDL alone (Table I). The full biological consequences of AA
metabolites are not known, but the inhibition data presented in
Table I suggest that the eicosanoids contribute to SMC SR-A
expression.

**TABLE I**

| Treatment | PGE2 (pg/ml) |
|-----------|--------------|
| None      | ND           |
| A23,187   | 278          |
| H/V       | 427          |
| H/V + A23,187 | 2,563  |
| H/V + A23,187 + NS-398 | 212  |
| H/V + A23,187 + Resveratrol | 384  |
| CM LDL*   | 8,995        |
| CM LDL + NS-398 | 2,487  |
| CM LDL + Resveratrol | 2,098 |
| CM LDL + A23,187 | 10,500  |
| CM LDL + A23,187 + NS-398 | 2,642  |
| CM LDL + A23,187 + Resveratrol | 2,357 |

* The medium containing cell-modified LDL before incubation with
the SMC contained 2,560 pg/ml PGE2.

therefore analyzed the conditioned medium for PGE2, a specific product of COX-mediated AA metabolism, to determine if the
increased COX-2 mRNA levels were associated with the pro-
duction of bioactive COX-2 metabolites (Table I). PGE2 production
did not always correlate with increased COX-2 mRNA
levels. Although COX-2 mRNA levels were similar in the pres-
ence of ionophore alone and ionophore with H/V (Fig. 9), PGE2
levels were 9.2-fold higher in the latter case (Table I). Increased
COX-2 activity as measured by PGE2 production correlated with increased SR-A expression. In SMC treated with H/V and
ionophore, both NS-398 and Resveratrol significantly inhibited
the ionophore-induced increases in PGE2 levels and, as
described above, the enhancement of SR-A up-regulation.

Similar results were obtained with cell-modified LDL (Table
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LDL alone (Table I). The full biological consequences of AA
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Table I suggest that the eicosanoids contribute to SMC SR-A
expression.

**FIG. 11.** Effect of treatment with cell-modified or Cu2+-oxi-
dized LDL on THP-1 cells. THP-1 cells in suspension were incubated
for 24 h at 37 °C with the indicated LDL treatments (all at 100 μg/ml)
and washed, and the adherent cells were incubated for 15–18 h with
DiI-labeled AcLDL (5 μg/ml). Both phase (left panels) and fluorescence
(right panels) photomicrographs are shown. The modified LDL in-
creased adhesion and SR-A activity; native LDL had no effect.

**Cell-modified LDL, but Not Native LDL, Induce Adhesion,**
**Monocyte-to-macrophage Differentiation, and SR-A Expression**
in **THP-1 Cells**—Next, we determined whether cell-modified or
chemically oxidized LDL, like H/V treatment (22), would in-
duced monocyte differentiation and up-regulate SR-A expres-
sion. Treatment of nonadherent THP-1 cells with cell-modified
LDL (50 μg/ml) induced both cell adherence and SR-A gene
expression (Fig. 11). Treatment with copper-oxidized LDL re-
sulted in the adherence of fewer cells but had a qualitatively
similar effect on SR-A activity; native LDL had no effect.
Interestingly, in THP-1 cells, treatment with H/V or cell-modified
LDL markedly increased both SR-AI and SR-AII mRNA levels.
Unlike the observation in SMC, calcium ionophore did not
appreciably enhance these high levels of induction (data not
shown).

