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Scavenger receptors of endothelial cells mediate the uptake and cellular pro-atherogenic effects of carbamylated LDL

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Abstract

Objective—Carbamylated LDL (cLDL) has been recently shown to have robust pro-atherogenic effects upon 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 so may cause the pro-atherogenic 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 translocation (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 while 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 utilizes unique pattern of scavenger receptors. They show that LOX-1 receptor, and partially, CD36, SREC-1 and SR-A1 receptors are essential for the pro-atherogenic effects of cLDL on human endothelial cells.

Keywords

carbamylated LDL; LOX-1; scavenger receptor; endothelial cells; atherosclerosis

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Disclosures

None.

Introduction

Modified low density lipoproteins (LDLs) play a significant role in atherosclerosis development 1. 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 2, is present in healthy individuals and elevated in patients with chronic kidney disease (CKD) 3. 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 4.

Carbamylated LDL has strong atherogenic effects on endothelial cells by impacting the cell cycle 5, causing cell injury 5, 6 and promoting monocyte adhesion through the overexpression of ICAM-1 and VCAM-1 molecules 7. The initial mechanisms of these cLDL-induced events are not known. In particular, there is no information regarding whether cLDL utilizes any specific receptors or messengers. Horkko and co-authors 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) 8. 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 8, 9. Other modified LDLs (i.e. oxidized LDL (oxLDL), glycated LDL, acetylated LDL (acLDL), ethylated LDL, myeloperoxidase-generated cLDL, and others) also bind preferentially to scavenger receptors compared to LDLR 4, 10-13.

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 pro-atherogenic 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 cLDL's pro-atherogenic effects. We demonstrated that several scavenger receptors are capable of binding cLDL, and that the LOX-1 receptor is up-regulated 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 www.ahajournals.org.

LDL labeling with I^{125} and AlexaFluor dyes

For details, please see www.ahajournals.org.

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-$Apoe^{tm1Unc/J}$ (background C57BL/6) mice were subjected to intravenous injections with ^{125}I-labeled cLDL (^{125}I-cLDL) or ^{125}I-labeled nLDL (^{125}I-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 www.ahajournals.org.

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. For details, please see www.ahajournals.org.

Cell culture and LDL treatment of cells

Human coronary artery endothelial cells (HCAECs) were supplied by Lonza Inc. (Walkersville, MD). 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 www.ahajournals.org.

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). For details, please see www.ahajournals.org.

Cytotoxicity assay

Cell death was measured using lactate dehydrogenase (LDH) release as described previously 5. 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, Madison, WI).

Immunocytochemical staining

For details, please see www.ahajournals.org.

Monocyte adhesion *in vitro*

Monocyte adhesion was determined in a static model as described previously 7. For details, please see www.ahajournals.org.

Cell ELISAs

The cells were washed, fixed and probed with one 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, Santa Cruz, CA). Protein expression in every sample was normalized by β-actin expression. For details, please see www.ahajournals.org.

Western blotting

For details, please see www.ahajournals.org.

LOX-1 siRNA silencing

LOX-1 silencing in HCAECs was performed as described before 7. For details, please see www.ahajournals.org.

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 $d(T)_{16}$. For details, please see www.ahajournals.org.

Statistical analysis

Results were expressed as mean ± standard error. Statistical analyses were performed using the ANOVA and Student's t test. Comparisons with multiple time points were performed by t-test with the Bonferoni's adjustment. Values of $P<0.05$ were considered significant.

Results

Accumulation of cLDL in the vascular system *in vivo*

While it was shown before that cLDL is removed rapidly from the bloodstream 8, 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 ^{125}I-cLDL or ^{125}I-nLDL, respectively. A profound decrease of both cLDL and nLDL in the plasma was observed (Fig. 1A) and plasma level of ^{125}I-nLDL was several times that of ^{125}I-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 (Fig. 1B).

