Mechanisms of enhanced macrophage apoE secretion by oxidized LDL

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Abstract Previous studies have demonstrated that atherosclerotic lesions contain apoE synthesized primarily by macrophages. As oxidized LDL has been implicated in the development of atherosclerosis, its effect on macrophage apoE synthesis and secretion was examined. Human monocytic leukemia cells, THP-1, and human monocyte-derived macrophages were exposed to various forms of oxidatively modified LDL for determination of their effect on apoE mRNA and protein levels. Extensively copper oxidized (Cu-oxidized) LDL resulted in a time- and concentration-dependent increase in apoE mRNA and protein as compared to other forms of oxidized LDL, i.e., LDL modified by soybean lipoxygenase (SLO), azoamidinopropane HCl (AAPH), and hypochlorite (HOCl). Consistent with these results, experiments using THP-1 cells transfected with the apoE promoter linked to a luciferase reporter gene indicated that Cu-oxidized LDL was the most potent stimulator of apoE transgene expression. Enhanced apoE expression due to Cu-oxidized LDL was shown to be due to cholesterol accumulation as well as additional factors. HPLC analysis of the various forms of modified LDL revealed that 7-ketocholesterol was the major oxysterol present in Cu-oxidized LDL. AAPH-oxidized LDL contained significantly less 7-ketocholesterol than Cu-oxidized LDL and virtually no 7-ketocholesterol was detected in SLO- or HOCl-oxidized LDL. Northern blot analysis indicated an increase in apoE mRNA in response to increasing concentrations of 7-ketocholesterol. These results elucidate a potential role of oxidized LDL, and specifically 7-ketocholesterol, in the stimulation of macrophage apoE secretion in atherosclerotic lesions.—**Cader, A. A., F. M. Steinberg, T. Mazzone, and A. Chait.** Mechanisms of enhanced macrophage apoE secretion by oxidized LDL. *J. Lipid Res.* 1997. **38:** 981–991.

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The complex processes that occur during atherogenesis remain largely unresolved. Several lines of evidence implicate oxidized lipoproteins as initiating and potentiating factors in atherosclerosis (1, 2). In particular, oxidized low density lipoprotein (oxLDL) has been shown to affect the expression of many proteins involved in atherosclerosis, e.g., adhesion molecules, monocyte chemotactic protein-1 (MCP-1), colony stimulating factors, and growth factors (3). Antibodies directed toward oxidation-specific epitopes indicate the presence of oxLDL in atherosclerotic lesions (4, 5). Further, autoantibodies against oxLDL have been detected in the circulation of humans with hypertension and atherosclerosis (6, 7).

Although endothelial cells, smooth muscle cells and macrophages are capable of oxidizing LDL, studies examining human and rabbit atherosclerotic lesions identified oxidation-specific epitopes primarily in macrophage-rich areas (5, 8). It is well established that macrophages readily take up modified LDL via the scavenger receptor (1, 9). Macrophages produce a plethora of compounds known to affect virtually all aspects of atherosclerosis. Of particular interest are modulators of lipid metabolism such as lipoxygenases (8), lipoprotein lipase (LPL) (10) and apolipoprotein E (apo E) (11).

ApoE is a 33–35 kD protein that functions in lipid transport and redistributes as a component of chylomicrons, very low density lipoproteins (VLDL), and high density lipoproteins (HDL). ApoE mediates the binding of lipoproteins to the apoB, E (LDL) receptor and LDL receptor-related protein (LRP) (12, 13). Apolipoproteins are synthesized primarily by the liver. However, apoE also is synthesized by both the liver and numerous extrahepatic tissues including brain, adrenal, and macrophages (14, 15) and is degraded in the lysosomal compartment (16). The functions of extrahepatically-synthesized apoE remain unclear, though it is likely that macrophage-derived apoE plays a significant role in vessel wall lipid homeostasis. Several important pieces of

Abbreviations: apoE, apolipoprotein E; LDL, low density lipoprotein; oxLDL, oxidized LDL; SLO, soybean lipoxygenase; AAPH, azoamidinopropane HCl; HOCl, hypochlorite; UC, unesterified cholesterol; EC, esterified cholesterol; TC, total cholesterol; MDM, monocyte-derived macrophages.

