Scavenger Receptors of Endothelial Cells Mediate the Uptake and Cellular Proatherogenic Effects of Carbamylated LDL

Eugene O. Apostolov, Sudhir V. Shah, Debarti Ray, Alexei G. Basnakian

Objective—Carbamylated LDL (cLDL) has been recently shown to have robust proatherogenic effects on human endothelial cells in vitro, suggesting cLDL may have a significant role in atherosclerosis in uremia. The current study was designed to determine which receptors are used by cLDL and thus cause the proatherogenic effects.

Methods and Results—In ex vivo or in vitro models as well as in intact animals, administration of cLDL was associated with endothelial internalization of cLDL and subendothelial transloca tion (transcytosis). In vitro recombinant LOX-1 and SREC-1 receptors showed the greatest cLDL binding. However, pretreatment of the endothelial cells with specific inhibiting antibodies demonstrated that cLDL binds mainly to LOX-1 and CD36 receptors. The transcytosis was dependent on SR-A1, SREC-1, and CD36 receptors whereas LOX-1 receptor was not involved. The cytotoxicity was mediated by several studied scavenger receptors, but cLDL-induced monocyte adhesion depended only on LOX-1. The cLDL-induced synthesis of LOX-1 protein significantly contributed to both cytotoxicity and accelerated monocyte adhesion to endothelial cells.

Conclusions—Our data suggest that cLDL uses a unique pattern of scavenger receptors. They show that LOX-1 receptor, and partially CD36, SREC-1, and SR-A1 receptors, are essential for the proatherogenic effects of cLDL on human endothelial cells. (Arterioscler Thromb Vasc Biol. 2009;29:1622-1630.)

Key Words: carbamylated LDL ■ LOX-1 ■ scavenger receptor ■ endothelial cells ■ atherosclerosis

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Modified low-density lipoproteins (LDLs) play a significant role in atherosclerosis development. Several proatherogenic modifications of LDL have been detected in humans. Recently, we showed that carbamylated LDL (cLDL), which is produced primarily by urea-derived cyanate modification of LDL, is present in healthy individuals and elevated in patients with chronic kidney disease (CKD). CKD patients are known to be at a significantly higher risk of atherosclerosis and other cardiovascular diseases (CVDs) than the general population. Carbamylated plasma proteins also correlate with CVD and predict risks of major adverse cardiac events.

Carbamylated LDL has strong atherogenic effects on endothelial cells by impacting the cell cycle, causing cell injury and promoting monocyte adhesion through the overexpression of ICAM-1 and VCAM-1 molecules. The initial mechanisms of these cLDL-induced events are not known. In particular, there is no information regarding whether cLDL uses any specific receptors or messengers. Horkko and coauthors proposed that cLDL lacks the ability to bind the LDL receptor (LDLR) on the endothelial surface, and so cLDL uptake is slow compared to native LDL (nLDL). With an increasing degree of carbamylation, cLDL binding switches from LDLR to scavenger receptors, which leads to faster uptake of cLDL from blood relative to nLDL. Other modified LDLs (ie, oxidized LDL [oxLDL], glyciated LDL, acetylated LDL [acLDL], ethylated LDL, myeloperoxidase-generated cLDL, and others) also bind preferentially to scavenger receptors compared to LDLR.

A variety of the scavenger receptors have been recently discovered and classified into several structure-based families. Although the function of the majority of the receptors is not clear, many of them have proatherogenic properties. For example, SR-A1 (class A receptor) binds oxLDL and acLDL; CD36 (class B receptor) scavenges a number of molecules, including oxLDL and nLDL, and promotes inflammation, cell adhesion, and foam cell formation; CD68 (class C receptor) binds both native and modified LDLs; LOX-1 (class E receptor) binds oxLDL and mediates or promotes oxidative...
stress and inflammation; SREC-1 (class F receptor) binds modified LDLs. The receptors responsible for binding cLDL are so far unknown. The present study was performed to determine which endothelial cell receptors may be responsible for cLDL binding and for mediating the proatherogenic effects of cLDL. We demonstrated that several scavenger receptors are capable of binding cLDL, and that the LOX-1 receptor is upregulated and is the receptor primarily involved with the biological effects of cLDL. The inhibition of the existing LOX-1 or prevention of LOX-1 protein synthesis protected endothelial cells from cLDL-induced cytotoxicity and from monocyte adhesion, suggesting that LOX-1 is involved in cLDL-mediated atherosclerosis. Importantly, the overall pattern of scavenger receptors involved in the cLDL effects seems to be unique and different from the one of oxLDL.

Materials and Methods

Native, Carbamylated, and Oxidized LDLs
For details, please see the supplemental materials (available online at http://atvb.ahajournals.org/).

LDL Labeling With 125I and AlexaFluor Dyes
For details, please see the supplemental materials.

In Vivo and Ex Vivo Experiments
All experiments with animals were approved by the Animal Care and Use Committee of the Central Arkansas Veterans Healthcare System. For cLDL or nLDL tracking, B6.129P2-Apoetm1Unc/J (background C57BL/6) mice from Jackson Laboratory, Bar Harbor, Maine were subjected to intravenous injections with 125I-labeled cLDL or 125I-labeled nLDL. To study the distribution of cLDL in the aorta, AF488-labeled cLDL was intravenously injected in mice (2 mg/kg). To study the rapid kinetics of cLDL, fluorescently labeled LDLs were used in an ex vivo working heart model as recommended by the Animal Models of Diabetic Complications Consortium. For details, please see the supplemental materials.

Cell-Free Fluorescent Ligand-Receptor Assay
To study the ability of the LDLs to bind receptors, the fluorescent ligand-receptor assay was performed. For details, please see the supplemental materials.

Cell Culture and LDL Treatment of Cells
Human coronary artery endothelial cells (HCAECs) were supplied by Lonza Inc. For cytotoxicity and monocyte adhesion experiments, the cells were treated with LDLs (200 μg/mL) in serum-free EGM-2 medium (Lonza) for 24 hours. For in vitro LDL binding/translocation assays, AF594-labeled LDLs (10 μg/mL) were used for designated periods of time. For details, please see the supplemental materials.