For tracking in cardiovascular tissues, cLDL was labeled with AF488 (Supplement Fig. 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 (Fig. 1C). Furthermore, we used the same compounds in *ex vivo* experiments where solutions were perfused through isolated hearts as described in the Supplement. In these experiments, cLDL was the only compound, which was localized under endothelial cells within 15 minutes of perfusion (Fig. 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). Prior to quantification, internalization of the cLDL was verified using the confocal microscopy (Fig. 2A). We found that cLDL was dose-dependently internalized in endothelial cells at a higher rate than the two other LDLs, reaching a plateau at 50 μg/ml (Fig. 2B). Endothelial cells demonstrated a time-dependent internalization of all three isoforms (25 μg/ml) in the period of time from 0 to 6 hours (Fig. 2C). Carbamylated LDL had the highest and nLDL had the lowest accumulation within the cells. The maximum cellular accumulation was detected after 6-8 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 remained to 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 (Supplement Fig. IIA). The use of k-carrageenan and polyinosinic acid (poly(I)), two commonly used chemical inhibitors of scavenger receptors 14, protected the endothelial cells from the accumulation of both cLDL and oxLDL (Supplement Fig. 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 semi-permeable membrane were treated with labeled cLDL using a modification of an *in vitro* system previously described 15. Within 30 minutes of treatment, a significantly higher proportion of cLDL than of oxLDL or nLDL was translocated beneath the cells (Fig. 2D). After one 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 www.ahajournals.org for results and Supplement Fig. III). We next investigated scavenger receptors' physiological ability to bind cLDL in intact endothelial cells. To investigate particular scavenger receptors, we blocked individual receptors using neutralizing antibody 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 (Supplement Fig. IV). Our results suggested that none of the receptors is solely responsible for cLDL internalization and subendothelial translocation (Fig. 3A). Pretreatment of the cells with antibody to LOX-1 and CD36 had the highest inhibition (25-30%) 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 four 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 (Fig 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, two 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 four scavenger antibodies significantly reduced the cytotoxicity induced by cLDL or oxLDL (Fig. 3C). The inhibition of LOX-1 provided the most prominent protection (39% for cLDL and 52% for oxLDL). 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 (data are not shown). 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 non-specific IgG, however oxLDL decrease did not reach statistical significance (Fig. 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 LOX-1 antibody alone (data are not shown).

Therefore these results suggest that, while 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 scavenger receptors' expression 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* 5, 16. 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 (Fig. 4A) and high (Fig. 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 (Fig. 4C). To determine if 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 (Fig. 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 pro-atherogenic 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 (Supplement Fig. V). While 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 (Fig. 5A). Inhibition of LOX-1 significantly protected against cLDL-accelerated monocyte adhesion, but did not affect oxLDL-mediated monocyte adhesion to endothelial cells (Fig. 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 (Supplement Fig. 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 (Supplement Fig. VII).

Our previous data suggested that ICAM-1 and VCAM-1 are important in cLDL-induced monocyte adhesion 7. 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 (Figs. 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 8, 9, 17. 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 9, 17. 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 18, 19.

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 two, 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 3, 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. While 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 collaboration and interference between different receptors in cells and necessity of di- or heteromerization of the scavenger receptors shown before 20. 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 four 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 21, 22.

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 utilizes the ICAM-1 and VCAM-1 adhesion molecules 7. Other reports have indicated that LOX-1 upregulates both ICAM-1 and VCAM-1 expression 23. Furthermore, our siRNA experiments suggested that cLDL-induced monocyte adhesion is mediated through LOX-1 as well as ICAM-1/VCAM-1 over-expression, thus, potentially linking cLDL with LOX-1 and ICAM-1/VCAM-1 molecules in a single pathway.

It is an interesting observation that while 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 21, 24, we can speculate that perhaps different affinity of cLDL and oxLDL to scavenger receptors and different biological properties of uptaken modified LDLs may explain this observation.

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 utilizes 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 25. Previously, it was proposed that oxLDL can be translocated through endothelium 26. Later studies determined that oxLDL is mainly formed subendothelially 27. Unlike oxLDL, most cLDL is synthesized in plasma by urea-derived cyanate 2. 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: while LDLR and SREC-1 are modestly increased, the LOX-1 receptor is dramatically over-expressed in response to cLDL. It has been shown before that LOX-1 is a stable protein, while siRNA may be used mostly to inhibit induction of new LOX-1 19. 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 19. 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 28. We speculate that inducible LOX-1 may be 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 utilizes 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 pro-atherogenic 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 anti-atherosclerosis 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 anti-atherosclerosis drugs toward the treatment and prevention of cLDL-induced atherosclerosis is a subject for future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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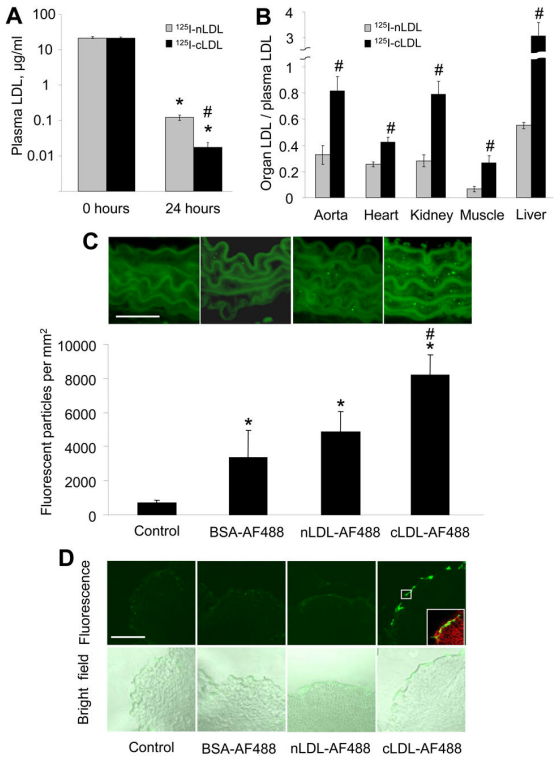