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evidence support this hypothesis. Macrophage apoE synthesis and secretion is stimulated by cholesterol loading in vitro via acetyl LDL (15). In vivo, macrophages of atherosclerotic lesions in humans and hypercholesterolemic and heritable hyperlipidemic rabbits overproduce apoE (17, 18). ApoE colocalizes with LPL in macrophages of human coronary atherosclerotic plaques (18). Together apoE and LPL may contribute to the altered lipid metabolism that occurs in atherosclerotic regions of the vessel wall. Recently, macrophage-specific expression of apoE has been shown to ameliorate atherosclerosis in apoE-deficient mice (19), suggesting that macrophage-derived apoE plays a role in reverse cholesterol transport in vivo. As the exposure of macrophages to oxLDL results in cellular free cholesterol accumulation (20), and apoE synthesis is regulated, at least in part, by intracellular free cholesterol levels (21), the objective of the present study was to clarify the effect of oxLDL on apoE synthesis and secretion.

METHODS

Macrophage cell culture

The human monocytic leukemia cell line, THP-1, was obtained from the American Type Culture Collection (Rockville, MD) and maintained as described previously (22). Cells were cultured in RPMI-1640 medium (Bio-Whittaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT), which contains very low levels of lipopolysaccharide. Most experiments were conducted using THP-1 cells 72 h after differentiation with 1.6×10^{-7} mol/L phorbol myristate acetate (PMA) (Sigma Chemical Co., St. Louis, MO) to allow cellular differentiation and induction of apoE expression. Human monocyte-derived macrophages cultured for 7–10 days in 20% autologous serum, as described previously (23), were utilized in some experiments.

Isolation and separation of mRNA

Macrophage mRNA was isolated by phenol/chloroform/isoamyl alcohol extraction of cell lysates according to the procedure of Chomczynski and Sacchi (24). RNA was separated on a 1.2% agarose–formaldehyde gel and transferred to a Hybond membrane (Amersham Corp, Arlington Heights, IL). Membranes were hybridized with [α-^{32}P]dCTP-labeled apoE (Sma 1 fragment, 986 bp) (18) and β-actin (Eco R1-Hind III fragment, 751 bp) (25) probes prepared using a random-prime labeling system (Amersham Corp). The data are expressed as apoE to actin mRNA content. Under the conditions used in these experiments, actin mRNA levels were not affected by lipid treatment. The bands were identified by autoradiography and quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

ApoE immunoprecipitation

Prior to immunoprecipitation, cells were washed twice with methionine-free RPMI-1640 medium (MF medium) then incubated with MF medium for 30 min at 37°C. Immediately thereafter, cells were pulse-labeled with 100 µCi [^{35}S]methionine for 45 min, after which the media were removed and the cells were chased for a further 45 min–4 h with MF medium containing 500 µmol/L methionine. The media and cells were collected and apoE was immunoprecipitated as described previously (21). For media samples, 2×10^5 counts per minute (cpm) were immunoprecipitated, whereas 2×10^6 cpm were immunoprecipitated for cell extracts in order to normalize apoE radioactivity for total protein secreted or synthesized, respectively. The samples were precleared overnight with non-immune serum and protein G agarose (Gibco, Grand Island, NY). ApoE was immunoprecipitated with goat anti-human apoE serum (Alpha Biomedical Laboratories, Bellevue, WA). Immune complexes were electrophoresed on 10% SDS-PAGE, after which fluorography was performed.