LDL Subendothelial Translocation Assay
LDL subendothelial translocation (transcytosis) assay was performed in 12-well plates equipped with 8-mm pore BD Biocoat inserts (BD Biosciences). For details, please see the supplemental materials.

Cytotoxicity Assay
Cell death was measured using lactate dehydrogenase (LDH) release as described previously. Briefly, HCAECs were treated with 200 μg/mL LDLs for 24 hours, and the activity of the released LDH was measured using CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega).

Immunocytochemical Staining
For details, please see the supplemental materials.

Monocyte Adhesion In Vitro
Monocyte adhesion was determined in a static model as described previously. For details, please see the supplemental materials.

Cell ELISAs
The cells were washed, fixed, and probed with 1 of the following antibodies: anti–LOX-1 (1:400), anti–SREC-1 (1:400), anti–CD36 (1:100), anti–SR-A1 (1:400; R&D Systems), anti–LDLr (1:75), or anti–VLDLR (1:100; Santa Cruz Biotechnology). Protein expression in every sample was normalized by β-actin expression. For details, please see the supplemental materials.

Western Blotting
For details, please see the supplemental materials.

LOX-1 siRNA Silencing
LOX-1 silencing in HCAECs was performed as described before. For details, please see the supplemental materials.

RNA Isolation and Real-Time RT-PCR
The total RNA was isolated using an RNeasy Mini kit from Qiagen. The reverse transcription reaction was performed using the GeneAmp Gold RNA PCR core kit (Applied Biosystems) using Oligo d(T)16. For details, please see the supplemental materials.

Statistical Analysis
Results were expressed as mean±SE. Statistical analyses were performed using the ANOVA and Student t test. Comparisons with multiple time points were performed by t test with the Bonferroni adjustment. Values of P<0.05 were considered significant.

Results
Accumulation of cLDL in the Vascular System In Vivo
Although it was shown before that cLDL is removed rapidly from the bloodstream, no one has studied cLDL accumulation in various organs and tissues. We started determining potential relationship between cLDL and vascular system with a series of experiments in mice. First, experimental and control mice were subjected to the intravenous administration of 2 mg/kg 125I-cLDL or 125I-nLDL, respectively. A profound decrease of both cLDL and nLDL in the plasma was observed (Figure 1A), and plasma level of 125I-nLDL was several times that of 125I-cLDL at the end of 24 hours. We next examined the distribution of nLDL and cLDL in different organs. The ratios comparing organ to plasma concentrations of cLDL (as an indication of the redistribution of cLDL from bloodstream to heart, aorta, kidney, liver, and skeletal muscle tissues) were significantly higher for cLDL than for nLDL (Figure 1B).

For tracking in cardiovascular tissues, cLDL was labeled with AF488 (supplemental Figure I). AF488-labeled nLDL or bovine serum albumin (BSA) served as controls. LDLs or BSA were injected intravenously in mice. We observed that more cLDL than either nLDL or BSA accumulated in the aortic subendothelial area (Figure 1C). Furthermore, we used the same compounds in ex vivo experiments where solutions were perfused through isolated hearts as described in the supplemental materials. In these experiments, cLDL was the only compound, which was localized under endothelial cells within 15 minutes of perfusion (Figure 1D).

These data suggest that cLDL has specific pattern of distribution and accumulation in vascular system.
Endothelial Internalization and Transcytosis of cLDL In Vitro

To analyze how the above localization of cLDL is mediated actively by the endothelium, we applied fluorescently labeled cLDL, nLDL, or oxLDL (0.25 to 250 μg/mL) to HCAECs for 24 hours. Because in some experiments the free FITC (green emission) was used as a reference dye, all lipoproteins for in vitro experiments were labeled with AF594 (red emission). Before quantification, internalization of the cLDL was verified using the confocal microscopy (Figure 2A). We found that cLDL was dose-dependently internalized in endothelial cells at a higher rate than the 2 other LDLs, reaching a plateau at 50 μg/mL (Figure 2B). Endothelial cells demonstrated a time-dependent internalization of all 3 isoforms (25 μg/mL) in the period of time from 0 to 6 hours (Figure 2C). Carbamylated LDL had the highest and nLDL had the lowest accumulation within the cells. The maximum cellular accumulation was detected after 6 to 8 hours for both cLDL and oxLDL, which indicates that either internalization was slowed or that equilibrium of internalization and release of LDL was reached. After the plateau was reached, cLDL was the isoform with the highest degree of accumulation. To ensure that LDL, as opposed to fluorescent LDL remnants, was assayed in the above experiment, cells were stained with ApoB antibody. ApoB content was significantly increased in endothelial cells treated with cLDL as compared to cells treated with nLDL or oxLDL (supplemental Figure IIA). The use of k-carrageenan and polyinosinic acid (poly(I)), 2 commonly used chemical inhibitors of scavenger receptors,14 protected the endothelial cells from the accumulation of both cLDL and oxLDL (supplemental Figure IIB). These data suggest that scavenger receptors are likely to be involved in the internalization of cLDL within endothelial cells.

To determine whether HCAECs can promote the subendothelial translocation of cLDL, confluent cells grown on
a semipermeable membrane were treated with labeled cLDL using a modification of an in vitro system previously described. Within 30 minutes of treatment, a significantly higher proportion of cLDL than of oxLDL or nLDL was translocated beneath the cells (Figure 2D). After 1 hour, the subendothelial cLDL concentration reached ≈50% of the maximum concentration found at 24 hours; and the cLDL concentration beneath the cells was greater than oxLDL and nLDL at all time points. Compared to nLDL, oxLDL had a higher rate of translocation during the first hour, but no difference was found by 24 hours. These data indicate that endothelial cells internalize cLDL and permit its transcytosis.

