Macrophage receptors responsible for distinct recognition of low density lipoprotein containing pyrrole or pyridinium adducts: models of oxidized low density lipoprotein

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Abstract Oxidation of low density lipoproteins (LDL) induced by incubation with Cu^{2+} ions results in the formation of a heterogeneous group of aldehydic adducts on lysyl residues (Lys) of apolipoprotein B (apoB) that are thought to be responsible for the uptake of oxidized LDL (oxLDL) by macrophages. To define the structural and chemical criteria governing such cell recognition, we induced two modifications of lysines in LDL that mimic prototypic adducts present in oxLDL; namely, ε-amino charge-neutralizing pyrrolation by treatment with 2,5-hexanedione (hdLDL), and ε-amino charge-retaining pyridinium formation via treatment with 2,4,6-trimethylpyrylium (tmpLDL). Both modifications led to recognition by receptors on mouse peritoneal macrophages (MPM). To assess whether the murine scavenger receptor class A-I (mSR-A) was responsible for recognition of hdLDL or tmpLDL in MPM, we measured binding at 4°C and degradation at 37°C of these modified forms of ^{125}I-labeled LDL by mSR-A-transfected CHO cells. Although uptake and degradation of hdLDL by mSR-A-transfected CHO cells was quantitatively similar to that of the positive control, acLDL, tmpLDL was not recognized by these cells. However, both tmpLDL and hdLDL were recognized by 293 cells that had been transfected with CD36. In the human monocytic cell line THP-1 that had been activated with PMA, uptake of tmpLDL was significantly inhibited by blocking monoclonal antibodies to CD36, further suggesting recognition of tmpLDL by this receptor. Macrophage uptake and degradation of LDL oxidized by brief exposure to Cu^{2+} was inhibited more effectively by excess tmpLDL and hdLDL than was more extensively oxidized LDL, consistent with the recognition of the former by CD36 and the latter primarily by SR-A. Collectively, these studies suggest that formation of specific pyrrole adducts on LDL leads to recognition by both the mSR-A and mouse homolog of CD36 expressed on MPM, while formation of specific pyridinium adducts on LDL leads to recognition by the mouse homolog of CD 36 but not by mSR-A. As such, these two modifications of LDL may represent useful models for dissecting the relative contributions of specific modifications on LDL produced during oxidation, to the cellular uptake of this heterogeneous ligand.—Podrez, E. A., G. Hoppe, J. O'Neil, L. M. Sayre, N. Sheibani, and H. F. Hoff. **Macrophage receptors responsible for distinct recognition of low density lipoprotein containing pyrrole or pyridinium adducts: models of oxidized low density lipoprotein. *J. Lipid Res.* 2000.** 41: **1455–1463.**

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Oxidation of lipoproteins such as low density lipoproteins (LDL) has attracted much attention because of its potential role in several disease processes including cardiovascular disease (1–3). Although oxidized LDL (oxLDL) has been shown to possess a myriad of biological properties, one in particular stands out, namely the ability of oxLDL to induce lipid loading of macrophages in culture, which has served as a useful model for the formation of lipid-laden macrophages or foam cells in vivo, the hallmark of early atherosclerotic lesions. The ability to induce high uptake of oxLDL by macrophages appears to be due to the recognition by scavenger receptors on such cells (4) of LDL that had been oxidized extensively with Cu^{2+} ions, and by the uptake via phagocytosis of aggregates of lipoprotein particles usually present in such samples of oxLDL (5). In more recent studies, oxLDL has been dem-

Abbreviations: acLDL, acetylated LDL; BHT, butylated hydroxytoluene; CHO cells, Chinese hamster ovary cells; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; FACS, fluorescence activation cell sorter; FCS, fetal calf serum; hdLDL, hexanedione-modified LDL; HNE, 4-hydroxy-2-nonenal; LDFCS, lipoprotein-deficient fetal calf serum; LDL, low density lipoproteins; MPM, mouse peritoneal macrophage; mSR-A, murine scavenger receptor, class A; oxLDL, oxidized LDL; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; REM, relative electrophoretic mobility; SR-A, scavenger receptor, class A; tmpLDL, trimethylpyrylium-modified LDL.

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onstrated to be recognized by a significant number of newly characterized scavenger receptors (6), the initial receptor termed SR-A having been cloned by Krieger and Herz (7) and possessing acetylated LDL (acLDL) as its prototypic ligand. Another receptor that appears to recognize both mildly and more extensively oxidized LDL is CD36, a receptor on macrophages that recognizes several ligands including thrombospondin (8). The importance of receptors other than SR-A in the uptake of oxLDL by macrophages is shown by the finding that oxLDL continued to be taken up by macrophages derived from SR-A knockout mice (9). However, it is still unclear which structural modifications occurring in oxLDL are responsible for its recognition by individual receptors.