Cholesterol mass determination

Cellular lipids were extracted with hexane–isopropanol 3:2 (v/v). The hexane phase of the lipid extract was dried under nitrogen and the lipid film was resuspended in chloroform. Cellular unesterified cholesterol (UC) and esterified cholesterol (EC) were separated by thin-layer chromatography using glass-backed silica gel H plates (Phase Separations, Norwalk, CT) with the solvent hexane–diethylether–acetic acid 130:40:1.5 (v/v/v). The UC and EC spots were identified by comparison with iodine-stained standards and scraped from the plates. For consistency, both the UC and EC spots were scraped from the plates and hydrolyzed with 1 N alcoholic KOH. Cholesterol was measured in each spot by reaction with cholesterol oxidase (26). Fluorescence was measured at an excitation wavelength of 325 nm and an emission wavelength of 415 nm. Data are expressed as cholesterol in the UC or EC fraction corrected for cell protein (27).

HPLC analysis

LDL 7-ketocholesterol content was measured using a Hewlett-Packard 1050 HPLC system with a diode array detector as described by Kritharides et al. (28). Lipid extracts were dissolved in HPLC-grade 2-propanol prior

to injection onto a reverse-phase C-18 column (Alltima, 250 × 46 mm, 5 μm particle size) equipped with a 50-mm guard column (Alltech Associates, Deerfield, IL). Compounds were isocratically eluted using a mobile phase solvent of 2-propanol–acetonitrile 70:30 (v/v) at a flow rate of 1 ml/min. Oxidized compounds were detected at a wavelength of 234 nm.

THP-1 cell transfection

Cell transfection and measurement of reporter gene activity were performed as described previously (29). Cells were transfected with an apoE-luciferase reporter construct (−2300/+24 apoE pGL2) using the modified dextran method. Some experiments also included a plasmid containing the β-galactosidase reporter gene under the control of the SV 40 promoter. The transfected cells were pooled before being exposed to experimental media in order to eliminate variability due to differential transfection efficiency. After treatment, the cells were lysed and reporter gene activity was measured using the Enhanced Luciferase Assay Kit (Analytical Luminescence Laboratory, San Diego, CA) or the Galactolyte Reporter Assay System (Tropix Laboratory, Bedford, MA). Cell protein concentration was determined using a DC protein assay kit (Bio Rad Laboratory, Richmond, CA). Luciferase/β-galactosidase ratios (RLU/β-gal) were calculated after correction for endogenous cellular β-galactosidase activity measured in mock-transfected cells.

LDL isolation and modification

LDL was isolated from human plasma and oxidatively modified by several methods designed to result in varying degrees of oxidation of fatty acids and oxysterol content. LDL was extensively oxidized by incubation with 5 μmol/L Cu for 24 h at 37°C (30). Less extensively oxidized forms of LDL were prepared by exposing LDL to either 2,2′-azobis (2-amidinopropane) hydrochloride (AAPH; Polysciences, Warrington, PA), soybean lipoxygenase (SLO; Sigma, St. Louis, MO) or sodium hypochlorite (HOCl; Aldrich Chemical Co, Milwaukee, WI). AAPH is a water-soluble, temperature-dependent free radical generator, which results in primarily oxidized lipids and only slight protein modification. AAPH-oxidized LDL was prepared by incubating LDL with AAPH (2 mmol/L final concentration) at 37°C overnight (31). A modification of the procedure of Parhami et al. (32) was used to prepare SLO-oxidized LDL. SLO was coupled to CNBr-activated Sepharose 4B beads (Pharmacia Biotech, Piscataway, NJ) in the presence of a coupling buffer (0.1 mol/L sodium bicarbonate, 0.5 mol/L sodium chloride, pH 8.3). Coupling occurred at room temperature for 2 h with gentle shaking followed by blocking with 0.1 mol/L Tris-HCl, pH 8.0, overnight at 4°C. SLO-oxidized LDL was prepared by incubating 1 mg LDL in 2 ml PBS containing 2 μl linoleic acid with the beads coupled to SLO for 2 days at room temperature with gentle shaking. HOCl-oxidized LDL was prepared by incubating LDL with diluted HOCl (1:5 dilution of reagent grade HOCl with water) in a fume hood on ice for 20 min, then dialyzing against PBS at 4°C overnight (33). Oxidation was stopped with 25 μmol/L BHT, after which the LDL was filtered through a 0.22-μm Millex-GV filter (Millipore, Bedford, MA).