Scavenger Receptors of Cultured Endothelial Cells Mediate the Internalization and Transcytosis of cLDL, as Well as cLDL-Induced Monocyte Attraction and Cytotoxicity

First, LOX-1 and SREC-1 receptors were found the most favorable candidates among scavenger receptors for cLDL in a cell-free system (please see supplemental Results and supplemental Figure III). We next investigated the physiological ability of the scavenger receptors to bind cLDL in intact endothelial cells. To investigate particular scavenger receptors, we blocked individual receptors using neutralizing antibodies and studied the ability of labeled LDL to bind to endothelial cells and cLDL accumulation in the subendothelial area. The inhibiting properties of the antibodies that we used have been successfully tested in fluorescent ligand-receptor assays with cLDL (supplemental Figure IV). Our results suggested that none of the receptors is solely responsible for cLDL internalization and subendothelial translocation (Figure 3A).

Pretreatment of the cells with antibodies to LOX-1 and CD36 had the highest inhibition (25% to 30%) of the cLDL internalization in endothelial cells. Antibodies to SREC-1 and SR-A1 equally suppressed the cLDL internalization (22%). Internalization of oxLDL was suppressed similarly. Furthermore, when all 4 antibodies were simultaneously applied to cells, internalization of about 55% of cLDL and 50% of oxLDL was inhibited. None of the antibodies affected nLDL internalization. Importantly, transcytosis of cLDL was significantly inhibited with anti-CD36, anti–SREC-1, and anti–SR-A1 antibody (Figure 3B). Although subendothelial transfer of oxLDL was significantly lower, it was partially diminished by anti–SR-A1 antibody. Movement of nLDL into the subendothelial space was not affected by any of the antibodies.

The same approach of using inhibiting antibodies was applied to define the functionality of the cLDL binding to the receptors. After treatment of the endothelial cells with modified LDLs, the endothelial cells were assayed for monocyte adhesion and cytotoxicity, 2 of the major atherogenic effects of modified LDLs on endothelial cells.6,7 In comparison to cells treated with nonspecific IgG, the use of any of the 4 scavenger antibodies significantly reduced the cytotoxicity induced by cLDL or oxLDL (Figure 3C). The inhibition of LOX-1 provided the most prominent protection (39% for cLDL and 52% for oxLDL) (Figure 3C). The inhibition of CD36 reduced endothelial cell LDH release by 39% and 11% after exposure of cells to cLDL and oxLDL, respectively. Cytotoxicity of cLDL or oxLDL mediated by SR-A1 and SREC-1 receptors was between 21% and 34%. The vehicle and nLDL did not induce significant cell death. Application of a mixture of all antibodies reduced LDH release by 57% and 61% from cLDL- and oxLDL-treated endothelial cells, respectively.
Importantly, monocyte adhesion to endothelial cells treated with cLDL and oxLDL was decreased in the presence of anti–LOX-1 antibody relative to cells treated with nonspecific IgG, however oxLDL decrease did not reach statistical significance (Figure 3D). Other antibodies either did not affect monocyte adhesion or slightly increased it. A mixture of all antibodies did not have any substantially greater protective benefit against monocyte adhesion compared to the effect of anti-LOX-1 antibody alone (data are not shown).

Therefore these results suggest that, although all of the studied receptors contribute to cLDL-induced endothelial injury/dysfunction, the LOX-1 receptor mediates both cytotoxicity and monocyte adhesion.

Carbamylated LDL Upregulates LOX-1 Expression in Endothelial Cells

The effect of cLDL on the expression of scavenger receptors in endothelial cells was studied next. HCAECs were treated with low (12.5 μg/mL) or high (200 μg/mL) concentrations...
of cLDL. These concentrations have induced proliferation and cytotoxicity of HCAECs in vitro. The protein expressions of major scavenger receptors were measured by cell ELISA at 2, 10, and 24 hours. Our results clearly indicate that only the expression of LOX-1 was significantly increased by cLDL treatment at both low (Figure 4A) and high (Figure 4B) concentrations of cLDL. Protein expressions of LDLR and SREC-1 were slightly upregulated after exposure to 200 μg/mL cLDL. Upregulation of LOX-1 expression after treatment with cLDL was confirmed by immunocytochemical staining (Figure 4C). To determine whether LOX-1 expression is induced at the transcription level, real-time RT-PCR was applied. Our results suggest that LOX-1 mRNA was already increased at 4 hours and reached maximal expression at 8 hours (Figure 4D). By 24 hours, LOX-1 mRNA expression was markedly decreased in comparison to its maximum level seen at 8 hours, possibly because of the beginning of cell death.

Our data suggest that cLDL induces expression (protein synthesis) of LOX-1 receptor in endothelial cells.

Both Inducible and Constitutive LOX-1 Mediate the Proatherogenic Effects of cLDL Toward Endothelial Cells

To evaluate whether cLDL-induced LOX-1 participates in the proatherogenic cellular effects in endothelial cells, HCAECs were transfected with anti-LOX-1 siRNA for 72 hours. The efficiency of the cell transfection and mRNA knockdown was validated by real-time RT-PCR (supplemental Figure V). Although constitutively expressed LOX-1 was intact, the induction of LOX-1 was completely prevented with siRNA transfection. Suppression of inducible LOX-1 completely protected endothelial cells from death induced by cLDL, but not by oxLDL, to the level of vehicle-treated cells (Figure 5A). Inhibition of LOX-1 significantly protected against cLDL-accelerated monocyte adhesion, but did not affect oxLDL-mediated monocyte adhesion to endothelial cells (Figure 5B).

Next, to determine whether inhibition of both constitutive and inducible LOX-1 expression provides additional protection of HCAECs treated with cLDL, the cells were transfected with anti-LOX-1 siRNA for 2 subsequent periods of 72 hours (supplemental Figure VI). Similar to the previous experiment, our data suggest that complete inhibition of the inducible form of LOX-1 and partial (about 30%) inhibition of the constitutive form of LOX-1 protects cells exposed to cLDL so that there is a statistically significant reduction in measures of cytotoxicity and monocyte adhesion down to the level of vehicle-treated cells (supplemental Figure VII).