Oxidation of LDL represents a complex mixture of chemical modifications to both the lipid and protein moieties of the lipoprotein particle (1–3, 10). The most commonly used method of oxidation of LDL is incubating with Cu^{2+} ions, which results in the free radical-induced formation of hydroperoxides in unsaturated fatty acids still esterified to phospholipids and cholesterol (11). These hydroperoxides can then decompose by β scission to form a variety of shorter chain aldehydes that contain either the methyl terminus or the carboxy-esterified end of the original fatty acid chain (12). Of particular consequence is the fact that many of these aldehydic scission products contain α,β unsaturation, epoxide moieties, and/or other groups that confer a high propensity toward formation of stable covalent adducts and cross-links with protein nucleophilic groups (3, 10). Much attention has focused on 4-hydroxy-2-nonenal (HNE), which is formed in abundance by the oxidation of arachidonate and linoleate (10, 13). The main reaction of HNE with proteins involves the formation of C-3 Michael adducts with the thiol, imidazole, and ε-amino side chains of cysteine, histidine, and lysine residues, respectively, that are stabilized in part by hemiacetal cyclization of the 4-hydroxy group (10–15). However, the HNE–lysine Michael adduct is formed reversibly (15), and the ability of HNE to react with the lysine ε-amino group also via Schiff base formation leads to a complex evolution of "advanced" adducts involving condensation, dehydration, and cyclization reactions (16) as well as lysine–lysine cross-linking (17–19). Theoretically, HNE can also cause inter- and intramolecular cross-linking of proteins by forming C-1 lysine–Schiff base/C-3 Michael adducts with cysteine thiols or histidine imidazoles (20), although the lysine–lysine Schiff base Michael cross-link requires reductive stabilization (15, 21). In addition to 4-hydroxy-2-alkenals such as HNE, other lipoxidation-induced modifiers including malondialdehyde (22, 23), 2-alkenals (24, 25), and 4,5-epoxy-2-alkenals (26) also modify protein lysine ε-amino groups, forming a large array of advanced adducts that alter the physicochemical properties of the side chain. This includes neutral pyrroles (16, 26, 27) and dihydropyridines (23, 28, 29) as well as cationic pyridiniums (24, 25, 29). It is unclear whether the formation of any such individual advanced adducts contributes to the biological properties of oxLDL, in part because any given lipoxidation-derived mediator (e.g., HNE) that can be reacted with LDL in vitro forms a greatly heterogeneous mixture of adducts.

To better define the structural and chemical criteria governing cell recognition of oxLDL, we chose two classic chemical modification agents that induce homogeneous modifications of apolipoprotein B (apoB) lysines in LDL that mimic two such "advanced" prototypic adducts believed to be present in oxLDL. They are *1*) the formation of a lysine charge-neutralizing pyrrole via treatment with 2,5-hexanedione, abbreviated as hdLDL; and *2*) the formation of a lysine charge-retaining pyridinium ring via treatment with 2,4,6-trimethylpyrilium, abbreviated as tmpLDL. Although few chemical modifications can be considered truly homogeneous, the reactions in these two have not been reported to generate significant by-products that could confuse the structural interpretation of our results. In this study we report that these two chemical models for oxidative modifications of LDL are recognized differently by receptors on MPM: hdLDL by both the scavenger receptor class A (SR-A) and by the mouse homolog of CD36, and tmpLDL by the mouse homolog of CD36 but not SR-A.

MATERIALS AND METHODS

Materials

Fatty acid-free bovine serum albumin (BSA), butylated hydroxytoluene (BHT), trichloroacetic acid (TCA), and Na_2EDTA were purchased from Sigma (St. Louis, MO). Carrier-free $Na^{125}I$ was from ICN Biomedical (Irvine, CA), RPMI 1640, F12 nutrition mixture, and Dulbecco's modified Eagle's medium (DMEM) were from GIBCO Laboratories (Grand Island, NY), and the bicinchoninic acid assay reagents were from Pierce (Rockford, IL). Tissue culture plates were obtained from Costar (Cambrige, MA); and C57BL/6 mice (16–20 weeks of age) were purchased from the Trudeau Institute (Saranac Lake, NY). The THP-1 macrophage cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD).

Lipoproteins

LDL was isolated from fresh plasma obtained from the Cleveland Clinic Blood Bank (Cleveland, OH) by sequential ultracentrifugation as a $1.019 < d < 1.063$ g/ml fraction, using the procedure of Hatch and Lees (30). The LDL was dialyzed against phosphate-buffered saline (PBS) containing 0.15 M NaCl, 20 mM sodium phosphate (pH 7.4), 0.5 mM Na_2EDTA, and 40 mM butylated hydroxytoluene (BHT), filter sterilized, and stored at 4°C. LDL was labeled with $Na^{125}I$ by the iodine monochloride procedure of McFarlane (31) as modified by Bilheimer, Eisenberg, and Levy (32); the specific activity of labeled preparations was between 100 and 200 cpm/ng protein. Lipoprotein concentrations are routinely expressed as micrograms of protein per milliliter. Acetylation of LDL was performed by repeated additions of acetic acid anhydride (33) and then extensively dialyzed against 0.15 M NaCl, 0.3 mM Na_2EDTA, pH 7.4. Oxidation of ^{125}I-labeled and unlabeled LDL was performed in the presence of 5 μM $CuSO_4$ in PBS at 37°C at a protein concentration of 200 μg/ml for 24 h. The concentration of EDTA in LDL preparations was reduced prior to oxidation by overnight dialysis against PBS. Oxidation was terminated by dialyzing samples into PBS containing 40 μM BHT and 0.5 mM EDTA for 24 h. Aggregated LDL was produced by vortexing solutions of LDL (500 μg/ml in 0.15 M NaCl, 0.3 mM Na_2EDTA, and 40 μM BHT). ^{125}I-Labeled hexanedione-modified LDL (hdLDL)

and ^{125}I-labeled trimethylpyrylium-modified LDL (tmpLDL) were prepared by incubation of ^{125}I-labeled LDL at a concentration of 500 μg/ml with 200 mM 2,5-hexanedione or 4 mM 2,4,6-trimethylpyrylium tetrafluoroborate for 3 days at 37°C in the presence of 40 μM BHT and 0.3 mM EDTA. Treatment of LDL with 2,5-hexanedione converts the positively charged lysine amino groups on apoB of LDL to neutral 2,5-dimethylpyrroles (34, 35), while treatment of LDL with 2,4,6-trimethylpyrylium (36) converts lysyl residues to a cationic pyridinium group. Because the 2,5-dimethylpyrroles formed on proteins by 2,5-hexanedione can over time result in autoxidation-dependent protein cross-linking (35, 37), it is important to obtain cell results on freshly treated samples. Modified LDL were centrifuged for 15 min at 10,000 *g* and filtered through 0.2-μm pore size filters prior to use.

