Minimally oxidized LDL inhibits macrophage selective cholesteryl ester uptake and native LDL-induced foam cell formation

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Abstract  Scavenger receptor-mediated uptake of oxidized LDL (oxLDL) is thought to be the major mechanism of foam cell formation in atherosclerotic lesions. Recent data has indicated that native LDL is also capable of contributing to foam cell formation via low-affinity receptor-independent LDL particle pinocytosis and selective cholesteryl ester (CE) uptake. In the current investigation, Cu²⁺-induced LDL oxidation was found to inhibit macrophage selective CE uptake. Impairment of selective CE uptake was significant with LDL oxidized for as little as 30 min and correlated with oxidative fragmentation of apoB. In contrast, LDL aggregation, LDL CE oxidation, and the enhancement of scavenger receptor-mediated LDL particle uptake required at least 3 h of oxidation. Selective CE uptake did not require expression of the LDL receptor (LDL-R) and was inhibited similarly by LDL oxidation in LDL-R⁻/⁻ versus WT macrophages. Inhibition of selective uptake was also observed when cells were pretreated or cotreated with minimally oxidized LDL, indicating a direct inhibitory effect of this oxLDL on macrophages. Consistent with the effect on LDL CE uptake, minimal LDL oxidation almost completely prevented LDL-induced foam cell formation. These data demonstrate a novel inhibitory effect of mildly oxidized LDL that may reduce foam cell formation in atherosclerosis.—Meyer, J. M., A. Ji, L. Cai, and D. R. van der Westhuyzen. Minimally oxidized LDL inhibits macrophage selective cholesteryl ester uptake and native LDL-induced foam cell formation. J. Lipid Res. 2014. 55: 1648–1656.

Supplementary key words  oxidized low density lipoprotein • LDL oxidation • selective lipid uptake

Atherosclerotic lesions are defined as lipid-rich plaques in the intima of susceptible arteries (1). This lipid is predominantly cholesterol and its esters and is localized both within cells (foam cells) and in the extracellular space (2). LDL is believed to play an essential role in plaque deposition, evidenced by the strong association between elevated serum LDL cholesterol and disease (3), and because LDL is the major lipoprotein that accumulates in lesions (4). As lesions progress, extracellular lipid accumulation predominates and therefore it has been proposed that the ability of macrophages and other cells to scavenge LDL is eventually overwhelmed, leading to accelerated lipid deposition and formation of the lipid-rich necrotic core (5). Therefore, understanding the mechanisms that control macrophage metabolism of LDL may be crucial to developing strategies to block atherosclerotic lesion progression. LDL is known to be vulnerable to oxidation and it is well established that atherosclerotic lesions contain oxidized LDL and lipids (4). Oxidized LDL (oxLDL) binds with high affinity to macrophage scavenger receptors thereby promoting efficient uptake of oxLDL by these cells (6, 7). Therefore, it is widely believed that oxidation of LDL is necessary for the generation of lipid-loaded foam cells, a theory that is supported by the identification of oxLDL in atherosclerotic lesions (8) and the protective effect of antioxidants in animal models of atherosclerosis (9). oxLDL is also known to contain many bioactive and cytotoxic substances that may promote inflammation and cell death in atherosclerotic plaques (10, 11). However, conflicting studies on the role of scavenger receptors in murine

Abbreviations:  BMM, bone marrow-derived macrophage; CE, cholesteryl ester; CL, cholesteryl linoleate; CO, cholesteryl oleate; CP, cholesteryl palmitate; FC, free cholesterol; LDL-R, LDL receptor; oxLDL, oxidized LDL; REM, relative electrophoretic mobility; SR-BI, scavenger receptor class B, type I; TC, total cholesterol.

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atherosclerosis (12) as well as disappointing results with antioxidants in human clinical trials have generated much controversy about the role of oxidized LDL in the disease (9).