Our previous data suggested that ICAM-1 and VCAM-1 are important in cLDL-induced monocyte adhesion. We next evaluated whether the LOX-1–dependent monocyte adhesion may be partially mediated through the ICAM-1 and VCAM-1 over expression using cell ELISA. Our data showed that both ICAM-1 and VCAM-1 expressions induced by cLDL are significantly reduced by anti-LOX-1 siRNA (Figure 5C and 5D).

Therefore our data, in support of the above observations, suggest that cLDL-induced LOX-1 expression is functionally important for the atherogenic effect of cLDL to endothelial cells.

Discussion

Our initial observation in this study was that when comparable amounts of chemically modified cLDL and nLDL are administered, the plasma levels of cLDL are lower than those of nLDL. However, the uptake of cLDL by endothelium is relatively higher than that of nLDL. This result is in agreement with previous studies that showed slow clearance of mildly modified cLDL and fast clearance of advanced cLDL from plasma in rabbits and humans. These phenomena have been explained by the lack of affinity of LDLR for cLDL, or by an acquired ability of scavenger receptors to bind cLDL. Our data support these observations. Furthermore, our data suggest there is both endothelial cellular and subendothelial accumulation of cLDL in the aorta and heart. A similar phenomenon has been observed with cultured human endothelial cells and other modified LDLs.

The current report is the first attempt to identify the receptor(s) that bind(s) cLDL and to compare cLDL and oxLDL binding to different types of endothelial cell scavenger receptors. Our results suggest that despite obvious similarities between these 2 abundant atherogenic modified LDLs, cLDL and oxLDL, their affinities and specificities for scavenger receptors may be different. It is very possible that the somewhat greater affinity of several scavenger receptors may drive the rate of cLDL binding and uptake by endothelial cells. Considering that the physiologically plausible concentration of cLDL is said to be higher than any other modified LDL, and based on the affinity seen in the current studies, we can speculate that cLDL may be a ligand for LOX-1 and SREC-1 receptors that is at least, if not more important, than oxLDL.

The inhibition of the scavenger receptors in cultured endothelial cells showed that the scavenger receptor binding of cLDL on endothelial cells is somewhat different from the receptor-ligand assay using recombinant receptors. Although LOX-1 and CD36 receptors seem to be involved in the binding of cLDL, the SREC-1 and SR-A1 inhibition have less effect. The partial discrepancy between the in vitro and cellular data may be from the different levels of expression, some difference in folding of recombinant proteins, the possibility of cooperation and interference between different receptors in cells and necessity of di- or heteromerization of the scavenger receptors shown before. Perhaps, monomeric LOX-1 and SREC-1 demonstrated higher binding rate in the cell-free systems because they maintain more ligand-binding activity than monomeric SR-A1 and CD36 receptors. Simultaneous use of all inhibiting antibodies had a greater extent of inhibition of cLDL or oxLDL internalization; however, it was never completely prevented. This may suggest that the presence of other alternative receptors/pathways for modified LDL internalization. We also observed that the same scavenger receptors were involved in cLDL-induced cytotoxicity and cLDL binding. The inhibition of all 4 receptors protected against cells death to different extents, and LOX-1 inhibition had the biggest impact. This result is in agreement with a number of studies on LOX-1–mediated cell injury.
Figure 4. Cell ELISA measurements of the LDLR, VLDLR, LOX-1, SREC-1, CD36, and SR-A1 protein expressions in HCAECs after treatment with nLDL or cLDL at concentration of 12.5 μg/mL (A) and 200 μg/mL (B) for 2, 10, or 24 hours as compared to baseline level (dashed line at 100%). n=3 to 4 per point; *P<0.05 as compared to 0-hour time point; #P<0.05 as compared to vehicle-treated cells; ¶P<0.05 as compared to nLDL-treated cells. C, Representative images of LOX-1 protein expression after cLDL or nLDL treatment (200 μg/mL, 24 hours) as determined by immunocytochemistry. Scale bar, 10 μm. D, Expression of LOX-1 mRNA in endothelial cells after cLDL or nLDL treatment (200 μg/mL, 24 hours) as measured by real-time RT-PCR. n=3 per point; *P<0.05 as compared to vehicle-treated cells; #P<0.05 as compared to nLDL-treated cells.
Our data clearly showed that cLDL-induced monocyte adhesion is mediated mostly through LOX-1 receptor. Our previous studies suggested that cLDL-induced monocyte adhesion uses the ICAM-1 and VCAM-1 adhesion molecules. Other reports have indicated that LOX-1 upregulates both ICAM-1 and VCAM-1 expression. Furthermore, our siRNA experiments suggested that cLDL-induced monocyte adhesion is mediated through LOX-1 as well as ICAM-1/VCAM-1 overexpression, thus potentially linking cLDL with LOX-1 and ICAM-1/VCAM-1 molecules in a single pathway.

It is an interesting observation that although several scavenger receptors are involved in the cLDL-induced cytotoxicity, LOX-1 seems to have the most significant effect on the cLDL-induced monocyte adhesion to endothelial cells. Considering that oxLDL may use both LOX-1 and CD36 receptors to induce monocyte adhesion, we can speculate that perhaps different affinity of cLDL with LOX-1 and ICAM-1/VCAM-1 molecules is the reason. This study, for the first time, demonstrated in vivo and in vitro that cLDL is rapidly translocated beneath endothelium and is accumulated there. Surprisingly, our data suggest that LOX-1, which mediates most of the cLDL binding to endothelial cells, is not involved in cLDL translocation. Instead, CD36, SREC-1, and SR-A1 are the receptors which mediate this event. Contrary to cLDL, oxLDL uses only SR-A1 and to a lesser extent, LOX-1. Our data suggest that cLDL is more prone to subendothelial transfer than oxLDL and that the processes involved are more diverse than oxLDL.