Figure 1.

Accumulation of ^{125}I-cLDL or ^{125}I-nLDL in tissues. The amounts of both LDLs remaining in plasma (**A**) or present in tissues (**B**) were measured 24 hours later. n=10; *P<0.05 as compared to 0 hours, #P<0.05 as compared to nLDL. Accumulation of AF488-labeled nLDL and AF488-labeled cLDL is shown in the aorta *in vivo* (**C**) and in the heart *ex vivo* (**D**). Scale, 150 μm. n=10; *P<0.05 as compared to control, #P<0.05 as compared to nLDL.

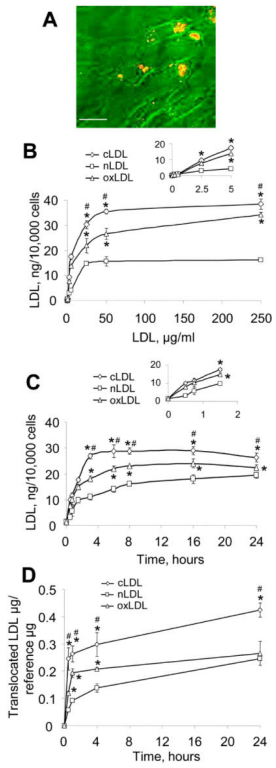

Figure 2.

Endothelial internalization and subendothelial transfer of cLDL *in vitro*. Internalization of AF594-labeled LDLs to HCAECs was detected by confocal microscopy (**A**) and fluorimetry in a dose-dependent (**B**) and a time-dependent manner (**C**). Small attached plots are presented to illustrate the data legibility at small doses and early time-points. n=4 per time/dose point; *P<0.05 as compared to nLDL; #P<0.05 as compared to oxLDL. Transfer of LDLs (25 μg/ml, 24 hours) through endothelial monolayer (**D**). n=6 per point, *P<0.05 as compared to nLDL, #P<0.05 as compared to oxLDL.

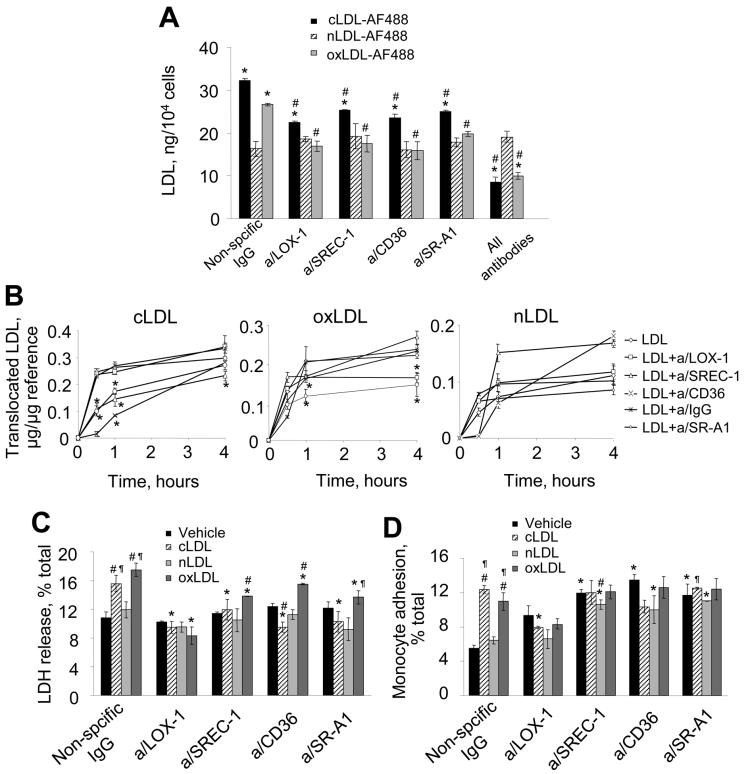

Figure 3.