More recently, native LDL has been implicated in foam cell formation (13). Many early studies demonstrated that native LDL induces little cholesterol accumulation in cultured macrophages compared with acetylated LDL (6) or oxLDL (14). However, these investigations were carried out at relatively low LDL concentrations (5–100 µg/ml LDL). At high concentrations (~1000 µg/ml LDL), non-saturable mechanisms including fluid-phase pinocytosis contribute significantly to native LDL uptake and result in foam cell formation (13). These concentrations are comparable to levels found in the serum and atherosclerotic lesions of patients with cardiovascular disease (often >200 mg/dl of LDL-cholesterol) (15).

In a recent study, we characterized the pathway of cholesterol delivery to macrophages during LDL-induced foam cell formation (16). Surprisingly, the majority of the cholesterol was acquired via selective uptake of cholesteryl ester (CE), with smaller amounts acquired by pinocytic uptake of whole LDL particles. Selective CE uptake has been previously reported in other cells and tissues and is the major mode of uptake of cholesterol from HDL as mediated by scavenger receptor class B, type I (SR-BI) (17). The mechanism of SR-BI-mediated selective CE uptake is poorly understood but is thought to involve transfer of CE from lipoprotein particles to the cell plasma membrane followed by internalization and hydrolysis by neutral cholesteryl esterase(s) (18). In macrophages, however, selective CE uptake is independent of SR-BI or the related scavenger receptor CD36 and LDL-derived CE acquired by this pathway appears to be hydrolyzed in lysosomes, indicating a pathway that is distinct from the established SR-BI-mediated pathway (16). The linear relationship of selective CE uptake to LDL concentration does not support the involvement of any high-affinity receptor binding in the selective uptake pathway and further studies are needed to define the mechanism of macrophage selective CE uptake.

One question that arose during the initial characterization of the macrophage selective CE uptake pathway was whether LDL modification would alter the rate of selective CE uptake. LDL acetylation resulted in increased LDL particle uptake and unchanged selective CE uptake, while LDL aggregation resulted in increased LDL particle uptake and completely eliminated selective CE uptake (16). Altered selective CE uptake may impact the processing of LDL-cholesterol and the development of foam cells within atherosclerotic lesions. Therefore, the current study was undertaken to investigate the effect of LDL oxidation, a physiologically relevant LDL modification, on macrophage selective CE uptake.

MATERIALS ANDETHODS

Animals and reagents

C57/BL6 mice were housed in the Veterans Affairs Medical Center (Lexington, KY) and all experiments were approved by the Institutional Animal Care and Use Committee. Animals were maintained in a pathogen-free facility with a 12 h light/dark cycle and free access to food and water. Iodide-125 radionuclide (125I), 1, 2, 3H(N)cholesterol ([1H]FC) and cholesteryl-1,2,3H(N)hexadecyl ether ([1H]CEt) were obtained from Perkin Elmer (catalog numbers NET139001MC and NET859001MC, respectively). 1, 2, 3H(N)cholesterol linoleate ([1H]CL) and 1, 2, 3H(N)cholesterol palmitate ([1H]CP) were synthesized from [1H]FC by reaction with excess linoleic anhydride (TCl, 24900-68-0) or palmitic anhydride (Sigma, 2865058), respectively, in toluene followed by purification by TLC. [1H]FC Cholesteryl 1, 2, 6, 7 H(N) oleate ([1H]CO) was purchased from American Radiola-beled Chemicals (catalog number ART 0432). Radiochemical purity of [1H]lipids was >95% as determined by TLC immediately before use in experiments. Silica-coated polyvinyl chloride (PVC) TLC plates were purchased from Macherey-Nagel (805013). Total cholesterol (TC) and CE E-kits were purchased from Wako (439-17501 and 435-35801, respectively).

