Lipoprotein receptor interactions are not required for monocyte oxidation of LDL

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Abstract Upon activation, human peripheral blood monocytes and U937 cells oxidized low density lipoprotein (LDL), converting it to a cytotoxin. The oxidized LDL loses its ability to interact specifically with the native LDL (apoB/E) receptor and becomes a ligand for the scavenger receptors and two other receptors, FcγRII (CD32) and CD36. We performed a series of studies to evaluate the potential contribution of each of these receptors to the process of monocyte-mediated LDL oxidation. To assess the participation of the apoB/E receptor, we tested the ability of activated human monocytes to oxidize LDL after up- and down-regulation of apoB/E receptors. Neither up-regulation nor down-regulation of the apoB/E receptor significantly modified the level of LDL lipid oxidation. Acetylated LDL, a ligand for scavenger receptors, was also oxidized by the activated monocytes. Methylated LDL, a chemically modified LDL that is not recognized by the apoB/E or scavenger receptors, was oxidized as well. Thus, LDL does not need to interact with either the apoB/E receptor or scavenger receptors in order to undergo lipid oxidation. Additionally, monoclonal antibodies to CD36 and CD32 were used to block these two receptors that recognize oxidized LDL. Although both antibodies interfered with oxidized LDL binding to these receptors, neither treatment interfered with LDL lipid oxidation mediated by activated human monocytes. Our results suggest that interaction with these receptors is not a requirement for LDL lipid oxidation by activated human monocytes.—**Cathcart, M. K., Q. Li, and G. M. Chisolm III.** Lipoprotein receptor interactions are not required for monocyte oxidation of LDL. *J. Lipid Res.* 1995. **36:** 1857–1865.

Supplementary key words lipoprotein oxidation • U937 cells • human monocytes • LDL receptors • oxidized LDL receptors • CD32 • scavenger receptors • CD36 • monocyte activation

Human peripheral blood monocytes are white blood cells that mediate important host defense and normal immune functions. Monocytes also serve as active participants in wound healing and inflammation. Our laboratory has shown that upon physiologic activation, human monocytes (1) and the monocytic cell line, U937 (2), acquire the capacity to oxidize the lipid components of low density lipoprotein (LDL). Both of these cell types are reported to express the apoB/E receptor which specifically recognizes native LDL (3, 4) and mildly oxidized LDL (5). Upon oxidation, LDL is recognized by other receptors; moderate oxidation results in recognition by CD36 (6) and further oxidation leads to recognition by FcγRII (CD32) and other scavenger receptors (6–8) while retaining recognition by CD36 (6). These receptors have been shown to be present on both U937 cells and monocytes/macrophages and can be regulated by activation (3, 4, 6, 7, 9–12).

In previous studies we have characterized some of the processes required for activated monocytes and monocytic cells such as U937 cells to oxidize LDL in culture systems devoid of added transition metals. We have shown that monocyte activation (1, 2), extracellular superoxide anion (13), calcium influx (14), calcium release from intracellular stores (14), cellular lipoxygenase activity (15, 16), and protein kinase C activity (17) are all required elements of monocyte-mediated LDL lipid oxidation. To understand this process in greater detail, we conducted a series of studies to determine whether LDL interaction with the apoB/E receptor is required for monocytes to mediate LDL lipid oxidation. Our previous data were obtained using cells that would have been expected to be down-regulated with regard to the apoB/E receptor; however, Aviram and Rosenblat (18) recently published a study showing that LDL interaction with the apoB/E receptor was required for murine monocyte oxidation of LDL in the presence of added metal ions. As our previous data have indicated that the oxidation of LDL by activated human monocytes involves an initiation phase followed by a propagation phase of lipid oxidation (13), we not only evaluated the potential role of apoB/E receptors but extended our investigations to examine whether lipoprotein receptors

Abbreviations: LDL, low density lipoprotein; Ac-LDL, acetylated LDL; Me-LDL, methylated LDL; MDA, malondialdehyde; TBARS, thiobarbituric acid-reactive substances; LPDS, lipoprotein-deficient serum; SOD, superoxide dismutase; PBS, phosphate-buffered saline; ZOP, opsonized zymosan; TCA, trichloroacetic acid; LPO, lipid peroxides.

that recognize oxidized forms of LDL contribute to the degree of LDL oxidation observed in this system.

METHODS

Lipoprotein preparation

Low density lipoprotein (LDL) was prepared according to previously described methods that minimize oxidation and exposure to endotoxin (14). Additionally, the LDL was protected from light and all solutions used for its isolation were prepared with Chelex-treated water. Each batch of LDL was assayed for endotoxin contamination by the limulus amoebocyte lysate assay (Whittaker Bioproducts, Walkersville, MD). Final endotoxin levels were always < 0.0015 unit/mg LDL cholesterol. LDL was stored in 0.5 mM EDTA and adjusted to 10 mg total cholesterol/ml. For some experiments, LDL was biochemically modified as described in the following section. Immediately before use, native or modified LDL was dialyzed in the dark at 4°C against phosphate-buffered saline without calcium or magnesium and containing Chelex (1 g/L). LDL was used at a final concentration of 0.5 mg LDL total cholesterol/ml.