Cells

Mouse peritoneal macrophages (MPM) were harvested 2 to 3 days after thioglycolate stimulation of female C57BL/6 mice by peritoneal lavage with ice-cold PBS. Primary cultures were prepared at a density of 10^6 cells/16-mm-diameter well in RPMI 1640 containing 10% fetal calf serum and were used 48 h after plating (38). CHO cells expressing mouse scavenger receptor class A type I (CHO-mSR-A) and mock-transfected parental CHO cells were a gift from M. Krieger (7). 293 cells (embryonic kidney epithelial cells transformed with adenovirus) were obtained from the ATCC (Rockville, MD) and maintained in DMEM with 5% fetal calf serum (FCS). The human CD36 cDNA was cloned into the pcDNAIneo expression vector (Invitrogen, Carlsbad, NY) as previously described (39). Transfected cells were grown in the presence of G418 (500 μg/ml), and clones were isolated and characterized for expression of CD36 by fluorescence activation cell sorter (FACS) analysis, using the monoclonal antibody (MAb) FA6-152 (Immunotech, Westbrook, ME). Human monocytes were isolated from whole blood by sequential centrifugation as described previously (40) and cultured at a density of 10^6 cells per well in DMEM with 10% FCS. The cell population consisted of more than 95% monocytes. THP-1 cells were plated at a density of 0.5×10^6 cells per well and incubated with 64 nM phorbol myristate acetate in RPMI 1640 with 10% FCS and 5×10^{-5} M 2-mercaptoethanol for 3 days prior to use.

Lipoprotein–cell interactions

Experiments were performed on confluent cell monolayers in Ham's F12 medium containing 3% lipoprotein-deficient FCS (LDFCS) when using CHO or CHO-mSR-A cells, and in RPMI 1640 containing LDFCS when using MPM. Cells were washed with serum-free medium, and the indicated concentration of ^{125}I-labeled native or modified LDL was added in 250 μl of medium containing fetal calf serum. After incubation for 5 h at 37°C, media were removed and assayed for trichloroacetic acid (TCA)-soluble, noniodide degradation products as described previously (38). Cells were washed three times with PBS, dissolved in 0.25 N NaOH, and assayed for cell-associated label and protein content. All determinations are reported as means ± SD of triplicate determinations. In competition experiments ^{125}I-labeled lipoprotein (5 μg/ml) was incubated with cells in the presence of unlabeled competitor (200 μg/ml). To assess binding, native and modified forms of ^{125}I-labeled LDL were incubated at the indicated concentrations with cells at 4°C for 4 h with (nonspecific binding) or without (total binding) a 40-fold excess of unlabeled lipoprotein as reported previously (41). Bound radioactivity was measured, and specific binding was calculated as total minus nonspecific binding. Incorporation of [^{14}C]oleate into cellular [^{14}C]cholesteryl oleate was assessed as described earlier (42). Foam cell formation in MPM was assessed microscopically after staining with oil red O.

Analytic procedures

The electrophoretic mobility relative to LDL (REM) was determined with premade 1% agarose gels (Ciba Corning, East Walpole, MA) as described elsewhere (43). The protein content of lipoproteins, cells, and cell extracts was measured in triplicate by the bicinchoninic acid (BCA) assay, using BSA as a standard (43). Samples were diluted in 1% sodium dodecyl sulfate (SDS) to minimize turbidity. Samples were incubated for 60 min at 60°C, and absorbance was measured at 595 nm. Blockage of lysyl residues was determined as described previously (44). All experiments reported were performed at least three times.

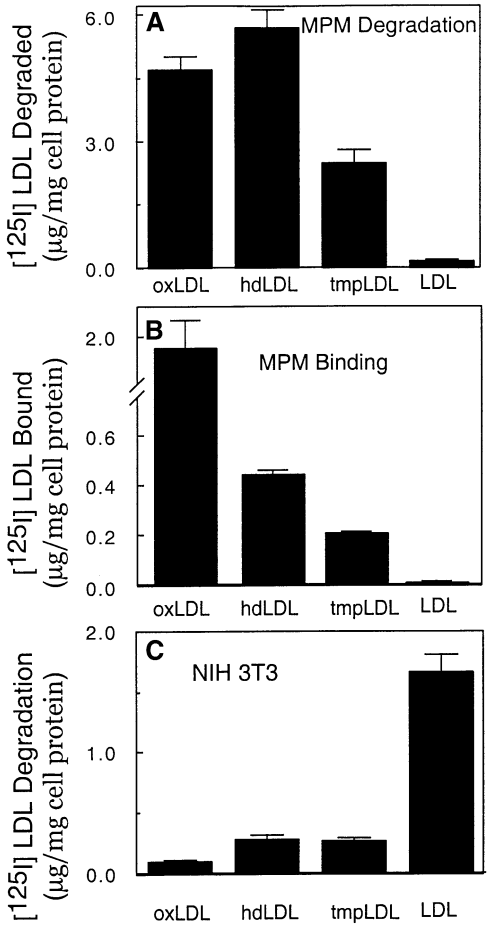
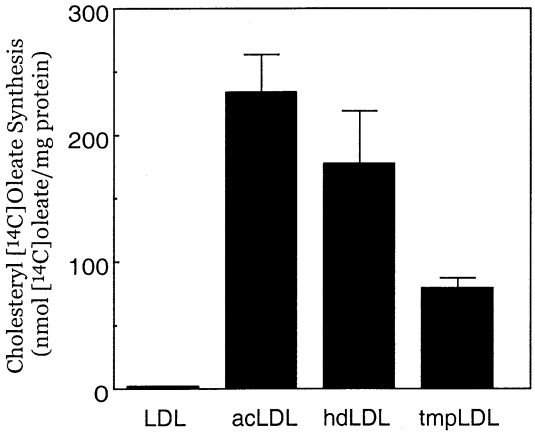