Statistical analysis

Analysis of variance was used to test differences between ordinal or categorical variables. If the data failed an equal variance test ($P < 0.05$), analysis of variance on ranks was performed. Student-Newman-Keuls or Dunn's methods of multiple comparisons were used to isolate specific groups that differed. Linear regression was used to test the relationship among quantitative variables. Sigmastat for Windows Version 1, Jandel Corp. (San Rafael, CA) was used for analysis of variance. Excel Version 7.0, Microsoft Corp. (Redmond, WA) was used for linear regression analysis. Data are presented as mean values ± standard deviation where appropriate. The level of significance was set at $P < 0.05$.

RESULTS

Alterations in apoE mRNA and protein expression in response to native and oxidized LDL

The effect of native and oxidized LDL on apoE mRNA expression was assessed by Northern blot analysis. Differentiated THP-1 cells were exposed to native and Cu-oxidized LDL for increasing amounts of time. Cu-oxidized LDL stimulated apoE mRNA expression to a greater extent than native LDL over time ($P < 0.05$; **Fig. 1A**). Cu-oxidized LDL also stimulated apoE mRNA level in a concentration-dependent manner (Fig. 1B). Immunoprecipitation techniques were used to determine whether the increase in apoE mRNA synthesis in response to Cu-oxidized LDL was associated with an accumulation of apoE protein within the cells and/or an increase in apoE protein secreted from the cells. THP-1 cells were pulse-labeled with [^{35}S]methionine followed by immunoprecipitation of apoE in the cell extracts and medium. Cu-oxidized LDL at 25 μg/ml resulted in a 2.5-fold increase in apoE present in the cell extract as compared to native LDL. Immunoprecipitation of apoE secreted by the cells, i.e., present in the media, indicated that 50 μg/ml Cu-oxidized LDL resulted in approximately a 3-fold increase in apoE as