LDL modification

LDL methylation. LDL was methylated according to a modification of previously described methods (19, 20). Briefly, 0.5 ml of 30 mM sodium borate buffer (pH 8.5) was added to 1 ml LDL solution (5–10 mg cholesterol/ml), then 1 mg of $NaBH_4$ was added to 1 ml of LDL. One μl of formaldehyde (37% w/v) per 10 mg LDL cholesterol was added every 6 min for six additions. After the final addition of formaldehyde, LDL was dialyzed against saline with 0.02% Na_2EDTA. All steps were performed at 4°C. A sham modification of LDL (sham Me-LDL) was identical except that the addition of formaldehyde was omitted. The sham Me-LDL was included as a control. The extent of LDL methylation was determined by the method of Fields (21) using trinitrobenzene sulfonic acid. More than 60% of lysines in LDL apoB/E protein were modified. Methylated LDL (Me-LDL) had less than 0.1 nmol MDA/ml as determined by the TBA assay and 5–7 nmol lipid peroxide/ml after 24 h incubation in RPMI in the absence of cells and at a concentration of 500 μg LDL chol/ml.

LDL acetylation. LDL was acetylated (Ac-LDL) using acetic anhydride as described previously by Basu et al. (22), after which LDL was dialyzed against saline with 0.02% Na_2 EDTA at 4°C. A sham treatment of LDL was performed in which no acetic anhydride was added. Antioxidant activity of Me-LDL and Ac-LDL was assessed by monitoring their inhibition of copper-mediated LDL lipid oxidation, as previously described (16). Ac-LDL exhibited low background levels of oxidation after 24 h incubation in RPMI that were similar to Me-LDL.

LDL radioiodination and binding assay. LDL was iodinated using the [^{125}I]iodine monochloride method of McFarlane (23) as modified by Bilheimer, Eisenberg, and Levy (24). After iodination, LDL was dialyzed against saline with 0.02% Na_2 EDTA and stored at 4°C. The specific radioactivity ranged from 120 to 200 cpm/ng of protein.

For lipoprotein binding studies, cells (5×10^6/ml) were incubated with 40 μg ^{125}I-labeled LDL protein/ml in the presence or absence of 20-fold excess unlabeled LDL for 4 h at 4°C. After incubation, the cells were centrifuged (14,000 *g* for 3 min at 4°C) through 0.15 M metrizamide to remove free labeled LDL from cell-bound ^{125}I-labeled LDL. The radioactivity of 100-μl aliquots of the cell pellet was determined.

Cell culture

U937 cells, obtained from the American Type Culture Collection (Rockville, MD), were cultured by methods reported previously (14). For experiments, log phase U937 cells ($3–6 \times 10^5$ cells/ml) were washed twice with RPMI-1640 without serum, and 5×10^5 cells/ml were plated into 12-well tissue culture plates (1 ml/well, Costar, Cambridge, MA) and cocultured with 0.5 mg LDL cholesterol/ml in the presence of opsonized zymosan (ZOP, 2 mg/ml). After the 24-h incubation, plates were centrifuged, supernatants were collected, and lipid peroxidation of LDL was determined. ApoB/E receptors were down- and up-regulated, respectively, by incubating the cells with LDL (100 μg/ml) and lipoprotein-deficient serum (LPDS, 4 mg protein/ml) or LPDS alone for 18 h prior to switching them to LPDS-free RPMI-1640 and initiating the coincubation with ZOP and LDL.

Human peripheral blood monocytes were freshly isolated and cultured by methods previous described (16). Experiments with monocytes were conducted similarly to those with U937 cells.

Detection of cell injury

Cell injury upon exposure to the various biochemically modified LDL particles was assessed by measuring ^{14}C release after preloading cells with [^{14}C]adenine (25). This method compares favorably with the more widely used chromium release assay (14).

Protein synthesis

U937 cells (5×10^5/ml) were incubated with LDL, Me-LDL, or sham-Me-LDL in the presence or absence of ZOP and with [^{3}H]leucine (10 μCi/ml). The incubations were carried out for various times, but the data shown in this article are from 24-h incubations. After

incubation the cells were washed twice in PBS and resuspended in PBS with 0.2% SDS and 0.1 mg/ml BSA. After 30 min at room temperature the proteins were precipitated on ice with an equal volume of ice-cold 20% TCA, collected on glass-fiber filters (GF/C, Whatman Intl., Maidstone, England) and rinsed with cold 10% TCA. The [^{3}H]leucine incorporated into the protein precipitate was then determined by counting the dried glass-fiber filters.

DNA synthesis

U937 cells (5×10^5/ml) were coincubated with LDL, Me-LDL or sham-Me-LDL in the presence or absence of ZOP and with [^{3}H]thymidine (1 μCi/ml) for 24 h. After incubation the cells were pelleted and the ZOP was removed by a density gradient centrifugation using ficoll-paque (2 min in a microfuge, 12,000 *g*). The cells were harvested from the interface and collected and washed on glass-fiber filters (GF/C). The [^{3}H]thymidine incorporation was determined by counting the dried filters.