Fig. 1. hdLDL and tmpLDL are bound and degraded by macrophages but not by NIH 3T3 fibroblasts. Mouse peritoneal macrophages (MPM) (A and B) were harvested 2 to 3 days after thioglycolate stimulation. Cells were plated at a density of 10^6 cells/16-mm diameter well in RPMI 1640 containing 10% fetal calf serum and were used 48 h after plating. NIH 3T3 cells (C) were grown to confluency in DMEM/Ham's F12 medium containing 5% fetal calf serum in 16-mm wells. Oxidation of LDL (oxLDL) was performed in the presence of 5 μM $CuSO_4$ in PBS at 37°C for 24 h. Hexanedione-modified LDL (hdLDL) and trimethylpyrylium-modified LDL (tmpLDL) were prepared by incubation of LDL at concentration 500 μg/ml with 200 mM 2,5-hexanedione or 4 mM 2,4,6-trimethylpyrylium tetrafluoroborate for 3 days at 37°C in the presence of 40 μM BHT and 0.3 mM EDTA. Cells were incubated with modified ^{125}I-labeled lipoproteins (5 μg/ml) either for 5 h at 37°C (A and C) or for 3 h at 4°C (B). Cells were washed two times with ice-cold PBS and three times with PBS/BSA. Subsequently, cellular degradation (TCA-soluble label) (A and C) or bound radioactivity (B) was measured. Data represent the mean ± SD of triplicate determinations.

RESULTS

hdLDL and tmpLDL share receptors with oxLDL on MPM

Modification of LDL with 2,5-hexanedione and with 2,4,6-trimethylpyrylium to form hdLDL and tmpLDL, respectively, was shown previously to induce specific chemical/structural modifications to LDL resulting in conversion of LDL into a high uptake form for macrophages (41). 2,5-Hexanedione converts positively charged lysine amino groups on apoB in LDL to neutral 2,5-dimethylpyrroles (41), while trimethylpyrylium converts lysyl residues to a cationic pyridinium group (41). For hdLDL and tmpLDL to mimic oxLDL in terms of receptor recognition one would anticipate them to be recognized by one or more of the scavenger receptors on macrophages but not by the LDL receptor on other cells. Consistent with this hypothesis, experiments on the binding at 4°C and degradation at 37°C demonstrated that both hdLDL and tmpLDL shared with oxLDL the ability to be recognized by some receptor(s) on thioglycolate-elicited mouse peritoneal macrophages (MPM) (**Fig. 1A** and **B**), although that of tmpLDL was lower. Similar results were obtained in human monocyte-derived macrophages and in the human monocyte-macrophage cell line THP-1 (data not shown). Degradation of hdLDL was comparable to that of oxLDL, while corresponding binding of oxLDL was significantly higher than that of hdLDL, reflecting the well-documented impaired degradation of oxLDL (38). Like oxLDL, both hdLDL and tmpLDL failed to be recognized by the LDL receptor on NIH 3T3 cells under conditions in which LDL is internalized (Fig. 1C). Similar results were obtained when measuring binding at 4°C (data not shown). Both labeled hdLDL and tmpLDL also bound to the surface of MPM in a concentration-dependent manner with high affinity and saturation kinetics (data not shown). Both hdLDL and tmpLDL appeared not to be aggregated, as indicated by agarose gel electrophoresis, and not to be internalized by phagocytosis. This is based on the observation that cytochalasin D, an inhibitor of phagocytosis (5, 45), failed to significantly inhibit the uptake and subsequent degradation of either hdLDL and tmpLDL, under conditions in which aggregated LDL uptake and degradation were inhibited (reduction in degradation relative to control values: of hdLDL, 11.2 ± 4.9%; of tmpLDL, 11.9 ± 5.9%; of acLDL, 16.6 ± 3.1%; and of vortex-aggregated LDL, 92.2 ± 0.4%). Because uptake of tmpLDL was only about one-half that of hdLDL, we asked whether



