Statins Modulate Oxidized Low-Density Lipoprotein-Mediated Adhesion Molecule Expression in Human Coronary Artery Endothelial Cells: Role of LOX-1

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Received February 18, 2002; accepted March 27, 2002

ABSTRACT

LOX-1, a receptor for oxidized low-density lipoprotein (ox-LDL), plays a critical role in endothelial dysfunction and atherosclerosis. LOX-1 activation also plays an important role in monocyte adhesion to endothelial cells. A number of studies show that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) reduce total LDL cholesterol and exert a cardio-protective effect. We examined the modulation of LOX-1 expression and its function by two different statins, simvastatin and atorvastatin, in human coronary artery endothelial cells (HCAECs). We observed that ox-LDL (40 μg/ml) treatment up-regulated the expression of E- and P-selectins, VCAM-1 and ICAM-1 in HCAECs. Ox-LDL mediated these effects via LOX-1, since antisense to LOX-1 mRNA decreased LOX-1 expression and subsequent adhesion molecule expression. Pretreatment of HCAECs with simvastatin or atorvastatin (1 and 10 μM) reduced ox-LDL-induced expression of LOX-1 as well as adhesion molecules (all P < 0.05). A high concentration of statins (10 μM) was more potent than the low concentration (1 μM) (P < 0.05). Both statins reduced ox-LDL-mediated activation of the redox-sensitive nuclear factor-κB (NF-κB) but not AP-1. These observations indicate that LOX-1 activation plays an important role in ox-LDL-induced expression of adhesion molecules. Inhibition of expression of LOX-1 and adhesion molecules and activation of NF-κB may be another mechanism of beneficial effects of statins in vascular diseases.

Endothelial dysfunction elicited by ox-LDL plays a critical role in the pathogenesis of atherosclerosis (Witztum and Steinberg, 1991). Ox-LDL changes the secretory activities of endothelium and causes endothelium to become dysfunctional (Erl et al., 1998). Ox-LDL inhibits the expression of endothelial nitric-oxide synthase (eNOS) (Keaney et al., 1996), induces expression of adhesion molecules on the endothelium, and facilitates monocyte adhesion to intima (Mehta et al., 1995).

Scavenger receptors on macrophages and smooth muscle cells are believed to mediate the biological role of ox-LDL (Sakai et al., 1998). Recent studies show that LOX-1, a novel lectin-like receptor for ox-LDL, facilitates the uptake of ox-LDL and mediates several of the biological effects of ox-LDL in endothelial cells (Sawamura et al., 1997; Mehta and Li, 1998). LOX-1 mediates ox-LDL-induced apoptosis in endothelial cells (Li and Mehta, 2000b) and phagocytosis of aged and apoptotic cells (Oka et al., 1998). Ox-LDL, angiotensin II, inflammatory cytokines, and shear stress up-regulate the expression of LOX-1 gene (Kume et al., 1998; Mehta and Li, 1998). LOX-1 mediates ox-LDL-induced cell death in endothelial cells (Li and Mehta, 2000b) and phagocytosis of aged and apoptotic cells (Oka et al., 1998). Ox-LDL, angiotensin II, inflammatory cytokines, and shear stress up-regulate the expression of LOX-1 gene (Kume et al., 1998; Mehta and Li, 1998). LOX-1 expression is up-regulated in atherosclerotic tissues in rabbits and humans (Kataoka et al., 1999; Chen et al., 2000).

The development of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) has been a major milestone in the primary and secondary prevention of coronary heart disease. These agents, besides lowering total and LDL cholesterol, have a multitude of other effects, which may have a bearing on the cardioprotective effect of these agents (Luscher et al., 1996). In a recent study (Li et al., 2001), we showed that two different statins, atorvastatin and simva-
statin, decrease LOX-1 expression and block LOX-1-mediated uptake of ox-LDL.

In the present study, we investigated 1) whether LOX-1 mediates ox-LDL-induced expression of genes for adhesion molecules; 2) whether statins inhibit the expression of adhesion molecules by ox-LDL; and 3) whether transcription factors NF-κB and AP-1 play a role in the interaction between ox-LDL and statins.

We carried out these studies in human coronary artery endothelial cells (HCAECs). As such, data from these studies may relate to the effect of statins in coronary heart disease in man.

Materials and Methods

Cell Culture. We have earlier described the methodology for culture of HCAECs (Mehta and Li, 1998; Li and Mehta, 2000a,b). The initial batch of HCAECs was purchased from Clonetics Corporation (San Diego, CA). The endothelial cells were pure based on morphology and staining for factor VIII-related antigen and acetylated LDL. These cells were 100% negative for α-actin smooth muscle expression.