Endothelial injury with subendothelial translocation and intravascular internalization of nLDL and modified LDLs are a leading cause for monocyte recruitment and transformation into macrophages, inflammation, and smooth muscle proliferation, which are all key processes for atherosclerosis development. Previously, it was proposed that oxLDL can be translocated through endothelium. Later studies determined that oxLDL is mainly formed subendothelially. Unlike oxLDL, most cLDL is synthesized in plasma by urea-derived cyanate. Therefore, because of the faster rate of subendothelial accumulation and strong proatherogenic properties, transcytosis of the cLDL seems to be extremely important for atherosclerosis.

Finally, our data suggest that cLDL causes changes in receptor protein expression: whereas LDLR and SREC-1 are modestly increased, the LOX-1 receptor is dramatically overexpressed in response to cLDL. It has been shown before that LOX-1 is a stable protein, whereas siRNA may be used mostly to inhibit induction of new LOX-1. In our experiments, the inhibition of LOX-1 expression using specific siRNA led to suppression of the inducible LOX-1 expression without significant effect on constitutive LOX-1. In another approach, we subjected cells to two subsequent long-term transfections and were able to knockdown about 30% of constitutive LOX-1 protein. Although partial inhibition of constitutive LOX-1 had some additional cell protection and prevented some of the "natural" cell death, both experimental settings demonstrated that cytotoxicity and monocyte adhesion are prevented to the level of the vehicle-treated cells. It may be concluded that newly synthesized LOX-1 is functionally active and responsible for both endothelial cell injury and monocyte adhesion. A similar phenomenon has been previously described for oxLDL. In addition, several other cell injury inducers were shown to facilitate the LOX-1 mechanism of injury, stimulate the LOX-1 expression, and exacerbate the injury. We speculate that inducible LOX-1 may be

![Figure 5](http://atvb.ahajournals.org/)

Figure 5. Inhibition of cytotoxicity (A) and monocyte adhesion (B) to HCAECs induced by cLDL, nLDL, or oxLDL (200 μg/mL, 16 or 24 hours for cytotoxicity and monocyte adhesion, respectively) using the siRNA to LOX-1 (50 nmol/L, 48 hours). In the same experiment, ICAM-1 (C) and VCAM-1 (D) expression was measured by cell ELISA. n=4 per point; *P<0.05 as compared to cells treated with control siRNA; #P<0.05 as compared to vehicle-treated cells; ¶P<0.05 as compared to nLDL-treated cells.
more important in cLDL-induced atherogenesis than constitutive LOX-1; however, the functional difference between induced and constitutive LOX-1s should be studied in future using other models.

In summary, cLDL uses several scavenger receptors, which facilitate its binding to endothelial cells, transcytosis, cytotoxicity, and monocyte adhesion to endothelial cells. In particular, cLDL upregulates LOX-1 protein expression, which may play a crucial role in mediating its proatherogenic effects. Despite some similarities in affinity and favorability for the individual scavenger receptors, the receptor pattern specific to cLDL seems to be unique and thus can be used for future cLDL-targeted antiatherosclerosis therapies. At this time it is unknown what part of the cLDL-induced atherogenesis could be the most efficient therapeutic target. Carbamylation is a passive process, and currently there is a lack of knowledge on whether cLDL production can be directly inhibited. The targeting of scavenger receptors seems to be more achievable, and clinically used statins and PPAR gamma ligands to control scavenger receptor expression/ functionality seem to be partially effective at least in some in vitro models. The effectiveness of currently used and prospective antiatherosclerotic drugs toward the treatment and prevention of cLDL-induced atherosclerosis is a subject for future studies.

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Disclosures
None.

References
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Apostolov et al. “Scavenger receptors…”

SUPPLEMENT MATERIAL

Materials and Methods

Native, carbamylated and oxidized LDLs

Human nLDL and all of the chemicals were purchased from Sigma (St. Louis, MO) unless stated otherwise. Carbamylated LDL and oxLDL were prepared by chemical modification and dialyzed as previously described \(^1\). Carbamylation of LDL was verified by the colorimetric method using diacetyl monoxime \(^2\). Oxidation of LDL was assayed by the thiobarbituric acid reactive substances assay \(^3\). The electrophoretic mobility of nLDL, cLDL and oxLDL was determined in 0.5% agarose gel, 0.2% bovine serum albumin (w/v) as described by Noble \(^4\). Modified and native LDLs were diluted with PBS containing 200 \(\mu\text{mol/L EDTA}\), kept at 4°C away from light, and used within 3 weeks after preparation.

LDL labeling with \(^{125}\text{I}\) and AlexaFluor dyes

Native and chemically carbamylated LDL were subjected to custom \(^{125}\text{I}\) labeling by Lofstrand Labs Limited (Gaithersburg, MD) using the Bolton-Hunter reagent. The specific activities of the preparations were adjusted to 0.66 \(\text{uCi/\mu g}\) using the appropriate unlabeled LDL isoform.

Fluorescent labeling of nLDL and modified LDLs were achieved by using the AlexaFluor 488 (AF488) Protein or AlexaFluor 594 (AF594) Protein labeling kits (Invitrogen, Carlsbad, CA). Briefly, 1 mg of each LDL isoform was coupled with AlexaFluor carboxylic acid, tetrafluorophenyl ester in the presence of 0.1 M sodium bicarbonate for 2 hours at 4 °C. Labeled LDLs were purified by gel filtration through Sephadex G-50 (Invitrogen). To equilibrate specific fluorescence (per mg protein) it was adjusted by adding appropriate unlabeled LDL isoforms.
Apostolov et al. “Scavenger receptors…”

**In vivo and ex vivo experiments**

All experiments with animals were approved by the Animal Care and Use Committee of the Central Arkansas Veterans Healthcare System (John L. McClellan Memorial VA Hospital). B6.129P2-Apoe\textsuperscript{tm1Unc}\textsuperscript{j} (background C57BL/6) mice were purchased from Jackson Laboratories (Bar Harbor, ME) (stock 002052). For cLDL or nLDL tracking, animals were subjected to intravenous injections with \textsuperscript{125}I-labeled cLDL (\textsuperscript{125}I-cLDL) or \textsuperscript{125}I-labeled nLDL (\textsuperscript{125}I-nLDL), respectively (2 mg/kg, 1.65 mCi/kg). The animals were sacrificed 24 hours later. Blood and internal organs were collected, weighed and the total radiation count per minute (CPM) was measured using a Packard Cobra Quantum gamma radiation counter (Shelton, CT). The data were presented as the amount of LDL per gram of tissue.