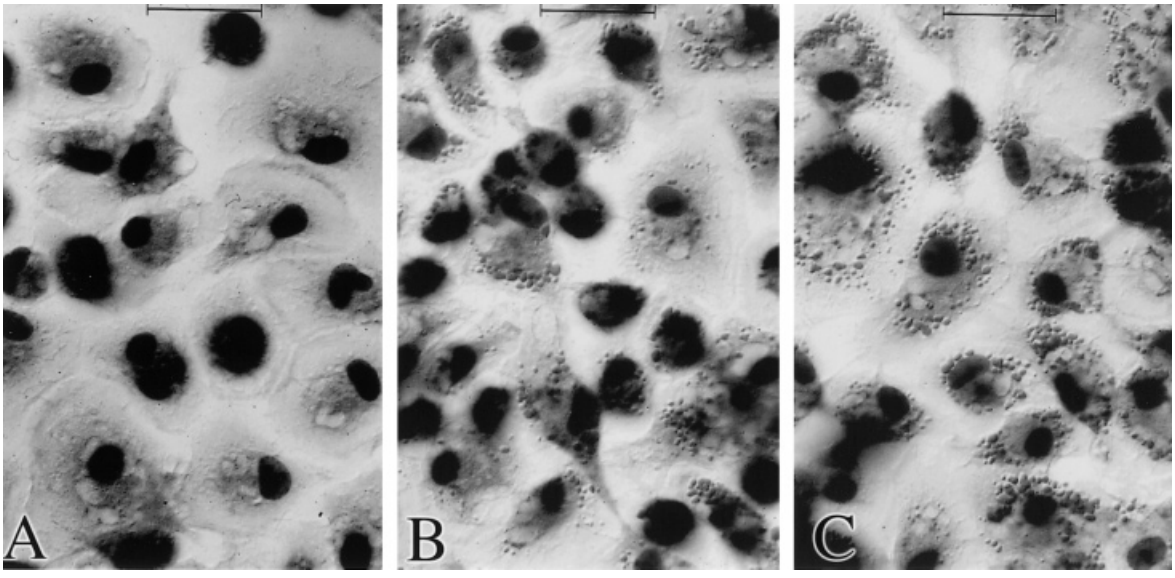


Fig. 2. hdLDL and tmpLDL can stimulate cholesterol esterification and induce foam cell formation in MPM. Top: Cells were incubated for 24 h at 37°C in the presence or absence of a 100-μg/ml concentration of unlabeled hdLDL, tmpLDL, acLDL (functioning as a positive control), and LDL (functioning as a negative control), and DMEM containing 0.27 mM [^{14}C]oleate-albumin (0.5 μCi/ml). After incubation, cells were washed with ice-cold PBS, cell neutral lipids were extracted with a hexane–isopropanol mixture 3:2 (v/v) and separated by TLC, and the incorporation of labeled oleate into the cholesteryl ester spot was determined. Data represent means ± SD of triplicate determinations. Bottom: Cells were incubated for 48 h at 37°C in the presence or absence of a 50-μg/ml concentration of unlabeled (A) LDL, (B) tmpLDL, or (C) hdLDL. Cells were fixed with 4% formaldehyde, stained with oil red O and hematoxylin, and viewed by interference contrast microsopy. Magnification bar: 10 μm.

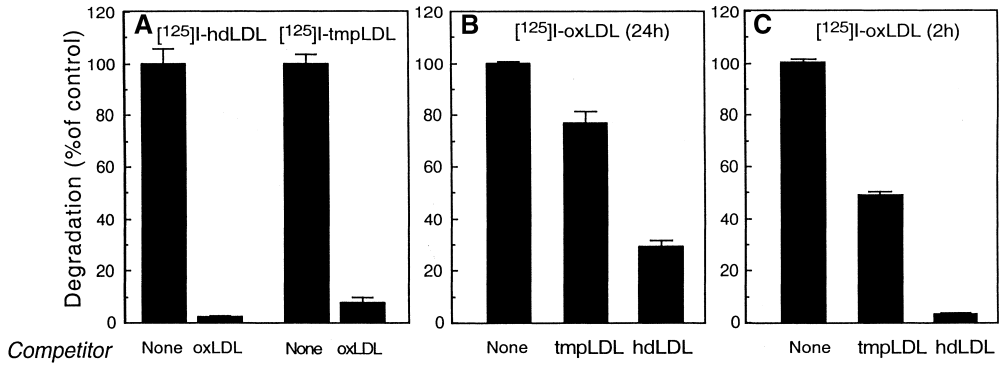


Fig. 3. tmpLDL competes better for the recognition by MPM of modestly oxidized LDL than of extensively oxidized LDL. (A) MPM were incubated for 5 h with ^{125}I-labeled hdLDL or tmpLDL (5 μg/ml) in the presence or absence of a 200-μg/ml concentration of unlabeled oxLDL (24 h), and cell degradation was determined as described in Materials and Methods. The 100% degradation values of hdLDL and tmpLDL were as follows: 6.41 ± 0.44 and 2.61 ± 0.23 μg/mg cell protein, respectively. MPM were incubated with ^{125}I-labeled LDL (5 μg/ml) that was oxidized with 5 μM Cu^{2+} for (B) 24 h = oxLDL (24 h), or for (C) 2 h = oxLDL (2 h), in the presence or absence of unlabeled hdLDL or tmpLDL (200 μg/ml), and degradation determined. The 100% values for oxLDL (24 h) and oxLDL (2 h) were as follows: 4.6 ± 0.1 and 2.3 ± 0.1 μg/mg cell protein, respectively. Data represent means + SD of triplicate determinations.

tmpLDL could induce foam cell formation in these cells by determining the stimulation of cholesterol esterification and accumulation of oil red O-positive neutral lipid droplets. Even though esterification levels were again higher for hdLDL than for tmpLDL (**Fig. 2, top**), reflecting the higher uptake and degradation (Fig. 1A), foam cell formation occurred in both, as determined by the presence of oil red O-positive droplets (Fig. 2, bottom).

tmpLDL is a poorer inhibitor of oxLDL recognition by MPM when the degree of oxidation is extensive than when it is modest

To assess whether hdLDL and tmpLDL shared the same recognition sites on MPM with oxLDL, we performed a cross-competition study for uptake and degradation in MPM. As seen in **Fig. 3A**, excess oxLDL almost quantitatively inhibited the uptake and degradation of both labeled hdLDL and tmpLDL. However, when competition was performed in the opposite direction, only excess hdLDL was an effective inhibitor of oxLDL uptake and degradation (Fig. 3B). To better understand why tmpLDL was a poor inhibitor of oxLDL, we asked whether labeled LDL, oxidized to a more modest degree, e.g., oxLDL (2 h) with Cu^{2+} rather than the 24 h routinely used to oxidize LDL throughout this study, would be inhibited more effectively than extensively oxLDL (24 h). Independent chemical studies on the formation of lipid hydroperoxides indicated that oxLDL (24 h) was oxidized to a higher degree than oxLDL (2 h) (data not shown). When competition studies were performed to assess the ability of excess tmpLDL and hdLDL to inhibit the degradation of modestly oxLDL (2 h), we now found that tmpLDL was a good inhibitor (50%), while hdLDL remained an effective inhibitor (Fig. 3C). These results suggest that tmpLDL share receptors on MPM with oxLDL (2 h) better than with oxLDL (24 h).