Lipoprotein isolation, characterization, radiolabeling, and modification

LDL (ρ = 1.019–1.063 g/ml) was isolated from human plasma by density gradient ultracentrifugation (19). Protein concentrations (mg/ml) were determined by the method of Lowry et al. (20), and TC, FC, and CE content (nmol/mg LDL protein) using cholesterol E-kits (Wako). LDL was analyzed by SDS-PAGE and nondenaturing gradient gel electrophoresis. LDL was 125I-labeled by the iodine monochloride method (21). To measure LDL CE uptake, [1H]CE was incorporated into 125I-LDL using the CETP method as previously described (22). After labeling, 125I- and 3H-specific activities were calculated by dividing the radioactivity (dpm/µL) by the LDL CE content (nmol CE/µL) and was between 3,000–8,000 and 100–1,000 dpm/nmol LDL CE for 125I and 3H, respectively. LDL (1 mg/ml) was oxidized with 20 µM CuSO4 at 37°C for 0.5–24 h as described (23) except that chloramphenicol and Chelalex-100 resin were not included in the preoxidation dialysis buffer. Oxidation was terminated by the addition of EDTA (10 mM final concentration) followed by dialysis into 0.3 mM EDTA/saline. oxLDL was analyzed by agarose gel electrophoresis (1% gel) to determine relative electrophoretic mobility (REM) and LDL aggregation. LDL aggregates were defined as the proportion of 125I in each lane that remained trapped within in the well. SDS-PAGE was used to analyze apoB fragmentation, quantified as the percent of 125I in each lane of the developed gel present as intact apoB. oxLDL lipids were analyzed by TLC with 3H quantification to determine [1H]CL, [1H]CO, and [1H]CP content as well as [1H]CL hydroperoxide ([1H]CLOOH) based on the positions of the corresponding authentic standards. oxLDL was stored at 4°C under argon gas and was used for experiments within 1 week of preparation, although it was found to be stable for at least 2 weeks by SDS-PAGE and agarose gel electrophoresis. Prior to use in experiments, LDL was dialyzed overnight against 50 vols of RPMI media to remove EDTA.

Bone marrow-derived macrophage isolation and culture

Murine bone marrow-derived macrophages (BMMs) were obtained from C57/BL6 mice (WT) or from LDL-R-deficient (LDL-R-/) mice (purchased from Jackson Laboratories) mice and cultured by standard procedures (24). Isolated cells were suspended in medium A [RPMI 1640 (Gibco, 11875) plus 15% (v/v) L-cell-conditioned medium (LCM)], 10% (v/v) heat-inactivated FBS, 50 IU/ml penicillin G, and 50 µg/ml streptomycin] and plated in 24-well plates (with or without glass cover slips for microscopy and cholesterol content determination, respectively) or in 48-well plates (for radiolabeled LDL uptake experiments). BMMs
were cultured in medium A for 7 days before being used for experiments, with culture media changed on days 3, 5, 6, and 7.

**Determination of CE and FC uptake**

LDL CE uptake was determined by treating BMMs with radio-labeled LDL or oxidized LDL, as indicated in the figure legends, in medium B (RPMI 1640 plus 15% (v/v) LCM, 0.5% (w/v) fatty acid-free BSA (Sigma), 10 mM HEPES, pH 7.4, 50 IU/ml penicillin G, and 50 µg/ml streptomycin). After the treatment period, 125I-LDL uptake, [3H]CE uptake and selective [3H]CE uptake were quantified as previously described (16). Briefly, cells were lysed by the addition of 0.1 M NaOH, collected, and counted for cell-associated 125I-LDL. Cell lysates were also used to determine cell protein content (mg/well) by BCA. Culture medium was precipitated with trichloroacetic acid, extracted with chloroform, and the aqueous phase counted to determine degraded 125I-LDL. Lipid was extracted from cell lysates using Dole reagent (isopropanol-heptane-1M sulfuric acid, 40:10:1) and counted for 3H by scintillation counting. Cellular 3H (dpm/mg cell protein) was used to quantify CE uptake (nmol CE/mg cell protein). 125I uptake (dpm/mg cell protein, the sum of cell-associated and degraded 125I) was used to calculate CE equivalents (nmol/mg cell protein) assuming whole-particle uptake with concomitant CE delivery, as previously validated (25). Selective CE uptake was calculated as the difference between total [3H]CE uptake and the CE uptake equivalents contributed by the uptake of whole-particle 125I-LDL and represents CE uptake that cannot be accounted for by whole-particle uptake.