To study the distribution of cLDL in the aorta, AF488-labeled cLDL was intravenously injected in mice (2 mg/kg). Two groups of control mice received similarly labeled nLDL or bovine serum albumin (BSA). Twenty four hours later the mice were sacrificed and the aortas were isolated, frozen and cryosected. Five \(\mu\)m aorta cross-sections were postfixfixed, mounted under coverslips and imaged under a fluorescent microscope as described below. The number and area of the AF488-positive compartments were blindly counted for each animal, and the data were presented as the average numbers of fluorescent particles per 1 \(\text{mm}^2\) of a section. To study the rapid kinetics of cLDL, fluorescently labeled LDLs were used in an ex vivo working heart model that was performed as recommended by the Animal Models of Diabetic Complications Consortium and described elsewhere \textsuperscript{5}. Briefly, every mouse was sacrificed using 200 mg/kg ketamine. The hearts were rapidly removed and stopped in ice-cold phosphate-buffered saline (PBS). Left atria were cannulated and connected with syringe pumps, and the hearts were quickly placed in Krebs-Henseleit bicarbonate (KHB) buffer pre-warmed at 37\(^\circ\)C. After the restoration of contractions (in \(\sim\)15-30 seconds), the hearts
Apostolov et al. “Scavenger receptors…”

were supplied with KHB (pH 7.4 at 37°C, equilibrated with a 5% CO₂ gas) for 2 minutes, and then with labeled cLDL (20 µg/ml) for 5 minutes. After this, the hearts were washed with KHB for an additional 3 minutes and rapidly frozen. Heart cross-sections were prepared as described above and analyzed under a microscope for the presence of fluorescent particles in the left ventricular endocardium.

Cell-free fluorescent ligand-receptor assay

In order to study the ability of the LDLs to bind receptors, the fluorescent ligand-receptor assay was performed as suggested by R&D systems and similarly to described elsewhere 6, 7. Briefly, 96-well plates were coated with 100 µl/well of recombinant protein (LOX-1, CD36, SR-A1, or SREC-1 from R&D Systems Inc., Minneapolis, MN) at 5 µg/ml in PBS overnight at 4°C. According to manufacturer instruction, the used Costar flat-bottom EIA plates can bind about 250 ng/cm² of protein. Because our recombinant protein concentration was at least 6 times higher than plastic capacity, we assumed that all of plastic surface was saturated with equally maximal mass of recombinant protein (80 ng/well).

For affinity and capacity studies, after blocking with 2% BSA containing PBS buffer and several washes with PBS, AlexaFluor 594-labeled LDLs (0.01-10 µg/ml) were applied for 2 hours at 37°C in absence or presence of 30-fold concentration of unlabeled cLDL. For comparison of cLDL-AF594, oxLDL-AF594 and nLDL-AF594 binding, all LDL isoforms were applied at concentration of 1 µg/ml for 2 hours at 37°C. For the study of competition, cLDL-AF594 (1 µg/ml) was applied for 2 hours to immobilized recombinant receptor proteins in presence of same, 10-fold and 30-fold amount of unlabeled oxLDL or nLDL.

The total fluorescence before and remaining fluorescence after the washings were measured at 530/645 nm. The amount of bound LDL was calculated based on label
specificity (fluorescence per 1 mol protein) and measured fluorescence numbers. The MWs of protein extracellular domains provided by R&D systems were used for final calculations of molar quantities of the recombinant receptor proteins and LDL: LOX-1 - 25.5 kDa; CD36 - 73 kDa; SREC-I - 69.1 kDa; SR-A1 – 42.5 kDa; and LDL particle - 3000 kDa. The data were presented as amounts of LDL (µmol) per amounts of receptor (mol) used for the LDL capturing.

Cell culture and LDL treatment of cells

Human coronary artery endothelial cells (HCAECs) were supplied by Lonza Inc. (Walkersville, MD) at passage 3 and used between passages 4 and 6. For cytotoxicity and monocyte adhesion experiments the cells were treated with LDLs (200 µg/ml) in serum-free EGM-2 medium (Lonza) for 24 h. The vehicle solution in the same medium was used as a control. For in vitro LDL binding/translocation assays, AF594-labeled LDLs (10 µg/ml) were used for designated periods of time. LDL binding experiments were performed in 96-well plates (10,000 cells per well). The total fluorescence was measured at 530/645 nm after the exposure of cells to LDLs. The cells were then washed three times with PBS, and the remaining fluorescence was re-measured.

In some experiments, blocking antibodies to LOX-1, SREC-1, anti-SR-A1 (R&D Systems) and CD36 (Abcam), (10 µg/ml) were applied to the cells 2 hours before the treatment. Control cells were pretreated with non-specific mouse or goat immunoglobulines G. The average of controls is used because there was no difference between the two controls. LOX-1 inhibitors, k-carrageenan and polyinosinic acid poly(I) were applied at concentrations of 250 µg/ml 4 hours prior to the LDL treatments of cells.

LDL subendothelial translocation assay
LDL subendothelial translocation (transcytosis) assay was performed in 12-well plates equipped with 8-nm pore BD Biocoat inserts (BD Biosciences, San Jose, CA) similarly to described by Dehouck and co-authors. Cells were plated for 24 hours in the inserts (30,000 cells per insert). The medium in the inserts and wells was kept at an even level. To ensure that cells formed a tight monolayer, the reference dye (free fluorescein (FITC), 1 µg/ml) was added into the inserts prior to the experiments and 30 minutes later the fluorescence in the wells was measured at 485/528 nm. The wells, which did not have the transmembrane leak of reference dye, were treated with AF594-labeled LDLs (10 µg/ml). Fluorescently labeled LDLs were measured at several time points in the media below the inserts at 530/645 nm. To control rapture of the cell monolayer and cell shrinkage, the reference dye (fluorescein) was also measured at 485/528 nm every time point. The amount of translocated LDL or reference was calculated based on label specificity (fluorescence per 1 ng protein) and measured fluorescence values. The data were presented as averages of translocated LDL amount normalized by reference dye amount in wells at every time point.