Study Design. Fourth generation HCAECs (~70% confluence) were incubated with ox-LDL (40 μg/ml) for 24 h to determine the expression of LOX-1, and adhesion molecules E- and P-selectins, VCAM-1 and ICAM-1.

To examine the receptor specificity of ox-LDL action, HCAECs were transfected with antisense or sense to LOX-1 mRNA (LOX-1-AS or LOX-1-S, each 0.5 μM) for 48 h (Li and Mehta, 2000a,b) and then exposed to ox-LDL for 24 h. The harvested cells were used to measure expression of adhesion molecules.

To determine the effects of statins on the expression of LOX-1 and adhesion molecules, we pretreated HCAECs with simvastatin or atorvastatin (each 1 μM) for 48 h (Li and Mehta, 2000a,b) and then exposed the cells to ox-LDL (40 μg/ml) for 24 h; thereafter, uptake of ox-LDL and activity of NF-κB and AP-1 were determined.

The concentration of all reagents and the duration of incubation were chosen based on previous studies (Hernandez-Perara et al., 1998; Mehta and Li, 1998; Li and Mehta, 2000a).

Preparation of Lipoproteins. We prepared native LDL and ox-LDL as described earlier (Mehta and Li, 1998; Li and Mehta, 2000a). The thiobarbituric acid reagents content of ox-LDL was 16.2 ± 0.28 versus 0.56 ± 0.16 mmol/100 μg of protein in the native-LDL preparation (P < 0.01). Ox-LDL was extensively dialyzed against Tris-saline, kept in 50 mM Tris-HCl, 0.15 M NaCl, and 2 mM EDTA at pH 7.4, and used within 10 days of preparation. The endothelin toxin level was measured by the E-Toxate kit (Sigma-Aldrich), membranes were incubated with 1:1000 dilution primary antibody (monoclonal antibody to LOX-1 (Sawamura et al., 1997); polyclonal antibody to E- and P-selectins, VCAM-1 or ICAM-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)) overnight at 4°C. Membranes were washed and then incubated with 1:2000 dilution secondary antibody (Amersham Biosciences, Inc., Piscataway, NJ) for 1 h, and the membranes were detected with the enhanced chemiluminescence system, and relative intensities of protein bands were analyzed by Scan-gel-it software (Mehta and Li, 1998; Li and Mehta, 2000a,b).

Electrophoretic Mobility Shift Assay. Isolation of nuclear fraction was accomplished following the previously published procedure (Li and Mehta, 2000a). Oligonucleotides containing the consensus sequence for AP-1 and NF-κB were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase and purified using Chroma Spin-10 columns. The labeled oligonucleotides were incubated with the nuclear fractions for 30 min at room temperature in 50 mM Tris-HCl buffer, pH 7.5, containing 20% glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, and 0.25 mg/ml poly(dI-dC). The products were separated by electrophoresis in a 4% nondenaturing polyacrylamide gel using 0.5× TBE (45 mM Tris/borate and 1 mM EDTA) as the running buffer. The gels were dried and exposed to a radiographic film.

Data Analysis. All data represent the mean of six independently performed experiments. Data are presented as mean ± S.D. Statistical significance was determined in multiple comparisons among independent groups of data in which analysis of variance and the F test indicated the presence of significant differences. A P value ≤0.05 was considered significant.

Results

Ox-LDL-Induced Expression of Adhesion Molecules and the Effect of Statins. Incubation of HCAECs with ox-LDL (40 μg/ml) for 24 h increased the expression of E- and P-selectins, VCAM-1 and ICAM-1 (mRNA and protein) (all P < 0.01 compared with control). Pretreatment of HCAECs with either simvastatin or atorvastatin (1 and 10 μM) for 30 min decreased the expression of these adhesion molecules (all P < 0.05). A high concentration of simvastatin or atorvastatin (10 μM) had a greater effect than the low concentration (1 μM) (both P < 0.05) (Fig. 1). In parallel experiments, incubation of HCAECs with simvastatin or atorvastatin (10 μM) alone or native LDL did not affect expression of these adhesion molecules.

Role of LOX-1 in the Expression of Adhesion Molecules. We have previously shown that LOX-1-AS blocks ox-LDL-mediated increase in LOX-1 (Li and Mehta, 2000a,b). We, therefore, postulated that LOX-1-AS might decrease LOX-1-mediated increase in adhesion molecule expression. As shown in Fig. 2, incubation of HCAECs with ox-LDL-transcribed material (1.5 μl) was amplified with Taq DNA polymerase (Promega) using specific human primers of LOX-1 and various adhesion molecules (Mehta and Li, 1998; Takami et al., 1998; Li and Mehta, 2000a,b). The products of PCR amplified samples were visualized on 1.5% agarose gels using ethidium bromide. Each specific mRNA band was normalized with a band of relative internal reference β-actin mRNA. Relative intensity of band of interest was analyzed by Scan-gel-it software (Silk Scientific, Inc., Orem, UT) and expressed as the ratio to β-actin mRNA band. The number of PCR cycles was selected so that the mRNA bands were clearly visible in the ethidium bromide-stained agarose gel to decrease the generation of postexponential phase quantification errors.