**DISCUSSION**

This study shows that coincubation with macrophages or
with macrophage-conditioned medium increases SMC SR-A
activity. Interestingly, this effect was mediated by cell-modi-
fied LDL, not by macrophage-secreted cytokines or growth
factors. Chemically oxidized LDL also increased SMC SR-A
activity. The SR-A gene expression mediated by cell-modified
LDL resulted from increased levels of the redox-sensitive tran-
scription factors AP-1/c-Jun and C/EBPβ and from increased
JNK activity. These transcription factors are important for
SMC SR-A up-regulation by phorbol esters and by ROS (22). In
addition, cell-modified LDL, but not native LDL, induced
THP-1 cell adhesion and SR-A expression. The increase in
SR-A activity in SMC was enhanced by calcium ionophore and
was associated with increased COX-2 activity. These findings
demonstrate that oxidized lipoproteins in the arterial wall can
up-regulate the scavenger receptors responsible for their clear-
Scavenger Receptor Regulation in SMC

The regulation of redox-sensitive transcription factors by cell-modified LDL suggests that oxidatively modified lipoproteins are a source of intracellular oxidative stress. This possibility is consistent with the increased levels of intracellular lipid peroxidation products and ROS in endothelial cells treated with oxidized LDL (34). Multiple lines of defense against oxidative stress in tissues and cells are overcome in pathological conditions such as atherosclerosis (35). The effect of oxidized LDL on redox balance in the vascular space depends upon the type and extent of oxidation (36). Minimally modified LDL induce the expression of genes encoding macrophage colony-stimulating factor (37), monocyte chemoattractant protein 1 (38), and tissue factor (39), all of which, like the SR-A gene (22), are induced by redox-sensitive transcription factors (40–42). While mounting evidence suggests that oxidants play a critical role in the regulation of gene expression, the pathways for redox-regulated signal transduction remain largely speculative (16). Oxidized LDL up-regulate the class B scavenger receptor CD36 and the SR-AI and SR-AII in macrophages (43, 44). In macrophages, CD36 expression, but not SR-A expression, results from peroxylipid activation of peroxisome proliferator-activated receptor-γ-dependent transcription (45). No treatment in the present study induced CD36 in SMC (data not shown). The transcriptional pathways leading to SR-A induction in either macrophages or SMC by oxidized LDL have not been defined. However, oxidized LDL induce C/EBPβ in monocyte/macrophages (46) and AP-1 in human arterial SMC (47). Both of these transcription factors are important for SMC SR-A induction by treatments that induce intracellular oxidative stress (11, 22) and, as shown here, are increased by moderate cell-mediated oxidation of LDL. AP-1 up-regulation by oxidized LDL in SMC has been attributed to the lysophosphatidylcholine content of the oxidized lipoprotein (47). Lysophosphatidylcholine also induces intracellular calcium flux (48). Extensive oxidation of LDL leads to cytotoxicity and apoptosis (36), cellular processes also associated with calcium flux (49).

Oxidants stimulate Ca²⁺ signaling by increasing cytosolic Ca²⁺ concentration (17, 18). The cellular oxidative stress generated by oxidized LDL has been associated with a rise in free cytosolic calcium (50). While the exact source of calcium release has yet to be defined, enhanced Ca²⁺ transport through Ca²⁺ channels (51), inhibition of Ca²⁺ pumps (52), and Ca²⁺ release from intracellular stores (17) have all been described in the presence of oxidants. Our current findings demonstrate that SMC SR-A gene activation is strikingly dependent on calcium flux. SR-A gene expression was effectively inhibited by the nonspecific calcium channel blocker nickel chloride and enhanced by the calcium ionophore A23,187. Specific calcium L and T type channel blockers, however, did not block SR-A up-regulation as NiCl₂ did. NiCl₂ is a potent, but nonspecific, calcium channel blocker whose mechanism of action is not completely understood. Besides blocking the influx of calcium into cells, NiCl₂ inhibits intracellular calcium mobilization and phospholipase C activation (53). The virtually complete suppression of SR-A up-regulation by NiCl₂ may result from a combination of these effects.