Antibody inhibition (10 μg/ml) of endothelial internalization (**A**) and subendothelial translocation (**B**) of fluorescently-labeled LDLs (25 μg/ml, 4 hours). n=4 per point; *P<0.05 as compared to an nLDL; #P<0.05 as compared to pretreated with non-specific IgGs. Inhibition of cytotoxicity (**C**) and monocyte adhesion (**D**) induced by cLDL, nLDL or oxLDL (200 μg/ml, 16 or 24 hours for cytotoxicity and monocyte adhesion, respectively) using the antibodies to LOX-1, SREC-1, CD36 or SR-A1 (10 μg/ml). n=4 per point; *P<0.05 as compared to cells pretreated with IgGs; #P<0.05 as compared to vehicle (PBS, 200 μM EDTA)-treated cells; ¶P<0.05 as compared to nLDL-treated cells.

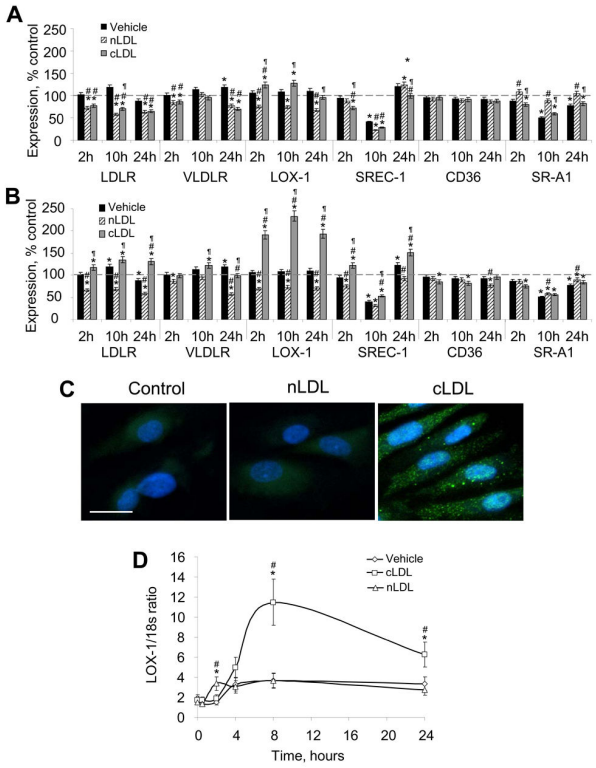

Figure 4.

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 = 3-4 per point; *P<0.05 as compared to 0 hours 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.

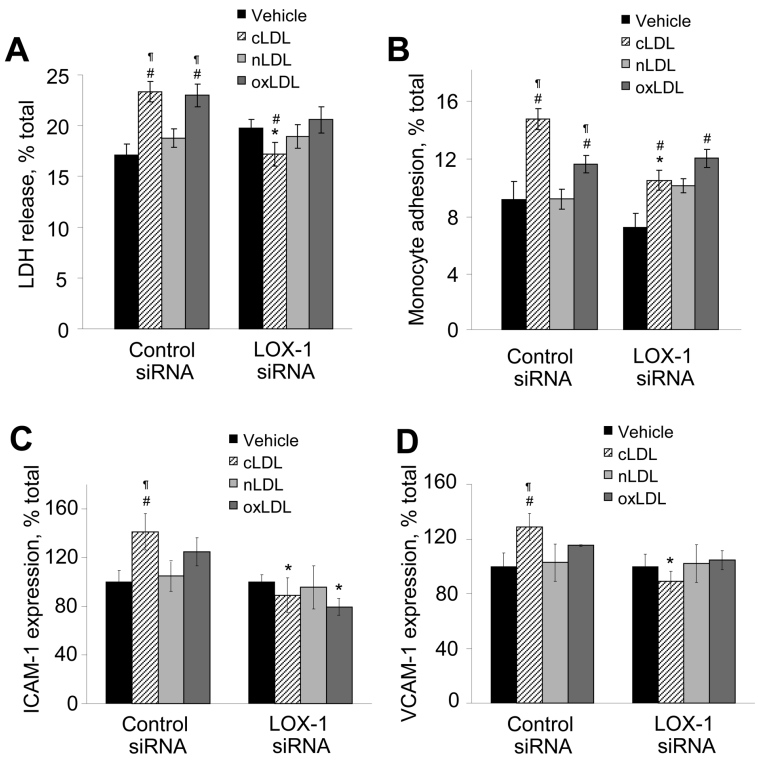

Figure 5.

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 nM, 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.