Superoxide anion production

Superoxide anion production was measured during the first hour after exposure to activator according to the method of Johnston (26). Briefly, U937 cells (5×10^5 cells/ml) and cytochrome C (160 mM) were incubated in the presence or absence of 150 units/ml superoxide dismutase in RPMI-1640 without phenol red. Incubations were conducted in the presence or absence of 2 mg/ml ZOP and incubated at 37°C in a humidified incubator with 10% CO_2. After 1 h incubation, the cell supernatant was collected, and the absorbance was measured at 550 nm. The following equation was used to determine the nmoles O_2^- produced:

$$O_2^- \text{ nmol/ml} = 47.6[OD_{550} \text{ (in absence of SOD)} - OD_{550} \text{ (in presence of SOD)}].$$

Measurement of lipid peroxidation

The peroxidation of LDL lipids was measured by both the thiobarbituric acid (TBA) assay and an assay for total lipid peroxides (LPO).

Thiobarbituric acid assay. The presence of lipid oxidation products on LDL was determined by a modification (14) of the assay described by Schuh, Fairclough, and Haschemeyer (27) that detects malondialdehyde (MDA) and MDA-like compounds reacting with TBA. Compounds that react with TBA are referred to as TBA-reactive substances (TBARS). Data represent the mean ± SEM of experimental results obtained in three similar experiments and are expressed in terms of MDA equivalents (nmol MDA/ml of sample) as MDA is used as a standard.

Total lipid peroxides. To detect lipid peroxides on LDL we performed the assay described by El-Saadani et al. (28) based on the oxidative capacity of lipid peroxides to convert iodide to iodine, which is measured spectrophotometrically at 365 nm. Data represent the mean ± SEM of experimental results obtained in three similar experiments and are expressed in nmol lipid peroxide/ml (LPO/ml).

Oxidation of LDL by copper

In some instances LDL was oxidized by incubating it in the presence of copper sulfate. This was performed as previously described (14, 16, 17). Briefly, LDL (500 μg cholesterol/ml) was incubated for 24 h at 37°C in RPMI 1640 with copper sulfate at the indicated concentrations (usually 5 μM). After incubation the level of oxidation achieved was similar to that obtained by incubating the LDL in the presence of activated U937 cells or activated human monocytes. To assess copper binding to LDL it was necessary to increase the concentration of the LDL and copper each 5-fold in order to obtain detectable levels of copper bound to LDL. For these studies LDL, Me-LDL, or sham Me-LDL (each at 2.5 mg cholesterol/ml) were incubated with 50 μM copper sulfate for

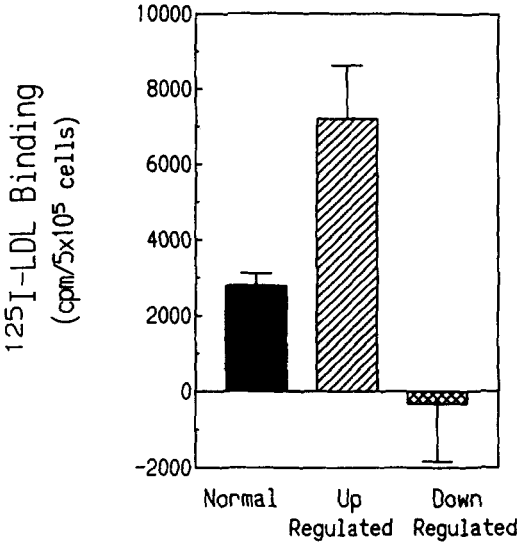

Fig. 1. Specific binding of ^{125}I-labeled LDL by U937 cells. U937 cells (5×10^6 cells/ml) were incubated with ^{125}I-labeled LDL (40 μg protein/ml) in the presence or absence of 20-fold excess unlabeled LDL for 4 h at 4°C. After incubation, free ^{125}I-labeled LDL was removed and the radioactivity of 100-μl aliquots of the cell pellet was determined as described in the Methods. The data represent the mean ± data range obtained from one of two similar experiments. Closed bar represents binding by normal cells (i.e., cells cultured by routine protocols given in Methods); hatched bar represents binding by cells that were preincubated with LPDS (4 mg protein/ml) for 24 h to up-regulate LDL receptors; and cross-hatched bar represents the binding by cells that were preincubated with LPDS (4 mg protein/ml) and LDL (100 μg/ml) for 24 h to down-regulate LDL receptors. These data verify that monocyte apoB/E receptors were up- and down-regulated by pretreatment without or with LDL, respectively.

24 h in RPMI or saline. After 24 h the samples were dialyzed against PBS to remove unbound copper. Dialyses were conducted in thin-walled dialysis tubing and brought to equilibrium three times against 500 × volume of PBS. The samples were then subjected to flame atomic absorption in the Section of Biochemistry, Laboratory Medicine, Cleveland Clinic Foundation.

RESULTS

We first conducted experiments to assess whether regulation of apoB/E receptor levels modulated the degree of LDL lipid oxidation mediated by activated human U937 cells. ApoB/E receptors were up-regulated by preincubating the cells in the presence of LPDS and down-regulated by preincubating the cells with LDL + LPDS according to previously published methods (29). Verification of the effects of these treatments was assessed by radioligand binding studies. Binding levels for ^{125}I-labeled LDL to untreated and pretreated cells are depicted in **Fig. 1.** Pretreatment with LPDS in the absence of added LDL substantially increased radioligand binding whereas pretreatment with LDL reduced radioligand binding to nondetectable levels. Cells designated as "normal" were treated as described in Methods. Cells with normal, down- or up-regulated receptors were then used to test whether this modulation affected their ability to oxidize LDL lipids. The results of these experiments are shown as open bars in **Fig. 2.** Analyses of these data by paired *t* test indicated that there was no significant difference between the level of LDL oxidation mediated by up- versus down-regulated cells ($P = 0.215$). If oxidation of LDL required LDL receptor interaction, up-regulation of apoB/E receptors would be expected to enhance LDL oxidation by activated U937 cells and/or down-regulation would inhibit oxidation. Neither of these effects was observed. These results suggested that the regulation of apoB/E receptor expression on these cells did not markedly affect the ability of the activated monocytic cells to oxidize LDL lipids. Similar results were obtained using freshly isolated human blood monocytes, as shown in **Fig. 3.** These data indicated either that only very low levels of receptor expression are required for the oxidation to proceed or that these receptors are not essential for oxidation of LDL lipids by activated U937 cells or monocytes.