**Cellular FC and CE content determinations and microscopy**

For cellular lipid determination, lipid was extracted from cells with hexane-isopropanol (3:2), dried, and resuspended in aqueous solution with 1% Triton X-100-H2O. TC and FC content (nmol cholesterol/mg cell protein) were determined using cholesterol E-kits. CE content was calculated as the difference between TC and FC content. Cell protein was determined using a BCA protein assay kit (Thermo Scientific). For microscopy, BMMs were treated as indicated in the figure legends then fixed for 5 min in freshly prepared 1% paraformaldehyde and stained with Oil Red O. Images were obtained from at least five randomly chosen fields for each condition.

**Statistics and data analysis**

Results are expressed as the mean ± SEM as indicated in the figure legends. Where not visible, error bars are contained within the symbols for results with n ≥ 2. Statistical analysis was carried out using unpaired, two-tailed t-tests. Best-fit curves were generated using GraphPad Prism4 software.

**RESULTS**

We previously demonstrated that selective CE uptake is a major pathway of cholesterol delivery to macrophages from native LDL (16). However, the effect of LDL oxidation on this selective CE uptake pathway is not known. In the current investigation, [3H]cholesteryl linoleate- and 125I-dual labeled LDL ([3H]CL/125I-LDL) was oxidized by treatment with Cu2+ at 37°C for up to 24 h and used to examine lipoprotein uptake in murine BMMs. As expected, LDL oxidation greatly increased subsequent 125I-LDL uptake in BMMs (Fig. 1A, white circles). Uptake, expressed as LDL CE uptake, was maximal with 6 h or more of oxidation. Oxidized LDL exhibited saturable uptake with respect to LDL concentration (Fig. 2A), consistent with the known involvement of the high-affinity scavenger receptors SRA and CD36 (7). Total [3H]CE uptake did not follow the same pattern (Fig. 1A, black circles). Instead, [3H]CE uptake was greatest with native unoxidized LDL (0 h) and was progressively decreased with LDLs that had been oxidized for 0.5 to 2 h. For LDLs oxidized for 3 to 6 h, [3H]CE uptake was increased, coincident with the increase in 125I-LDL uptake, followed by a gradual decrease in [3H]CE uptake from LDLs oxidized 6–24 h. The difference between [3H]CE uptake and 125I-LDL uptake ([3H]CL/125I) is defined as selective CE uptake, and indicates CE acquired by cells independent of LDL particle uptake. Selective CE uptake was progressively impaired by LDL oxidation and was reduced by >85% in 24 hr oxLDL (Fig. 1A, gray circles). As we previously reported (16) and in contrast to 125I-oxLDL uptake, selective CE uptake was non-saturable with respect to LDL concentration (Fig. 2B).

This is the first report of reduced selective CE uptake from oxidized LDL. To investigate the mechanism by which this occurs, two hypotheses were considered: 1) LDL oxidation alters the chemical composition and/or structure of LDL particles, thereby preventing cell-surface binding or selective CE transfer, and 2) reactive products generated during oxidation inhibit the cell-mediated selective uptake pathway.