hdLDL, but not tmpLDL, is recognized by cells transfected with SR-A

To more directly identify the receptor(s) on MPM recognizing hdLDL and tmpLDL, we asked whether these ligands, like acLDL and oxLDL (24 h), would be recognized by CHO cells that had been stably transfected with the mouse scavenger receptor class A (SR-A). Both hdLDL and oxLDL demonstrated increases in degradation relative to the degradation in mock-transfected CHO cells (**Fig. 4**). In contrast, we found that tmpLDL failed to show any increases in degradation relative to the mock-transfected cells. Results on the binding at 4°C of hdLDL to SR-A-transfected and mock-transfected cells and cell association values at

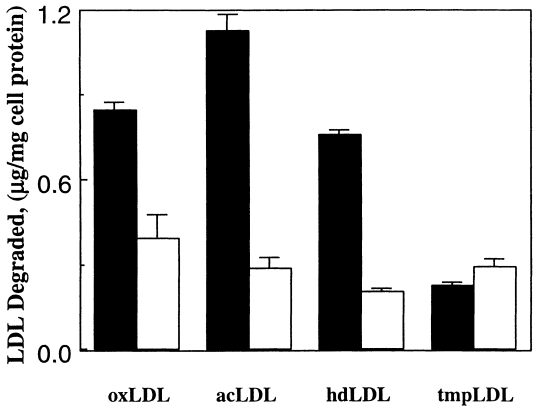


Fig. 4. hdLDL but not tmpLDL is recognized by mSR-A. CHO cells expressing SR-A (solid columns) or parental CHO cells (open columns) were grown to confluency in 16-mm well plates in F12 nutrition mixture containing 5% fetal calf serum. Cells were incubated for 5 h at 37°C with ^{125}I-labeled lipoproteins (5 μg/ml) modified as described in Materials and Methods. Subsequently, cells were washed two times with ice-cold PBS and three times with PBS/BSA and cellular degradation (TCA-soluble noniodine radioactivity) was determined. Data represent means + SD of triplicate determinations.

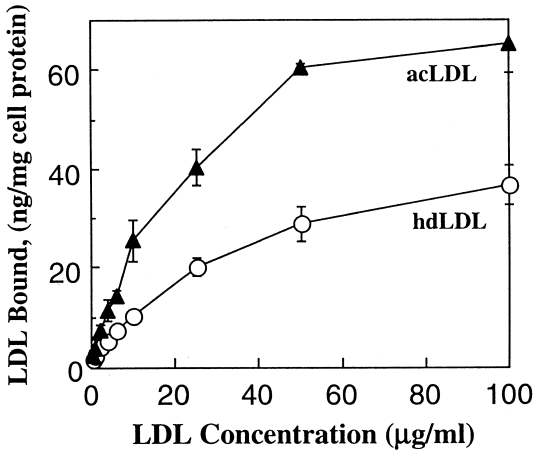


Fig. 5. hdLDL is not recognized to the same degree by SR-A as acLDL. ^{125}I-labeled hdLDL (open symbols) or acLDL (solid symbols) were incubated at the indicated concentrations with SR-A or vector-transfected CHO cells at 4°C for 3 h. Cells were washed two times with ice-cold PBS and three times with PBS/BSA. Subsequently, cells were solubilized in 0.1 M NaOH. Bound radioactivity was measured and SR-A-dependent binding was calculated as the difference between binding to CD36-transfected cells and binding to vector-transfected cells. Data represent means ± SD of triplicate determinations.

37°C paralleled those on the degradation at 37°C (data not shown). These data suggest that hdLDL, like oxLDL (24 h), is recognized by the SR-A, whereas tmpLDL is not. To assess whether the degree of recognition of hdLDL by the SR-A matches that of the positive control, acLDL, we performed a dose–response study (**Fig. 5**). These data on the concentration dependence of binding of hdLDL to CHO (mSR-A) cells gave a K_d of 35 ± 3.3 μg/ml, and a B_{max} of 49.6 ± 2.0 μg/mg cell protein for hdLDL, and a K_d of 24.4 ± 2.9 μg/ml and a B_{max} of 83.7 ± 3.8 μg/mg cell protein for acLDL.

Both hdLDL and tmpLDL are recognized by cells transfected with CD36

It had been shown previously that CD36 is partially responsible for the recognition of Cu^{2+}-oxidized LDL by macrophages both at low and high degrees of oxidation (8). To determine whether this receptor plays a role in the recognition of hdLDL or tmpLDL by macrophages, we assessed whether binding at 4°C of hdLDL or tmpLDL occurred in 293 cells that had been stably transfected with CD36. Surface expression of CD36 on CD36-transfected cells was confirmed by FACS analyses (data not shown). This experiment was conducted in the presence of excess unlabeled LDL to exclude any contribution of the LDL receptor possibly present on the mock-transfected cells. As seen in **Fig. 6A**, binding of both hdLDL and tmpLDL was increased in CD36-transfected as compared with mock-transfected cells. OxLDL (24 h), a prototypic ligand for CD36, showed significantly higher binding to CD36-transfected cells than to mock-transfected cells. Native LDL did not bind significantly under these conditions to cells transfected with CD36. Figure 6B demonstrates the concentration dependence at 4°C of binding of hdLDL and tmpLDL to CD36-transfected cells, demonstrating different K_d levels (K_d of 14.8 ± 1.9 μg/ml for hdLDL, K_d of 20.9 ± 4.6 μg/ml for tmpLDL). Likewise, B_{max} levels were significantly higher for hdLDL (B_{max} of 0.97 ± 0.04 μg/mg cell protein) than for tmpLDL (B_{max} of 0.61 ± 0.04 μg/mg cell protein). Because binding of tmpLDL to CD36-transfected cells was relatively low compared with hdLDL and oxLDL, we additionally verified the ability of