To discriminate between these possibilities, we conducted experiments to determine whether activated U937 cells or activated human monocytes could oxidize Me-LDL, a modified form of LDL that is not recognized by the apoB/E receptor (20, 30, 31). The degree of lysine modification achieved upon methylation was above that previously shown to eliminate recognition of LDL by apoB/E receptors (30). The data presented in the

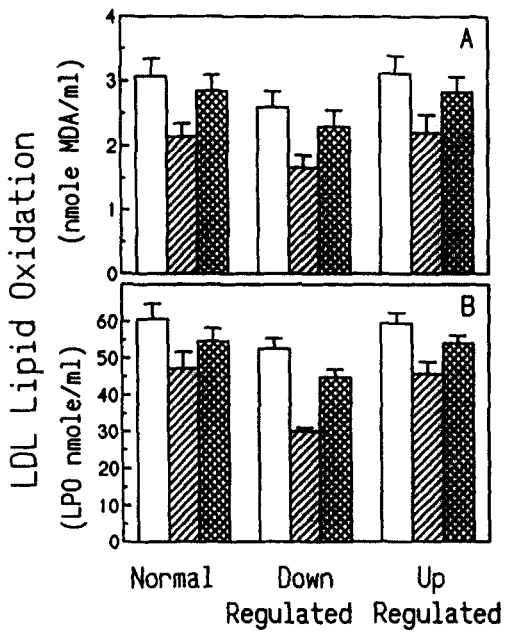

Fig. 2. Down- or up-regulation of apoB/E receptors did not substantially modulate cell-mediated LDL lipid peroxidation. U937 cells (5 × 10^5 cells/ml) in RPMI-1640 were incubated with 0.5 mg chol/ml of native LDL, Me-LDL or sham-Me-LDL and 2 mg/ml of ZOP at 37°C for 24 h. After incubation, peroxidation of LDL lipids was determined by both the TBA assay (A) and the LPO assay (B) as described in Methods. Open bars indicate data obtained from LDL oxidized in the presence of U937 cells and activated with opsonized zymosan. Hatched bars represent the oxidation of Me-LDL under similar conditions. Cross-hatched bars reflect the degree of lipid oxidation observed with sham-Me-LDL. The data are the means ± SEM of experimental results obtained in three similar experiments.

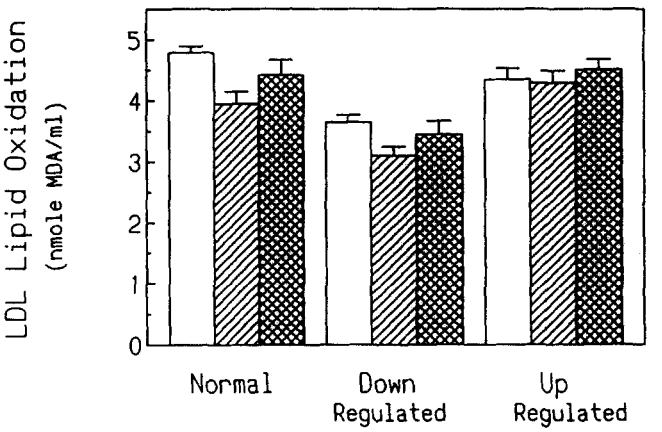

Fig. 3. Human monocyte apoB/E receptors are not required for cell-mediated LDL lipid oxidation. Human monocytes (1 × 10^6 cells/ml) in RPMI-1640 were incubated with 0.5 mg chol/ml of native LDL, Me-LDL, or sham-Me-LDL and 2 mg/ml of ZOP at 37°C for 24 h in a humidified incubator with 10% CO_2. After incubation, LDL lipid oxidation was determined by the TBA assay as described in Methods. The data are the means ± data range of experimental results obtained from one of two similar experiments. Open bars represent monocyte-mediated native LDL lipid oxidation; hatched bars represent monocyte-mediated Me-LDL lipid oxidation; and cross-hatched bars represent monocyte-mediated, sham-Me-LDL lipid oxidation.