Western Analysis. HCAEC lysates from each experiment (30 μg per lane) were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After incubation in blocking solution (4% nonfat milk; Sigma-Aldrich), membranes were incubated with 1:1000 dilution primary antibody (monoclonal antibody to LOX-1 (Sawamura et al., 1997); polyclonal antibody to E- and P-selectins, VCAM-1 or ICAM-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C. Membranes were washed and then incubated with 1:2000 dilution secondary antibody (Amersham Biosciences, Inc., Piscataway, NJ) for 1 h, and the membranes were detected with the enhanced chemiluminescence system, and relative intensities of protein bands were analyzed by Scan-gel-it software (Mehta and Li, 1998; Li and Mehta, 2000a,b).

Electrophoretic Mobility Shift Assay. Isolation of nuclear fraction was accomplished following the previously published procedure (Li and Mehta, 2000a). Oligonucleotides containing the consensus sequence for AP-1 and NF-κB were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase and purified using Chroma Spin-10 columns. The labeled oligonucleotides were incubated with the nuclear fractions for 30 min at room temperature in 50 mM Tris-HCl buffer, pH 7.5, containing 20% glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, and 0.25 mg/ml poly(dI-dC). The products were separated by electrophoresis in a 4% nondenaturing polyacrylamide gel using 0.5× TBE (45 mM Tris/borate and 1 mM EDTA) as the running buffer. The gels were dried and exposed to a radiographic film.

Data Analysis. All data represent the mean of six independently performed experiments. Data are presented as mean ± S.D. Statistical significance was determined in multiple comparisons among independent groups of data in which analysis of variance and the F test indicated the presence of significant differences. A P value ≤0.05 was considered significant.
markedly increased the expression of P-selectin, VCAM-1, and ICAM-1 protein. In contrast, native LDL (40 μg/ml) had no effect. LOX-1-AS reduced the effects of ox-LDL on the expression of these adhesion molecules (all \( P < 0.01 \)). In contrast, LOX-1-S had no effect (Fig. 2).

**Statins and the Ox-LDL Receptor.** Pretreatment of HCAECs with simvastatin or atorvastatin (1 and 10 μM) markedly decreased ox-LDL-induced up-regulation of LOX-1 protein and mRNA. High concentration of simvastatin and atorvastatin (10 μM) had a more pronounced effect than the low concentration of statins (1 μM). Adhesion molecule mRNA was determined by semiquantitative reverse transcription-PCR. Each band density of adhesion molecules was normalized by \( \beta \)-actin and expressed as ratio of adhesion molecule mRNA to \( \beta \)-actin mRNA. Adhesion molecule protein was determined by Western analysis. Each band density was normalized by its own control. The left panel is representative of six independent experiments. The right panel is the summary of data (mean ± S.D.) from these six experiments.

**Discussion**

We show that ox-LDL up-regulates the expression of E- and P-selectins, VCAM-1 and ICAM-1 in HCAECs. These effects of ox-LDL are mediated via activation of LOX-1. Two different statins simvastatin and atorvastatin attenuate ox-LDL-induced activation of LOX-1 and subsequent up-regulation of expression of adhesion molecules. Last, ox-LDL activates NF-κB signaling pathway, and this pathway can be blocked by statins.

**Ox-LDL and Its Receptor LOX-1.** Traditionally, it is believed that ox-LDL exerts its biological effects via activation of scavenger receptors on the surface of macrophages and smooth muscle cells (Zhou et al., 1996). Endothelial cells are generally devoid of these scavenger receptors (Kume et al., 1991; Bickel and Freeman, 1992). LOX-1, found predominantly on endothelial cells, has a different biochemical structure from the scavenger receptor (Sawamura et al., 1997). Several investigators (Sawamura et al., 1997; Kume et al., 1998; Mehta and Li, 1998; Oka et al., 1998; Li et al., 1999b; Li and Mehta, 2000b) have demonstrated that endothelial cells take up ox-LDL by LOX-1 activation, which results in endothelial activation and/or injury. For example, studies from our laboratory (Li and Mehta, 2000a) showed that LOX-1 participates in ox-LDL-induced apoptosis in HCAECs.