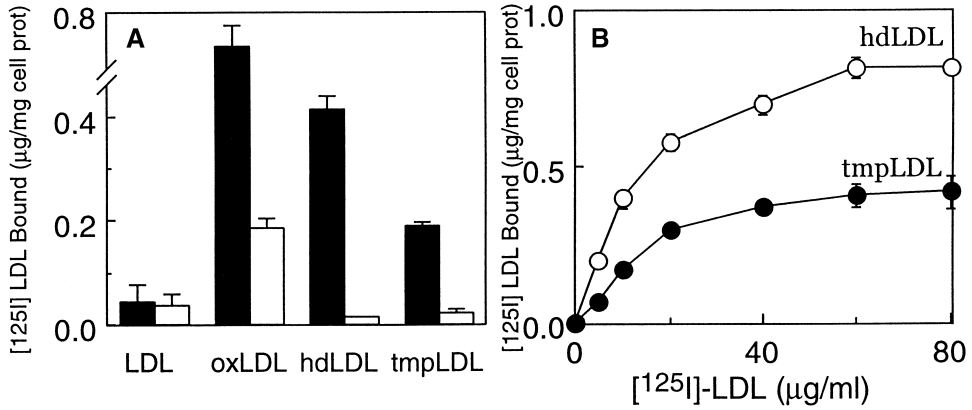


Fig. 6. CD36 recognizes both hdLDL and tmpLDL. (A) 293 cells stably transfected with CD36 (solid columns) or vector-transfected 293 cells (open columns) were grown to confluency in 16-mm well plates in DMEM containing 5% FCS. Cells were incubated with ^{125}I-labeled hdLDL or tmpLDL (10 μg/ml) for 3 h at 4°C. Cells were washed two times with ice-cold PBS and three times with PBS/BSA. Subsequently, cells were solubilized in 0.1 M NaOH and bound radioactivity was measured. Data represent means + SD of triplicate determinations. (B) ^{125}I-labeled hdLDL (open symbols) or tmpLDL (solid symbols) were incubated at the indicated concentrations with CD36- or vector-transfected 293 cells at 4°C for 3 h. Cells were washed two times with ice-cold PBS and three times with PBS/BSA. Subsequently, cells were solubilized in 0.1 M NaOH. Bound radioactivity was measured and CD36-dependent binding was calculated as the difference between binding to CD36-transfected cells and binding to vector-transfected cells. Data represent means ± SD of triplicate determinations.

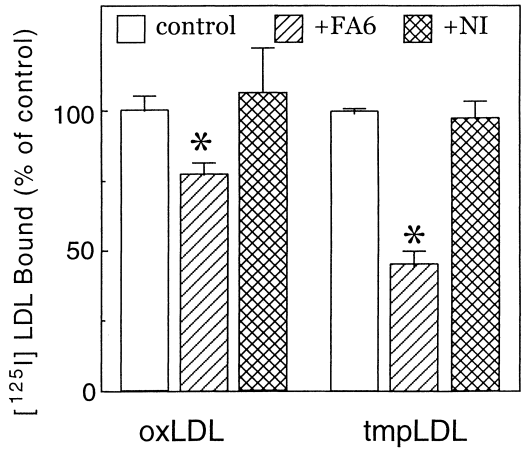


Fig. 7. A blocking antibody to CD36 inhibits the binding of tmpLDL to a human monocyte-macrophage cell line. THP-1 cells were plated at a density of 0.5×10^6 cells per well and incubated with 64 nM phorbol myristate acetate in RPMI 1640 with 10% FCS and 5×10^{-5} M 2-mercaptoethanol for 3 days prior to use. Cells were incubated with a 5-μg/ml concentration of ^{125}I-labeled tmpLDL or oxLDL (24 h) for 3 h at 4°C in the presence (hatched columns) and absence (open columns) of a 20-μg/ml concentration of the blocking anti-CD36 monoclonal antibody FA6 or isotype-matched nonimmune antibody (NI; cross-hatched columns). Cells were washed two times with ice-cold PBS and three times with PBS/BSA. Subsequently, cells were solubilized in 0.1 M NaOH and bound radioactivity was measured. The 100% values for oxLDL (24 h) and for tmpLDL were as follows: 94.8 ± 4.8 and 29.0 ± 0.3 μg/mg cell protein, respectively. Data represent means ± SD of triplicate determinations. * Significant differences compared to control values ($P < 0.05$).

tmpLDL to be recognized by CD36 in independent studies using the blocking monoclonal antibody (FA6) to CD36. As seen in **Fig. 7**, FA6 showed significant inhibition of binding at 4°C of tmpLDL by phorbol 12-myristate 13-acetate (PMA)-treated THP-1 cells, while isotype-matched nonimmune antibody had no effect. Collectively, these results clearly indicate that both hdLDL and tmpLDL are recognized by CD36.