TABLE 1. Effects of methylated LDL on cell metabolism and viability

Treatment	Cell Injury [^{14}C]Adenine Release (10^3 cpm/well)	Protein Synthesis [^{3}H]Leucine Incorporation (10^4 cpm/well)	DNA Synthesis [^{3}H]Thymidine Incorporation (10^4 cpm/well)
Cells + 0.2% SDS	100.9 ± 1.0		
LDL			
Cells	6.6 ± 0.1	7.4 ± 0.2	16.8 ± 1.7
Cells + ZOP	5.3 ± 0.1	14.5 ± 0.5	15.7 ± 2.6
Me-LDL			
Cells	4.9 ± 1.3	8.4 ± 0.1	17.1 ± 1.8
Cells + ZOP	3.5 ± 0.2	13.7 ± 1.1	17.7 ± 0.2
Sham-Me-LDL			
Cells	8.7 ± 1.3	9.1 ± 1.0	15.2 ± 0.2
Cells + ZOP	7.1 ± 0.8	15.2 ± 1.5	16.6 ± 0.2

U937 cells (5×10^5) were incubated with LDL, Me-LDL, or sham-Me-LDL in the presence or absence of opsonized zymosan. After 24 h incubation, cell injury, protein synthesis, and DNA synthesis were assessed as described in Methods. Cell injury was assessed by measuring the release of ^{14}C after preloading cells with [^{14}C]adenine. The data for this assay represent the mean ± SD of triplicate determinations from a representative experiment of three performed. Protein synthesis was evaluated by incorporation of [^{3}H]leucine. The data represent the average ± data range from a representative experiment of three performed. DNA synthesis was evaluated by [^{3}H]thymidine incorporation. These data are the averages ± data range of duplicate determinations from one of two experiments performed.

hatched bars in Figs. 2 and 3 indicate that the lipids of Me-LDL became substantially oxidized upon exposure to activated U937 cells or activated human monocytes. This finding was consistent with the idea that recognition by the apoB/E receptor is not required for oxidation of lipoprotein lipids.

In repeated experiments, however, Me-LDL, although always substantially oxidized, was modified to a lesser degree than native LDL or the sham-methylated control (sham Me-LDL). On average, Me-LDL lipids were oxidized 25% less by U937 cells than were sham Me-LDL. As shown in Figs. 2 and 3, this reduction in oxidation was independent of up- or down-regulation of apoB/E receptors. We therefore conducted a series of experiments designed to identify the mechanisms accounting for this decreased oxidation. The effects of Me-LDL on cell integrity, proliferation, and protein synthesis were quantified. Representative experiments are shown in **Table 1** and indicate that Me-LDL did not injure the cells or alter their rates of total protein synthesis or DNA synthesis from that observed using native LDL. We then tested whether Me-LDL was less oxidizable by subjecting it to oxidation mediated by copper ions using methods previously described (16). As shown in **Fig. 4,** fewer lipid oxidation products were formed on Me-LDL compared to that measured on native LDL after exposure to copper oxidation, suggesting that Me-LDL was not as susceptible to metal ion oxidation as native LDL. Next, we assessed whether this was due to impaired copper binding by Me-LDL. Copper contents of LDL, Me-LDL, and sham Me-LDL, under conditions similar to copper-mediated oxidation, were measured by flame atomic absorption (in the Department of Biochemistry, Cleveland Clinic Foundation). Three times more copper bound to the LDL incubated in RPMI (4 μg Cu/mg LDL cholesterol) compared to that bound after incubation in saline, but equal quantities of copper were bound to LDL regardless of its methylation, suggesting that reduced oxidation was not due to reduced copper binding.

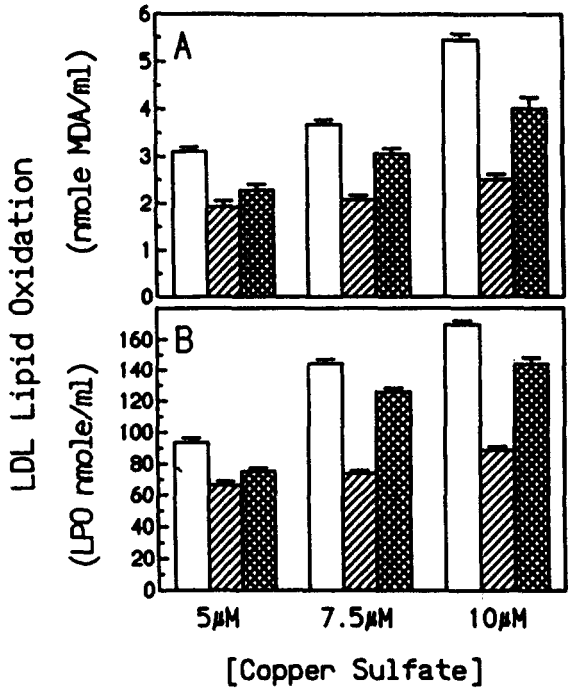

Fig. 4. Copper sulfate-mediated lipid oxidation of native or sham-Me-LDL exceeded that observed with Me-LDL. Native LDL or Me-LDL and sham-Me-LDL were incubated in RPMI-1640 at 37°C for 24 h with different concentrations of copper sulfate as indicated. After incubation, LDL lipid peroxidation was assayed by both the TBA assay (A) and the LPO assay (B) as described in Methods. Lipid oxidation of LDL is represented by open bars, while that of Me-LDL and sham-Me-LDL is represented by hatched and cross-hatched bars, respectively. The data are the means ± SEM of three similar experiments.