First, the relationship between LDL oxidation and selective CE uptake was examined by correlating reductions in selective CE uptake to several established modifications that occur during LDL oxidation: increased relative electrophoretic mobility (REM), LDL aggregation, apoB fragmentation, and LDL CE oxidation (Fig. 1B–D). REM and LDL aggregation were determined by agarose gel electrophoresis (Fig. 1B, gel shown in supplementary Fig. IA). LDL REM increased progressively during oxidation after a short lag phase (0.5 h) and reached a maximum of ~2.35 after 24 h. In contrast, LDL aggregation was not detected during the first 4 h of oxidation and did not exceed 16% at any stage of oxidation. ApoB fragmentation was measured by SDS-PAGE (Fig. 1C; gel shown in supplementary Fig. IB). As previously described (26), modification of apoB was the earliest change detected during LDL oxidation and was characterized by a rapid loss of intact apoB (molecular weight ~517 kDa), followed by the appearance of apoB fragments. CE oxidation was determined by TLC (Fig. 1D, plate shown in supplementary Fig. IC) and was quantified as the loss of intact [3H]CL and the generation of cholesteryl linoleate hydroperoxide ([3H]CL-OOH). CE oxidation was observed after a lag phase of 2 h and reached ~85% by 24 h. Taken together, the inhibition of selective uptake during LDL oxidation correlated well with apoB fragmentation and to a lesser degree with increased electrophoretic mobility but did not correlate with CE oxidation or LDL aggregation.

While the extent of CE oxidation did not correlate with reduced selective uptake, this modification is especially relevant to CE uptake because the CE oxidation products may themselves have altered selective uptake capacity.
Oxidized LDL inhibits macrophage selective CE uptake.

1. **Fig. 1.** LDL oxidation impairs macrophage selective CE uptake. \(^{3}H\)CL/\(^{125}\)I-LDL was oxidized by treatment with Cu\(^{2+}\) for the indicated time period, then incubated with BMMs (500 µg/ml) in medium B for 4 h. A: \(^{125}\)I-LDL uptake (white circles, expressed as LDL CE uptake), total \(^{3}H\)CE uptake (black circles), and selective CE uptake (gray circles) were determined as described in Materials and Methods. B: REM (black circles) and LDL aggregation (agglLDL, white circles) of the oxLDL preparations were determined by agarose gel electrophoresis. C: Intact apoB (gray diamonds) was determined by SDS-PAGE. D: \(^{3}H\)CL (black circles) and \(^{3}H\)CLOOH (white diamonds) were determined by TLC. Values are the mean ± SEM. (n = 3 for A and n = 2 for B–D) and are representative of two independent experiments.

2. **Fig. 2.** Concentration dependence of LDL and oxLDL uptake. \(^{3}H\)CL/\(^{125}\)I-LDL (LDL, white circles) or \(^{3}H\)CL/\(^{125}\)I-LDL that was oxidized by treatment with Cu\(^{2+}\) for 2 hr (2 hr-oxLDL, gray circles) or 24 h (24 hr-oxLDL, black circles) was incubated with macrophages at the indicated concentration in medium B for 4 h. After the treatment period, \(^{125}\)I-LDL uptake (A, expressed as LDL CE uptake), and selective CE uptake (B) were determined as described in Materials and Methods. Values are the mean ± SEM. (n = 3) and are representative of two independent experiments.
is not the result of reduced selective uptake of oxidized CE derivatives.

In contrast to the relationship to CE oxidation, reduced selective uptake did correlate well with apoB fragmentation (Fig. 1). Chemical or oxidative apoB modification is known to alter binding of LDL to the LDL receptor (LDL-R) (27, 28). Therefore, the possible contribution of LDL-R to selective CE uptake from LDL and oxLDL was investigated using BMMs from LDL-R−/− mice (Fig. 4). 125I-LDL uptake was significantly lower in LDL-R−/− BMMs compared with WT, with uptake of LDL, 1 hr-oxLDL, and 4 h-oxLDL reduced by 48%, 34%, and 24%, respectively (Fig. 4A). In contrast, selective CE uptake from LDL was at most 14% lower in LDL-R−/− BMMs (Fig. 4B). LDL oxidation impaired selective CE uptake similarly in LDL-R−/− compared with WT BMMs. Therefore, LDL-R likely does not contribute to selective CE uptake and reduced binding to this receptor does not explain loss of selective uptake with LDL oxidation.