**Immunocytochemical staining and microscopy**

The immunocytochemical staining of cells with mouse monoclonal anti-human LOX-1 receptor (1:400) antibody (R&D Systems) was performed as described by us previously. Goat anti-mouse-AF488 secondary antibody (Invitrogen) was used at a dilution of 1:400 for the detection of the primary antibodies. Slides were mounted under the Prolong medium with DAPI (Invitrogen) and imaged using an Olympus IX-81 microscope (Olympus America Inc., Center Valley, PA) equipped with a digital camera Hamamatsu ORCA-ER (Hamamatsu Photonics K.K., Hamamatsu City, Japan). Slidebook 4.1 software was used for the image grabbing and analysis (SciTech Pty Ltd., Australia). For
the confocal microscopy Zeiss LSM 410 microscope equipped with 594 laser source and Zeiss LSM software (v. 3.99) was used.

**Monocyte adhesion in vitro**

Monocyte adhesion was determined in a static model as described previously \(^9\). Briefly, the endothelial cells were seeded in a 96-well plate, treated with LDLs (200 µg/ml) for 24 hours and then exposed to U937 cells labeled with 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) for 30 minutes. The total fluorescence before and after the washings was detected using a Synergy-HT-I plate reader (BioTek, Winooski, VT) at 485/528 nm. The background of LDL-treated cells without monocytes was subtracted from the experimental values.

**Cell ELISAs**

Cell ELISA was performed as described by Frahm et al. \(^10\). Immune reaction conditions were elaborated prior to the experiment for every antibody separately. HCAECs were seeded in a 96-well plate, treated with 12.5 or 200 µg/ml for 2, 10 or 24 hours, washed and fixed (4% w/v paraformaldehyde, 0.012% saponin, PBS) for 10 minutes at room temperature. The cells were washed and probed with one of the following antibodies: anti-LOX-1 (1:400), anti-SREC-1 (1:400), anti-SR-A1 (1:400) (R&D Systems), anti-CD36 (1:100) (Abcam), anti-LDLR (1:75) or anti-VLDLR (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer (2% bovine serum albumin, PBS) for 2 h. The primary antibodies were detected with anti-mouse or anti-goat IgGs (1:1000) conjugated with horse radish peroxidase (HRP) (Santa Cruz Biotechnology). The HRP activity was measured using 3,3',5,5'-tetramethylbenzidine substrate solution (Sigma) at delta 450-540 nm. Protein expression in every sample was normalized by β-actin expression.
measured with the rabbit polyclonal anti-β-actin antibody conjugated with FITC (Santa Cruz Biotechnology).

All measurements were done in quadruplicates per one marker per one time/concentration point and were repeated at least three times in different plates. The negative controls of the primary antibody were done by their substitution with blocking buffer.

**Western blotting**

LOX-1 antibodies for Western blotting were purchased from R&D Systems. Cell lysates were prepared using the lysis buffer from Cell Signaling Technology, Inc. (Danvers, MA). The total protein was extracted from cells, run through the 12% SDS-PAGE at 10 µg/lane and transferred to nitrocellulose membranes. The membranes were stained with Ponseau S to control equal protein load as described elsewhere 11. After incubation in blocking solution (4% nonfat milk), membranes were incubated with polyclonal antibody to LOX-1 (1:500) overnight at 4°C. Membranes were washed and incubated with a 1:1000 dilution of secondary antibody for 1 hour, and tested with the chemiluminescence system (Sigma).

**LOX-1 siRNA silencing**

LOX-1 silencing in HCAECs was performed as described before 9. For this, HCAECs were seeded in 6-well or 96-well plates at the density of 10,000 cells per well and subjected to transfection (50 nM) with the following siRNA duplex (Dharmacon, Lafayette, CO): sense, 5’-CAGCCAAGAGAAGUGCUUGdTdT-3’, and antisense, 5’-CAAGCACUUCUCUUGGCUUGdTdT-3’. The complex was prepared using the TransIT-TKO reagent (Mirus, Houston, TX) and applied to the cells for 4 h. Control Non-
Targeting siRNA #1 (Dharmacon) and transfection reagent alone were used as controls. RNA isolation or cell treatments were performed 48 hours after the start of transfection.

**RNA isolation and real-time RT-PCR**

The total RNA was isolated using an RNeasy Mini kit from Qiagen (Valencia, CA). The reverse transcription reaction was performed using the GeneAmp Gold RNA PCR core kit (Applied Biosystems) using Oligo d(T)16. Briefly, 0.5 µg of total RNA was used for reverse-transcription and following real-time PCR using SmartCycler (Cepheid, Sunnyvale, CA) as described previously 9, 12. The reaction mix was prepared using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen) and the following primers: forward: 5’- GGCTCTGGGAGGACGGTT-3’; reverse: 5’- TGTCTGGGAGACAGCGCC-3’. 18s ribosomal subunit RNA was amplified in a parallel reaction using primers: 5’-TTGAACGTCTGCCCTATCAA-3’ and 5’-ATGGTACGGCGCGCCTCA-3’. Two-temperature cycles with an annealing/extension temperature at 62°C for LOX-1 and 64°C for 18s were used. The fluorescence was measured at the end of the annealing step. The melting curve analyses were performed between 55°C and 95°C to evaluate the quality of the final PCR products. Reactions were performed in triplicates for each sample. The standard curve of the reaction effectiveness was calculated using the serially diluted mixtures of experimental cDNA samples for LOX-1 and 18s separately. The calculation of the relative RNA concentration was performed using Cepheid SmartCycle software (Version 2.0d). Data are presented as the ratio of EndoG/18s mRNA.