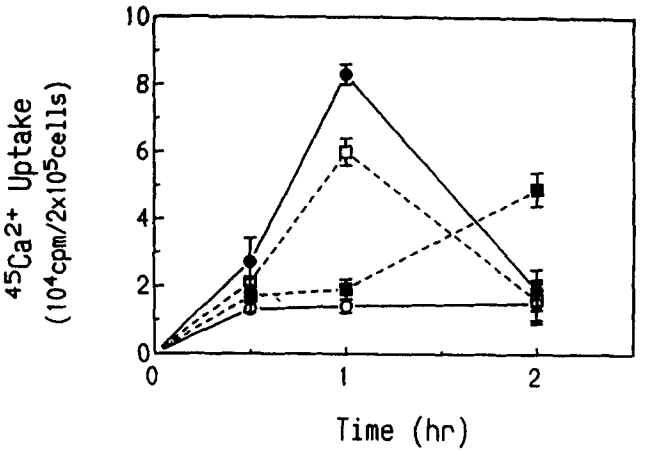

Fig. 5. $^{45}Ca^{2+}$ uptake by ZOP-activated U937 cells was altered in the presence of methylated LDL. U937 cells (2×10^6 cells/ml) in HBSS were incubated with 0.5 mg cholesterol/ml of native LDL (closed circles) or Me-LDL (closed squares) or sham-Me-LDL (open squares). At zero time, 2 mg/ml of ZOP was added together with 2 μCi $^{45}Ca^{2+}$/ml. At the given time intervals, 2×10^5 cells were separated, filtered, and counted as described in Methods. A control group consisting of unactivated U937 cells was also examined. The data from this group are indicated by open circles. All experiments were performed with triplicate samples in each group. The results are presented as the mean ± SD of data obtained in one of two similar experiments.

We have previously shown that LDL oxidation by activated human monocytes and U937 cells is dependent on calcium influx and that calcium influx is required for superoxide anion production upon monocyte activation (14). From studies depicted in **Fig. 5,** we found that calcium influx in response to cell activation was diminished or delayed by Me-LDL in comparison to that observed in the presence of native or sham-treated LDL. In addition, superoxide anion production was dramatically inhibited in activated cells exposed to Me-LDL (**Fig. 6**), a result consistent with lower calcium influx (14). This inhibition of superoxide anion production was not caused by methylated albumin (data not shown) and is therefore not due to effects of methylated proteins in general. Thus, the diminished oxidation of Me-LDL is likely related to its interference with cell activation signals and its diminished susceptibility to oxidation, not to apoB/E receptor-dependent events.

As Me-LDL is not recognized by either the apoB/E receptor or the scavenger receptor, the results presented in Figs. 2 and 3 additionally suggested that scavenger receptor recognition is not required for the optimum oxidation of LDL lipids by activated U937 cells. To corroborate this finding, we examined whether polyinosinic acid (poly-I), an inhibitor of scavenger receptor recognition, altered LDL lipid oxidation by activated U937 cells. In these studies, Ac-LDL, avidly recognized by scavenger receptors, was oxidized by activated U937 cells to levels similar to Me-LDL (data not shown). Oxidation was measured by both the TBA and LPO assays. While poly-I did not inhibit the oxidation of native LDL, it also did not affect the oxidation of Ac-LDL or Me-LDL, suggesting that the oxidation is independent of scavenger receptor recognition. Poly-A, the control for poly-I, was also without effect. Findings were similar using human monocytes (data not shown).

Two receptors that recognize oxidized but not native LDL have been identified and characterized, CD32 (FcγRII) (7) and CD36 (10, 11). To assess the potential involvement of these receptors in the optimal oxidation of LDL lipids by activated human monocytes, we examined the effects of OKM5 (a monoclonal antibody to CD36) and IV.3 (a monoclonal antibody to FcγRII, also designated CD32; Medarex, West Lebanon, NH). As shown by the data presented in **Table 2,** neither of these treatments affected the oxidation, suggesting that interaction with these receptors does not influence the level of oxidation observed. In other studies (data not shown), exposure of U937 or human monocytes to the same concentrations of these monoclonal antibodies inhibited the binding of oxidized, iodinated LDL.

Our laboratory has observed that monocytes must be activated to oxidize LDL when cultured in RPMI 1640. In contrast, other laboratories report that monocyte/macrophages can oxidize LDL without requisite activation when cultured in the presence of free metal ions, such as copper (e.g., refs. 18, 32). We therefore performed a set of experiments to evaluate the role of apoB/E receptors in the oxidation of LDL by monocytes cultured in the presence of 1 μM copper. The results of these studies are illustrated in **Fig. 7.** The upper panel, panel A, of this figure shows the ZOP-induced activation

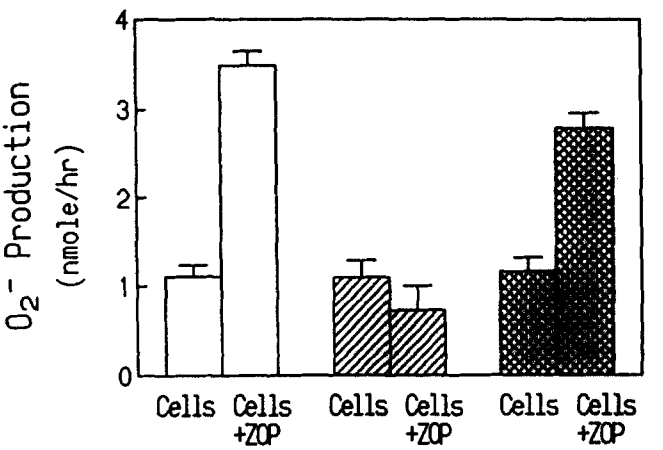

Fig. 6. Cell-mediated superoxide anion production was decreased by exposure to methylated LDL. Superoxide anion release was measured in U937 cells during the first hour after stimulation with opsonized zymosan in the presence of 0.5 mg cholesterol/ml LDL (open bars), Me-LDL (hatched bars), or sham-Me-LDL (cross-hatched bars). The data are the means ± SEM of data obtained in three similar experiments.