As stated in hypothesis (2) above, it is possible that bioactive products generated during LDL oxidation inhibit the cellular pathway of selective uptake. In fact, oxidized lipids including oxidized CE are known to exert many activities in macrophages including induction of pinocytosis (29). To test this, BMMs were incubated simultaneously with [3H]CL/125I-LDL and unlabeled oxLDL to determine if the presence of oxLDL would inhibit uptake of the coincubated unoxidized [3H]CL/125I-LDL (Fig. 5). Interestingly, oxLDL inhibited selective CE uptake by as much as 94%, depending on the extent of oxidation of the oxLDL used in coinoculation (Fig. 5A). In contrast, only 24 hr oxLDL caused a significant reduction in 125I-LDL uptake and caused only ~50% inhibition (Fig. 5A). In an independent experiment, the inhibitory effect of oxLDL on selective CE uptake was found to be dose-dependent and was observed at concentrations of 2 hr oxLDL as low as 25 µg/ml (supplementary Fig. III). While it was possible that the labeled [3H]CL/125I-LDL could have been oxidized by exposure to oxLDL and cells during the treatment period, this is thought to be unlikely because of the high concentration of antioxidants and absence of Cu2+ in RPMI medium. This was confirmed by collecting the treatment medium from cells at the end of the experiment and quantifying the extent of [3H]CL/125I-LDL oxidation as described for Fig. 1. There was no significant [3H]CL oxidation by TLC (data not shown); however, there was a small but significant amount of apoB fragmentation as determined by SDS-PAGE (Fig. 5B). Media containing 4 hr oxLDL or 24 hr oxLDL were associated with a ~10% and 18.5% reduction, respectively, in intact apoB of the coincubated [3H]CL/125I-LDL. However, this corresponds to a lesser degree of LDL oxidation than that observed with LDL oxidized by Cu2+ for 30 min (Fig. 1C), and therefore is not sufficient to explain the reduction in selective uptake observed (Fig. 5A). These data suggest that oxLDL inhibits selective CE uptake from coincubated native LDL, possibly via cell-mediated effects (hypothesis 2 above).

The contribution of a direct inhibitory effect of oxLDL on cells was further investigated by determining the initial rates of LDL uptake and selective uptake in cells pretreated with or without oxLDL (Fig. 6; time course curves shown in supplementary Fig. IV). BMMs were pretreated with either LDL or 2 hr oxLDL (unlabeled) for 4 h, washed to remove these ligands and then treated with [3H]CL/125I-LDL or [3H]CL/125I-oxLDL. Compared with LDL, pretreatment with oxLDL had a modest inhibitory effect on subsequent 125I-oxLDL uptake but no effect on 125I-LDL uptake (Fig. 6A). In contrast, pretreatment with oxLDL
resulted in pronounced inhibition of selective CE uptake from both LDL as well as oxLDL (Fig. 6B). These results indicate that oxLDL exerts a strong effect on selective CE uptake by modulating the macrophage pathway responsible for such uptake.

The effect of LDL oxidation on selective uptake would be predicted to influence cholesterol accumulation and foam cell formation. However, the cholesterol content of cells is not always by cholesterol efflux during treatments and does not always relate quantitatively to measured CE uptake (16). Therefore, net cholesterol accumulation was examined by measuring cellular cholesterol content after a 24 h loading period with native or oxLDL (Fig. 7). BMMs treated without lipoproteins contained ~117 nmol free cholesterol (FC) per mg cell protein and did not contain a significant amount of CE (calculated as the difference between total cholesterol and FC content). LDL treatment resulted in significant concentration-dependent cholesterol accumulation, with FC and CE increasing 1.7 and 4.7-fold, respectively, in cells treated with 500 µg/ml LDL. In contrast, 1.5 hr oxLDL induced only minimal changes in FC (<30%) and did not significantly alter CE content. As expected, 8 h oxLDL or 24 hr oxLDL treatment resulted in pronounced accumulation of both FC and CE, which increased 5.8- and 26.7-fold, respectively. The greater degree of cholesterol accumulation induced by oxLDL compared with native LDL is consistent with previous work demonstrating lysosomal trapping and reduced efflux of CE derived from extensively oxidized LDL (30, 31). In contrast, CE derived from selective CE uptake is efficiently hydrolyzed and subject to efflux from macrophages (16).