The importance of calcium mobilization for the redox regulation of SR-A gene expression is underscored by the ability of calcium chelators to suppress SR-A induction by H/V plus A23,187 (Fig. 7). It is not evident why SR-A up-regulation by cell-modified LDL was only partially inhibited by the same combination of intra- and extracellular calcium chelators. Possibly, the THP1-generated eicosanoids already present in medium containing THP1-cell-modified LDL (see Table I) can promote intracellular oxidative stress without additional calcium mobilization. The mechanism of further calcium mobilization by cell-oxidized LDL has not been established. It is possible that bioactive lipid peroxides affect cell membranes, physically disrupting ionic homeostasis (54, 55). Because oxidized LDL induce a significant calcium flux, calcium ionophore augments SR-A expression to a lesser extent than was observed after treatment with H/V.

Membrane lipid peroxidation is associated with phospholipid hydrolysis by Ca²⁺-dependent phospholipase A₂ (PLA₂) (56). An increased net negative charge of membranes increases the binding affinity for Ca²⁺ (57), serving to catalyze PLA₂ (58). In macrophages, a sustained increase in intracellular calcium induced by A23,187 is sufficient to activate PLA₂ and release AA from phospholipids (59). Reasoning that this may also be true in SMC induced to take on macrophage-like properties, such as SR-A gene expression, we evaluated certain bioactive products of AA metabolism. AA is specifically metabolized by COX-1 and COX-2. Normally, the majority of COX expressed within vessels is in the constitutive form, COX-1, found mainly in the endothelial layer, with much lower levels being present in the underlying smooth muscle (60). However, data from animal models have suggested that when the endothelium is compromised or vessels are damaged, the inducible isoform, COX-2, can be expressed in vascular smooth muscle (61). Furthermore, the COX-2 promoter, like the SR-A promoter, contains a critical redox AP-1/c-Jun binding site as well as an upstream CEBP/β binding element (33). And the COX-2 gene, like the SR-A gene, is markedly induced after stimulation with cytokines, growth factors, or tumor promoters (62–64).

We found that factors that increase SR-A activity up-regulated COX-2 mRNA and that COX-2 activity correlated with increased SR-A expression. Furthermore, COX-2 inhibitors blocked the SR-A up-regulation. Calcium ionophore may at least in part mediate the enhancement of SR-A gene expression by providing AA substrate for COX-2. COX-2 induction alone does not greatly increase prostanoid production in vivo. A second, AA-liberating stimulus is also required (65). A concomitant increase in calcium-dependent PLA₂ activity and mobilization of AA is presumed to supply the COX-2 enzymatic system with substrate.

The extracellular group II PLA₂ (sPLA₂) has been implicated in numerous inflammatory conditions, and transgenic mice overexpressing sPLA₂ exhibit dramatically increased atherosclerosis on a high fat diet (66). This increase has been attributed to the finding that polyunsaturated free fatty acids, which are liberated by sPLA₂, increase the formation of bioactive phospholipids in oxidized LDL (67). PLA₂ has also been implicated in the up-regulation of SR-A expression in cultured aortic SMC (68). Interestingly, although a sustained increase in calcium is sufficient to induce AA release (59), we found that ionophore alone is not sufficient to induce significant SR-A expression. The ability of calcium ionophore to up-regulate COX-2 expression in SMC indicates that the presence of COX-2 and AA together is also insufficient for SR-A induction. Ionophore treatment alone did not affect AP-1/c-Jun binding (data not shown) or JNK activity (Fig. 5B). Therefore, concomitant up-regulation of AP-1/c-Jun and PLA₂-mediated AA release and COX-2 induction appear to be necessary for maximal up-regulation of SMC SR-A activity. The mechanism for this synergy remains under investigation. It is tempting to speculate that COX-2-mediated metabolism of AA contributes to intracellular oxidative stress by the generation of pro-inflammatory eicosanoids. Indeed, cytomegalovirus-induced generation of oxidative stress in SMC has been attributed to COX-2 activity (69).
Scavenger Receptor Regulation in SMC

Acknowledgments—We thank James McGuire for technical assistance, Stephen Ordway and Gary Howard for editorial support, September Plumlee for manuscript preparation, and Stephen Gonzales and John Carroll for photography and graphics.

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Scavenger Receptor Regulation in SMC

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17670

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