TABLE 2. Oxidized LDL receptors, CD36 and CD32, are not required for monocyte-mediated LDL lipid oxidation

Treatment	TBARS (nmol MDA/ml)	Total Lipid Peroxides (nmol/ml)
A. U937 cells		
Cells + ZOP + LDL	2.9 ± 0.1	50.1 ± 2.5
+ anti-CD36 (OKM5)	2.8 ± 0.1	48.7 ± 1.5
Cells + ZOP + LDL	2.7 ± 0.1	51.4 ± 0.6
+ anti-CD36 (OKM5)	2.7 ± 0.1	50.1 ± 1.5
+ anti-CD32 (IV.3)	2.8 ± 0.1	50.5 ± 0.6
Cells + ZOP + LDL	3.9 ± 0.1	n.d.
+ anti-CD32 (IV.3)	3.8 ± 0.2	n.d.
B. Human monocytes		
Cells + ZOP + LDL	2.2 ± 0.1	42.7 ± 2.1
+ anti-CD36 (OKM5)	2.0 ± 0.2	40.2 ± 2.1
Cells[a] + ZOP + LDL	4.6 ± 0.3[a]	n.d.
+ anti-CD32 (IV.3)	5.7 ± 0.1[a]	n.d.

U937 cells (5×10^5) or monocytes (5 or 10×10^5) were preincubated with either OKM5 (antibody to CD36) or IV.3 (antibody to FcγRII, CD32) at an antibody concentration of 2 μg/ml for 30 min at 37°C. LDL (0.5 mg chol/ml) and ZOP (2 mg/ml) were then added and the incubations were continued for 24 h. After incubation, supernatants were collected and the lipid oxidation products were assessed by both the TBA assay and the LPO assay. Results are expressed as the mean ± SD of triplicate determinations from a representative experiment of three performed; n.d., not determined.

[a]Monocytes in this particular experiment were at the higher concentration (1×10^6/ml).

of monocyte oxidation of LDL and the levels of oxidation of LDL obtained after up- and down-regulation of apoB/E receptors in monocytes cultured in RPMI 1640. When 1 μM copper was included (panel B), monocytes were induced to oxidize LDL, even in the absence of activation, to levels higher than those observed with activation by ZOP. Up- or down-regulation did not modulate the level of oxidation differently for native LDL than for Me-LDL. The bottom panel shows similar results obtained with monocytes incubated in a different medium, DMEM, with added copper (1 μM), also in the absence of activation by ZOP. These experiments highlight that the presence of copper circumvented the requirement for ZOP activation and again demonstrate that the oxidation appeared to be independent of the apoB/E receptor.

DISCUSSION

Human monocyte/macrophages and monocytic cell lines express receptors for various forms of LDL. As LDL has been shown to be oxidized by these cells, we explored whether the receptors that recognize these various forms of LDL were required for the lipoprotein lipid oxidation. Although variations in the absolute level of LDL lipid oxidation were observed among these experiments, several points are clear. *1)* Methylated and acetylated LDL were readily oxidized by activated human monocytes and U937 cells. *2)* Increasing or decreasing apoB/E receptor expression did not significantly affect the level of oxidation of native LDL. *3)* Inhibitors of scavenger receptors did not decrease the oxidation of native or acetylated LDL. *4)* Blocking the oxidized LDL receptors, CD36 and CD32, did not interfere with LDL lipid oxidation. *5)* The moderate inhibition of oxidation observed with methylated LDL compared to