**Protein measurement**

Protein was measured using the BCA (bicinchoninic acid) protein assay (Pierce, Rockford, IL). Bovine serum albumin was used as a standard.
Results

LOX-1 and SREC-1 are the most favorable candidates among scavenger receptors for cLDL in a cell free system

The effectiveness of chemical inhibitors of scavenger receptors for the prevention of cLDL internalization in endothelial cells justified a more thorough search for scavenger receptors responsible for cLDL binding. Receptors for cLDL-AF594 were tested by a fluorescent ligand-receptor assay using a set of recombinant human scavenger receptor proteins. Previous studies suggested that scavenger receptors LOX-1, CD36, SR-A1 and SREC-1 play physiological roles for modified LDLs. Because we used oversaturating concentrations of all receptor proteins, we assume equal coating of recombinant receptor proteins on test plate wells (please see www.ahajournals.org). Under such assumptions, our experiments indicate that although all studied scavenger receptors are capable of specific binding of cLDL, LOX-1 and SREC-1 receptors appear to have the highest affinity for cLDL (Supplement Fig. IIIA). In a separate experiment, LOX-1 bound more oxLDL than cLDL (Supplement Fig. IIIB), while the binding to SREC-1 was not statistically different between these two LDL isoforms. SR-A1 and CD36 bound significantly more cLDL and oxLDL than nLDL (Supplement Fig. IIIB). Binding of labeled cLDL to LOX-1, SREC-1 and SR-A1 was only modestly diminished by competition from unlabeled oxLDL (Supplement Fig. IIIC) suggesting that perhaps all three receptors have higher affinity for cLDL than for oxLDL. The binding of cLDL to CD36 was substantially inhibited only by unlabeled oxLDL. Pretreatment of LOX-1 with poly(I) or k-carrageenan significantly inhibited binding of both modified LDLs (Supplement Fig. IIID).

Taken together, these data suggest that while cLDL and oxLDL appear to bind to the same scavenger receptors, scavenger receptor affinity may differ.


Supplement Figure I. AlexaFluor 488 (A) and AlexaFluor 594 (B, upper panel) labeled LDLs and BSA prepared as described in Materials and Methods. The gel images were taken in UV light. To verify that fluorescent bands belong to lipoproteins, the gel was stained with Sudan black (B, lower panel).
Supplement Figure II. Detection of ApoB protein using cell ELISA in HCAECs after treatment with LDL (25 µM, 4 hours). n=4 per point; *P<0.05 as compared to nLDL (A). Inhibition of LDL internalization by HCAECs using the chemical inhibitors of scavenger receptors, k-carrageenan and poly(I) (250 µg/ml each). n=4 per group; *P<0.05 as compared to vehicle (water) (B).
Supplement Figure III. Results of the cell-free in vitro receptor-ligand binding assay. (A) The affinity of the AF594-labeled cLDL (0.01-10 µg/ml, 2 hours) to the several recombinant scavenger receptor proteins in the absence or presence of 30-fold concentration of non-labeled cLDL was evaluated as described in Materials and Methods; n=4 per point. (B) Comparison of cLDL, oxLDL and nLDL binding to the same recombinant scavenger receptor proteins (1 µg/ml, 2 hours); n=4 per point; *P<0.05 as compared to a buffer; #P<0.05 as compared to nLDL; ¶P<0.05 as compared to oxLDL. (C) To evaluate the competition between cLDL and oxLDL or nLDL, in separate experiments, the same, 10-fold and 30-fold amount of unlabeled oxLDL or nLDL was added to the labeled cLDL (1 µg/ml, 2 hours) and immobilized scavenger receptors; n=4 per point; *P<0.05 as compared to a cLDL-AF594. (D) Pretreatment of the immobilized recombinant LOX-1 receptor protein with k-carrageenan and poly(I) (250 µg/ml) significantly inhibited cLDL and oxLDL binding to LOX-1. n=4 per point; *P<0.05 as compared to vehicle (water).
Supplement Figure IV. Ability of the antibodies (10 µg/ml) to inhibit cLDL (1 µg/ml) binding to scavenger receptors immobilized to 96-well plate (5 µg/ml) as tested in cell-free in vitro receptor-ligand binding assay described in Materials and Methods. n = 3 per point, *P<0.05 vs. cLDL-AF594+non-specific IgG.
Supplement Figure V. One-time transfection of HCAECs with siRNA to LOX-1. Cells were transfected with anti-LOX-1 siRNA for 48 hours and then exposed with 200 μg/mL cLDL for 24 hours. At the end of the experiment, some cells were harvested, total RNA was extracted and real-time RT-PCR performed (A). Another part of the cells were fixed and cell ELISA was performed using the anti-LOX-1 antibody as described in Methods (B). Control cells were treated with vehicle or 200 μg/mL nLDL. n = 3 per point, *P<0.05 vs. cells before treatment, #P<0.05 vs. control siRNA-pre-treated cells.
Supplement Figure VI. Double transfection of HCAECs with siRNA to LOX-1. Cells were transfected with anti-LOX-1 siRNA for 72 hours, then re-tranfected for 48 hours, and then exposed with 200 μg/mL cLDL for 24 hours. At the end of the experiment, cells were fixed and cell ELISA (A) or Western blotting (B) was performed using the anti-LOX-1 antibody as described in Methods. Control cells were treated with vehicle or 200 μg/mL nLDL. n = 3 per point, *P<0.05 vs. cells before treatment, #P<0.05 vs. control siRNA-pre-treated cells.
Supplement Figure VII. Inhibition of cytotoxicity (A) and monocyte adhesion (B) was studied after double transfection of HCAECs with siRNA to LOX-1. The cells were transfected with anti-LOX-1 siRNA for 72 hours, then re-transfected for additional 48 hours, and then exposed with 200 μg/mL cLDL, oxLDL or nLDL for 24 or 16 hours for cytotoxicity or monocyte adhesion assays, respectively. To control specificity of siRNA, all experimental cells were compared to cells transfected with control siRNA. *P<0.05 as compared to cells treated with control siRNA; #P<0.05 as compared to vehicle-treated cells; ¶P<0.05 as compared to nLDL-treated cells.