DISCUSSION

Several lines of evidence provided in this article support the conclusion that hdLDL is recognized by the SR-A on MPM. Both hdLDL and oxLDL cross-compete in MPM, suggesting recognition by the same receptor, but neither is recognized by the LDL receptor. Both forms of modification lead to charge neutralization of the particle. Such charge neutralization of critical lysine residues of apoB is required for SR-A-mediated recognition of modified LDL (22). Both were recognized by a high affinity receptor-mediated process that did not involve phagocytosis of aggregated particles, and that induced stimulation of cholesterol esterification over a 24-h incubation period leading to foam cell formation. In CHO cells transfected with the SR-A, uptake of both hdLDL and oxLDL, as well as acLDL, the prototypic ligand for the SR-A (33), was increased relative to mock-transfected CHO cells. Furthermore, in these transfected cells hdLDL and acLDL effectively cross-competed. However, the B_{max} for acLDL was significantly higher than that of hdLDL. One interpretation of these data is that acetylated LDL is recognized by more binding sites on SR-A than is hdLDL. In contrast, tmpLDL was not recognized by SR-A-transfected cells. Because the characteristic chemical modification in hdLDL is the formation of a neutral pentyl pyrrole–lysine adduct (46) and a charged pyridinium–lysine adduct in tmpLDL (36), respectively, recognition by the SR-A of the former and not the latter is consistent with a preference of this receptor for lysine charge neutralization, as occurs in acLDL. Additional studies analyzing the effect of varying local charge density in other modifications should better define the structural basis of receptor recognition.

The results from the interaction of hdLDL or tmpLDL with cells transfected with CD36, another class of scavenger receptors present on macrophages (8), clearly demonstrate that both ligands were recognized by this receptor. Recognition of tmpLDL by CD36 was confirmed by our studies showing that an anti-CD36-blocking monoclonal antibody inhibited binding to the THP-1 human monocyte-macrophage cell line. However, the B_{max} for binding of hdLDL to CD36 was higher than that of tmpLDL. One interpretation is that hdLDL is recognized by more binding sites on CD36 than tmpLDL. What factors are responsible for the recognition of ligands by individual binding sites on CD36 still needs to be clarified. CD36 is known to have broad specificity, recognizing such diverse proteins as thrombospondin and oxLDL by separate binding sites (47).

CD36 was previously shown to be a receptor for oxLDL (8). Monocytes derived from humans who lacked CD36 demonstrated a reduced uptake of oxLDL (48). Although only extensively oxidized LDL was recognized by the SR-A, both modestly and extensively oxidized LDL were recognized by CD36 (8). Our studies in this article showed that both excess tmpLDL and hdLDL were better competitors for the uptake and degradation in MPM of modestly oxidized LDL, for example, oxLDL (2 h) than of extensively oxidized LDL, for example, oxLDL (24 h). Taking these data together with those obtained with transfected cells, our interpetation is that recognition of oxLDL (2 h) by receptors on MPM occurs more through CD36 than through SR-A, when compared with the relative contribution of CD36 recognition of oxLDL (24 h).

Recognition of mildly oxidized LDL by CD36 suggested the possibility that lipid loading in tissue macrophages expressing this receptor could still occur when surrounded by LDL that was only slightly modified. Long-term incubation of MPM with tmpLDL in this study led to the formation of foam cells. The possible role of CD36 in foam cell formation, the hallmark of early atherosclerotic lesions, was highlighted by our results showing that LDL oxidized by reactive nitrogen species produced by myeloperoxidase, a hydrogen peroxide-generating system, and nitrite, induced foam cell formation in macrophages (49) through an uptake mechanism mediated by CD36 ligation (50). A potential role of CD36 in atherogenesis is supported by

data showing that the mouse homolog of CD36 is expressed in atherosclerotic lesions from SR-A knockout mice (51).

Although modification of LDL by 2,5-hexanedione is not physiologic, it closely mimicks the lysine-based pyrroles formed when lysines are interacted with HNE (16) or 3,4-epoxy-2-alkenals (26, 27). The 2-pentylpyrrole that forms in relatively low yield from reaction of lysines with HNE (16) can be formed in higher yield, although not exclusively, by treatment with 4-oxononanal. An antibody recognizing protein-bound 2-pentylpyrroles (52) was demonstrated to react with plasma proteins and lipoproteins, suggesting a physiologic relevance of such structures (L. M. Sayre, R. G. Salomon, and H. F. Hoff, unpublished studies). Such a 2-pentylpyrrole was also induced to form on LDL by modification with 4-oxononanal, leading to recognition by a receptor on macrophages that could be competed with oxLDL (38). However, because 4-oxononanal-modified LDL was prone to aggregate, presumably as a result of a cross-linking reaction that competes with 2-pentylpyrrole formation (53), the physiologic significance of this modification of LDL remains uncertain. Although dimethylation of lysine ε-amino groups on apoB in LDL retains the positive charge and generates a modified LDL with the same electrophoretic properties as tmpLDL, this modification does not lead to recognition by either the SR-A or CD36 scavenger receptors on macrophages. Although the charges are the same, the cationic pyridinium group in tmpLDL differs markedly from the protonated dimethylamino group in terms of size, charge density, and hydrogen-bonding potential.

In summary, we have demonstrated that two types of chemical homogeneous modifications to LDL represent model ligands for scavenger receptors. One, induced by 2,5-hexanedione and resulting in the formation of a pyrrole ring, is recognized by both the mSR-A and CD36 on MPM, and might function as a model for chemical changes in oxLDL after pyrrolation by lipoxidation-derived aldehydes such as 4-hydroxy-2-alkenals (e.g., HNE) and 3,4-epoxy-2-alkenals. The other, induced by 2,4,6-trimethylpyrylium and forming a pyridinium ring, is recognized by CD36, but not by SR-A, and might function as a model for pyridinium modifications on LDL induced by 2-alkenals and MDA. These two modifications of LDL may represent useful tools for dissecting out the relative contributions to cell uptake via specific receptors of different types of modifications of LDL occurring during oxidation. In contrast, evaluation of known lipoxidation products such as 2-alkenals, 4-hydroxy-2-alkenals, and 3,4-epoxy-2-alkenals in this regard is complicated by the multiplicity of adduct types formed from each product. Additional chemical agents that induce homogeneous modifications prototypical of other physicochemical types of protein modification occurring during LDL oxidation would be worthy of further study.

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