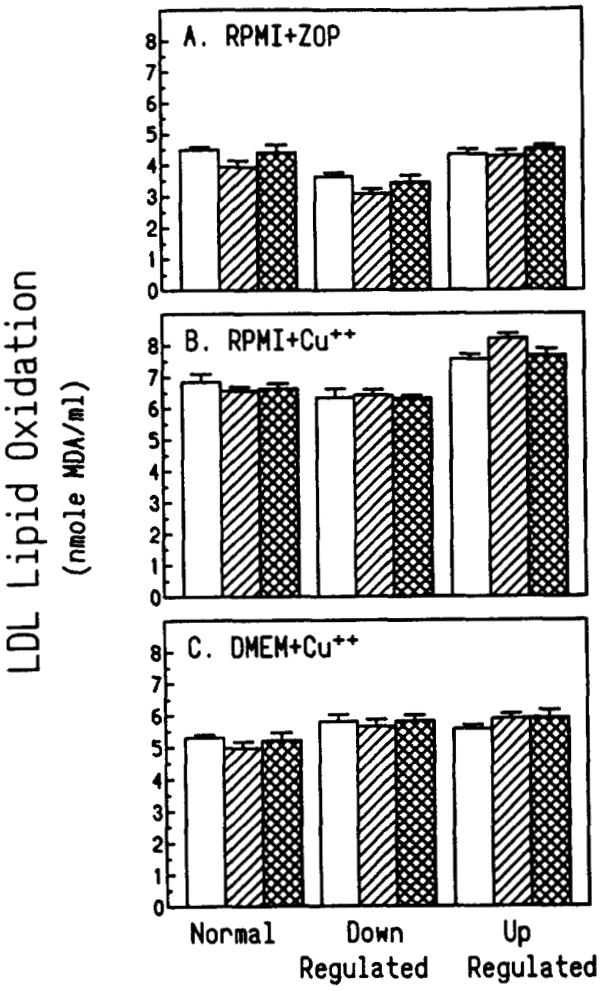

Fig. 7. Copper induces human monocyte-mediated LDL lipid oxidation in the absence of ZOP activation: Absence of B/E receptor influence on oxidation. Human monocytes (1×10^6 cells/ml), treated with ZOP (2 mg/ml) or 1 μM copper were incubated with native LDL (open bars), Me-LDL (hatched bars), and sham-Me-LDL (cross-hatched bars). In parallel groups, the monocytes were pretreated to up- or down-regulate apoB/E receptors as described in Methods. After 24 h incubation, cell-mediated LDL lipid oxidation was determined by the TBA assay as described in the Methods. LDL oxidation in the absence of cells was 0.67 nmol MDA/ml (panel A), 1.83 nmol MDA/ml (panel B), and 1.67 nmol MDA/ml (panel C). These values were subtracted from the values obtained with cells to determine the cell-mediated LDL lipid oxidation. The data represent means ± data range. A: Human monocytes were activated by ZOP (2 mg/ml) in RPMI-1640; B: human monocytes were incubated in the absence of ZOP, but with copper in RPMI-1640; C: human monocytes were incubated with copper in the absence of ZOP under the same conditions as B, but in DMEM.

that with native LDL did not appear to be related to its failure to be recognized by the apoB/E receptor.

Recently, Aviram and Rosenblat (18) reported that LDL receptor recognition is required for LDL oxidation by mouse peritoneal macrophages, a mouse cell line, J774.A1, and human macrophages. Our results, obtained with human monocytes, lead us to reach a different conclusion about the role of LDL receptors in LDL oxidation. The reasons for these discrepancies might be related to the different culture systems being used or due to species differences or stages of cell differentiation. In our studies, we used freshly isolated human monocytes and a human monocytoid cell line, U937, whereas the majority of the data presented by Aviram and Rosenblat (18) were obtained with mouse cells. An additional difference is the stage of differentiation of these cells, as 7–10 day macrophages and mouse peritoneal exudate cells are likely more differentiated than the human monocytes in our studies. Under our culture conditions, the human monocytes and U937 cells did not oxidize LDL lipids unless they were exposed to an activator such as opsonized zymosan, LPS, or T cell supernatants (1, 2). We have characterized this culture system in considerable detail (1, 2, 13–17). We routinely use media that has no transition metal ions in its formulation, whereas Aviram and Rosenblat (18) added 1 μM $CuSO_4$. We know oxidation of LDL in our culture system to be dependent on transition metal ions because we previously showed that extracellular chelators blocked the oxidation (33) in studies including agents that chelated copper and iron. We have tentatively concluded that the source of the metal is cell-derived upon activation. We hypothesize that the participating metals are likely protein-bound as concentrations of free transition metals are extremely low in physiological fluids (34, 35). This leads us to speculate that metal ions contributed by activated cells, rather than those added free in the medium, may represent a more physiological situation. The mechanism of LDL oxidation by monocyte/macrophages in the presence of free metal ions appears distinct from that mediated by physiologically activated monocyte/macrophages. Indeed, we have observed that when free metal ions are added to our cultures, we can circumvent the requirement to activate the monocytes to induce LDL oxidation and the oxidation is not dependent on superoxide anion generation (Fig. 7 and V. Folcik, Q. Li, and M. K. Cathcart, unpublished observations). Our results suggest that under the culture conditions used in our studies, where monocyte activation is required for LDL oxidation to proceed, interactions of LDL with the apoB/E receptor, the scavenger receptors, or two oxidized LDL receptors, CD36 or CD32, are not required for monocyte-mediated LDL lipid oxidation.

In the course of these studies we showed that Me-LDL reduces monocyte activation and oxidation of LDL lipids. Me-LDL decreased the calcium influx and superoxide anion production induced by immunologic activation. These effects were not related to cell injury and another methylated protein, methylated albumin, failed to decrease the superoxide anion production. This suggests that Me-LDL can interact with cells and influence their metabolism. Me-LDL may interfere with monocyte interaction with opsonized zymosan thus influencing the signal transduction pathways triggered by this activator. Such alterations in cellular processes should be considered when evaluating results obtained by using this modified lipoprotein. In spite of these effects, Me-LDL was substantially oxidized by activated U937 cells and human monocytes and the level of oxidation was unaffected by altered apoB/E receptor expression or interference with other receptors for modified lipoproteins, thus supporting our conclusions that recognition by the apoB/E receptor or scavenger receptors is not required for oxidation of LDL.

In summary, the results of these studies support the concept that interaction with receptors that have been identified and characterized as interacting with native or modified forms of LDL is not required for native, acetylated, or methylated LDL to become oxidized by activated human monocytes or U937 cells.

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