We now demonstrate that ox-LDL up-regulates the expression of leukocyte adhesion molecules. This effect of ox-LDL is mediated by LOX-1 activation, since a specific antisense to LOX-1 mRNA decreased LOX-1 expression and attenuated the up-regulation of expression of leukocyte adhesion molecules. Although the precise pathophysiological consequences of ox-LDL uptake by endothelial cells through LOX-1 are not clear, it appears that the expression of this novel ox-LDL receptor may be important in the development of atherosclerotic disease. It is of note that LOX-1 expression is markedly increased in rabbit (Chen et al., 2000) and human (Kataoka et al., 1999) atherosclerotic tissues.
Statins and Expression of LOX-1 and Adhesion Molecules. We observed that both simvastatin and atorvastatin inhibited the expression of LOX-1 gene elicited by ox-LDL. Since endothelial cells express traditional scavenger receptors CD36 and SR-B1 in extremely small amounts (Uittenbogaard et al., 2000), we believe that LOX-1 is the primary receptor for the uptake of ox-LDL in HCAECs, and its inhibition by statins is a major factor in reduced ox-LDL uptake by HCAECs (Li et al., 2001).

Expression of adhesion molecules and subsequent monocyte adhesion to endothelial cells is an early step in atherogenesis (Ramos et al., 1998). Statins have been shown to decrease CD11b expression in humans (Weber et al., 1997) and leukocyte-mediated reperfusion injury in the rats (Lefer et al., 1999). We now provide direct in vitro evidence that ox-LDL increases the expression of several adhesion molecules (E- and P-selectins, ICAM-1 and VCAM-1) on HCAECs. We (Li and Mehta, 2000a) have earlier described that monocyte adhesion to HCAECs is mediated by LOX-1 activation. We now extend these observations by showing that two different statins decrease the expression of these adhesion molecules elicited by ox-LDL, an effect similar to that of LOX-1 antisense.

Intracellular Mechanism of Action of Statins. The expression of adhesion molecules on endothelial cells is also regulated by eNOS (Iwata et al., 2001). It is noteworthy that statins have been shown to up-regulate eNOS expression (Hernandez-Perera et al., 1998). It is possible that statins inhibit ox-LDL-induced monocyte adhesion, at least in part, by modulating eNOS expression.

### Experimental Results

**Fig. 2.** Role of LOX-1 in the action of ox-LDL. Incubation of HCAECs with ox-LDL (40 μg/ml) markedly increased the expression of adhesion molecules determined by Western blot. In contrast, native LDL (40 μg/ml) did not affect the expression of these adhesion molecules. Pretreatment of HCAECs with antisense to LOX-1 mRNA (LOX-1-AS) (0.5 μM) for 48 h markedly reduced the effects of ox-LDL on the expression of these adhesion molecules, but sense-LOX-1 (LOX-1-S) (0.5 μM) had no effect. The top panel is representative of six independent experiments. The lower panel is the summary of data (mean ± S.D.) from these six experiments.

**Fig. 3.** Statins and LOX-1 expression. Ox-LDL (40 μg/ml) increased the expression of LOX-1 mRNA and protein. Pretreatment of HCAECs with simvastatin or atorvastatin (1 and 10 μM, respectively) decreased ox-LDL-induced up-regulation of LOX-1. A high concentration of statins (10 μM) exerted a more pronounced effect than the low concentration (1 μM).

**Fig. 4.** Activation of transcription factors by ox-LDL. Incubation of HCAECs with ox-LDL induced activation of NF-κB but not AP-1. Both simvastatin and atorvastatin (10 μM each) attenuated this effect of ox-LDL on NF-κB activity. These gels are representative of six independent experiments.

Experimental studies have shown that ox-LDL causes injury to the endothelial cells via activation of different signal transduction pathways, such as protein kinase C (Li et al.,...
1999a) and mitogen-activated protein kinase (Li and Mehta, 2000b). ox-LDL also activates NF-κB as well as AP-1 in several cell lines (Roebuck, 1999; Matsuqhita et al., 2000). In the present study, we found that ox-LDL activates NF-κB in HCAECs. Importantly, we found that both simvastatin and atorvastatin inhibited the activation of NF-κB in response to ox-LDL in HCAECs.

Summary

We provide evidence that LOX-1 plays a critical role in ox-LDL-induced expression of adhesion molecules on endothelial cells. Statins inhibit the expression of LOX-1 and subsequently attenuate the uptake of ox-LDL and expression of adhesion molecules. We also show a modulatory effect of statins on the activation of NF-κB. These observations indicate that inhibition of LOX-1 by statins may contribute to the beneficial effect of these agents in atherosclerosis.

References


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