LDL-induced foam cell generation was assessed by Oil Red O staining of neutral lipid droplets (Fig. 8). Cells treated for 24 h without lipoproteins did not contain Oil Red O-positive lipid droplets (data not shown). 8 hr oxLDL or 24 hr oxLDL induced foam cell generation at either 100 µg/ml or 500 µg/ml, consistent with our previous observation of lipid loading by native LDL through a low-affinity and nonsaturable process (16). Interestingly, 1.5 h oxLDL induced much less lipid deposition compared with native LDL. The morphology of the lipid-loaded cells was examined at higher magnification and revealed distinct differences in the type and distribution of the Oil Red O-stained droplets (Fig. 8B). The droplets formed during treatment with native LDL were less numerous but much larger than those formed during treatment with oxidized LDL and appeared localized toward the cell periphery. In contrast, the droplets induced by oxLDL were widely spread throughout the cell.

**Fig. 5.** Effect of oxLDL cotreatment on selective CE uptake. BMMs were treated for 4 h with 500 µg/ml of [3H]CL/125I-LDL or 500 µg/ml unlabeled LDL (0 h Cu²⁺ oxidized) or LDL oxidized by treatment with Cu²⁺ for the indicated time period. After the treatment period, 125I-LDL uptake (expressed as LDL CE uptake) and selective CE uptake were determined as described in Materials and Methods (A). Media was collected from cells after the experiment and apoB fragmentation of the [3H]CL/125I-LDL determined by SDS-PAGE (B). Values are the mean ± SEM. (n = 3 for A and n = 2 for B). *, P < 0.05 compared with LDL; **, P < 0.01; ***, P < 0.001.

**Fig. 6.** Effect of oxLDL pretreatment on the initial rate of selective CE uptake. BMMs were pretreated for 4 h with the indicated addition of unlabeled LDL or 2 h oxLDL (250 µg/ml) in medium B, washed twice with 0.1% BSA/PBS to remove ligands then treated for 20, 60, or 120 min with 250 µg/ml of either [3H]CL/125I-LDL or [3H]CL/125I-2 hr oxLDL. After the treatment period, the initial rate of 125I-LDL uptake (A, expressed as LDL CE uptake) and selective CE uptake (B) were calculated as the 20 min values divided by 1/3 h. Values are the mean ± SEM. (n = 3) and are representative of two independent experiments. **, P < 0.01 compared with the corresponding LDL pretreated group; a, b, P < 0.001 and P < 0.05, respectively, compared with the corresponding 3H/125I-LDL treated group.
cytoplasm. Therefore, cells loaded with native LDL were easily distinguished from those treated with oxLDL.

DISCUSSION

We recently demonstrated that high-capacity selective CE uptake is a major pathway of cholesterol delivery to macrophages during native LDL-induced foam cell formation (16). The current results extend the previous study by showing that LDL oxidation results in progressive inhibition of this pathway, in contrast to the well-known enhancement of LDL particle uptake that occurs with LDL oxidation due to recognition by macrophage scavenger receptors. The mechanism is unknown, but our studies suggest that this occurs via inhibition of macrophage uptake activity, resulting in reduced selective uptake from both oxLDL as well as native LDL. The impact of altered CE uptake at each stage of LDL oxidation was reflected by significant differences in macrophage foam cell formation. Native LDL cholesterol was acquired largely via low-affinity or nonsaturable selective CE uptake, leading to the formation of foam cells containing large lipid droplets. Minimally oxidized LDL was associated with reduced selective CE uptake and induced significantly less lipid accumulation. Extensively oxidized LDL was taken up with high affinity as whole particles and resulted in profuse deposition of small lipid droplets, similar to previous reports of scavenger receptor-mediated uptake (32).

LDL oxidation involves a complex series of chemical and physical changes; therefore, in this study, special attention was given to the degree of oxidation and its relationship to the CE uptake. A significant reduction in selective CE uptake was observed with as little as 30 min of oxidation while altered macrophage LDL particle uptake, increased LDL electrical charge, LDL aggregation, and LDL CE oxidation required at least 3 h of oxidation. These results indicate that the impairment of selective uptake is not associated with the modifications that promote scavenger receptor-mediated uptake of extensively oxidized LDL. Instead, the impairment of selective CE uptake correlated closely with apoB fragmentation, a well-known feature of apoB that occurs early in the oxidation process (33). Oxidative peptide fragmentation is not fully understood; however, it is thought to involve direct scission of peptide bonds by free radical-containing phospholipid oxidation products. Phospholipid and protein forms the shell through which CE must traverse to be transferred to cells by selective uptake, so it is conceivable that derangement of this surface layer in minimally oxidized LDL could impact the rate of selective CE uptake.

We also considered the possibility that oxidation or fragmentation of apoB may alter binding of LDL to LDL-R or another cell-surface binding site. The current investigation provides evidence that LDL-R is not involved in macrophage selective CE uptake from LDL. Interestingly, oxLDL and oxidized phospholipids were previously shown to inhibit selective uptake of HDL CE in SR-BI expressing cells, with a variable effect on HDL binding depending on cell type (34, 35). The current study is consistent with a similar effect of oxidized phospholipids or other bioactive lipids in macrophages. However, we recently demonstrated that macrophage selective CE uptake from LDL is not dependent on SR-BI (16). It is possible that the SR-BI-independent selective uptake pathway in macrophages shares a similar oxLDL-sensitive step in common with the SR-BI-mediated pathway in other cell types.
This is the first report of an oxLDL (minimally oxidized LDL) inducing less macrophage lipid accumulation than native LDL, a finding that was apparent only at a high LDL concentration and was likely overlooked in previous investigations due to the lower LDL concentrations used. Several conclusions may be inferred from this observation. First, this provides additional evidence that oxidation is not involved in native LDL-induced lipid loading, because mild oxidation of LDL that may occur during LDL isolation and during treatment of cells would be expected to decrease the observed loading. Second, while lipid loading was almost completely eliminated by minimal oxidation of LDL, LDL particle uptake including pinocytosis was not decreased, suggesting an essential role for selective CE uptake in LDL-induced foam cell formation. Previous studies established that macrophage LDL pinocytosis is required for foam cell formation (13) but did not rule out the possibility that another pathway also contributed to cholesterol uptake. In fact, selective CE uptake was reduced by minimal LDL oxidation, suggesting that the selective uptake pathway is also required. This hypothesis is supported by the large proportion of cholesterol supplied by the selective uptake pathway and by previous studies of the metabolism of CE following selective uptake (16).

Finally, reduced foam cell formation with minimally oxidized LDL suggests that LDL oxidation that occurs in atherosclerotic lesions may reduce cellular lipid uptake and accumulation in some circumstances in which the extent of oxidation is limited. We propose that this effect is relevant to atherosclerosis because analysis of oxidized lipids in human lesions suggests that LDL is minimally oxidized (<2.5% and <1% of cholesteryl linoleate and cholesterol oxidized, respectively) (36). As stated above, in the current study, a significant inhibitory effect of oxLDL on macrophages was observed that persisted even after the oxLDL was removed. This result is important because it indicates that the presence of oxLDL in lesions and other tissue sites may influence not only its own metabolism but also that of native LDL. It is not known whether this effect is mediated by intact oxLDL particles or by one or more chemical constituents such as oxidized lipid products. The isolation and identification of such products would be of great interest because they may serve as pharmacologic agents for reducing foam cell formation in the treatment of atherosclerosis.

In summary, this study demonstrates that oxLDL inhibits selective LDL CE uptake, resulting in reduced foam cell generation in macrophages treated with minimally oxidized LDL. Further studies are needed to elucidate the mechanism of this phenomenon and to determine the significance of these pathways in atherosclerosis.

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