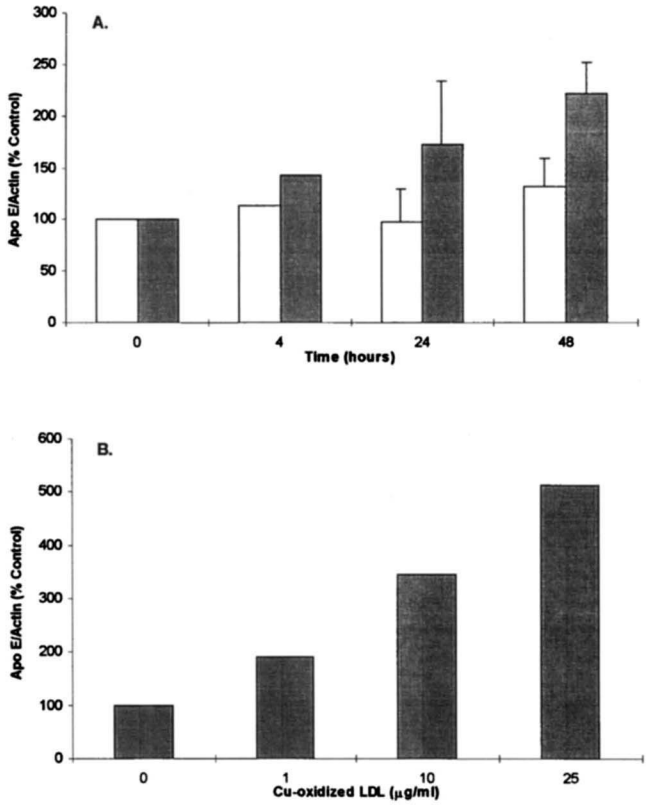

Fig. 1. A: Time-dependent effect of native and Cu-oxidized LDL on apoE mRNA. Differentiated THP-1 cells were cultured for 72 h in medium containing 10% FBS. Subsequently, the cells were exposed to 10 μg/ml LDL without serum for 4, 24, and 48 h. Cellular RNA was extracted, electrophoresed on a 1.2% agarose gel, and transferred to a nylon filter membrane as described in Materials and Methods. The filter was hybridized to ^{32}P-labeled cDNA probes for apoE and actin. The data are expressed as apoE to actin mRNA content. Northern blot analysis revealed that Cu-oxidized LDL (filled bars) stimulated apoE mRNA synthesis to a greater extent than native LDL (open bars). B: Concentration-dependent effect of Cu-oxidized LDL on apoE mRNA. Cells were cultured for 72 h after which they were exposed to increasing concentrations of Cu-oxidized LDL for 24 h in serum-free medium. Membrane hybridization with a ^{32}P-labeled apoE cDNA probe showed that Cu-oxidized LDL stimulated apoE mRNA synthesis in a concentration-dependent manner. The data are expressed as apoE to actin mRNA content.

compared to native LDL. These results indicate that Cu-oxidized LDL not only stimulated apoE mRNA expression but also apoE protein accumulation within cells and secretion from cells.

Alterations in apoE mRNA expression in response to different forms of oxidized LDL

In an attempt to identify the component(s) of oxidized LDL that may be responsible for the increase in apoE mRNA expression, various methods were used to oxidize LDL. These methods modified different components of the LDL particles, i.e., lipid versus protein modification (**Table 1**). Oxidation of LDL by AAPH (a temperature-dependent free radical generator) and SLO (soybean lipoxygenase) produced LDL particles that contained some of the early measurable products of oxidation, such as lipid peroxides and conjugated dienes. Only slight increases in the later oxidation indicators, e.g., increased TBARS formation and alterations in the relative electrophoretic mobility, an index of protein modification, were seen with AAPH or SLO oxidation of LDL. Treatment with HOCl led to changes primarily associated with the protein component of LDL as indicated by the increase in relative electrophoretic mobility (increased electronegativity), with little modification of lipid. Lastly, Cu-oxidized LDL resulted in the greatest alteration of all the oxidation parameters measured, i.e., exposure to Cu produced extensive modification of both the lipid and protein components of LDL.

THP-1 cells were incubated with the various forms of oxidized LDL described above and the levels of apoE mRNA were determined after 48 h (**Fig. 2**). Cu-oxidized LDL resulted in a 3-fold increase in apoE mRNA relative to native LDL ($P < 0.05$). However, AAPH-, SLO-, and HOCl-oxidized LDL did not affect apoE mRNA expression. It appears from these analyses that Cu-oxidized LDL is a potent stimulator of apoE mRNA expression.

Effect of native and oxidized LDL on apoE mRNA expression and cholesterol accumulation

Synthesis of apoE by macrophages has been reported to be regulated, at least in part, by cellular cholesterol content, especially unesterified cholesterol (21). To determine whether oxidized LDL stimulation of apoE expression was a result of cellular cholesterol accumulation, as has been shown to be the case for acetyl LDL, THP-1 cells were incubated with increasing concentrations of native, HOCl-, and Cu-oxidized LDL (**Fig. 3**). Cells exposed to Cu-oxidized LDL accumulated slightly greater amounts of UC than cells incubated with native LDL. However, HOCl-oxidized LDL led to the greatest UC accumulation (Fig. 3A). HOCl-oxidized LDL also resulted in the greatest increase in EC accumulation compared to native and Cu-oxidized LDL, which had virtually no effect on EC (Fig. 3B). Thus, HOCl increased total cholesterol (TC) accumulation to a greater extent than Cu-oxidized LDL (Fig. 3C).

The relationship between apoE mRNA expression and cholesterol accumulation appears to be different for Cu- versus HOCl-oxidized LDL (**Fig. 4**). After exposure of THP-1 cells to HOCl-oxidized LDL, a significant relationship also was observed between apoE mRNA expression and cellular UC ($r = 0.84$; $P < 0.001$; Fig. 4A), EC ($r = 0.83$; $P < 0.01$; Fig. 4B), and TC ($r = 0.86$; $P < 0.001$; Fig. 4C). A significant relationship was observed

TABLE 1 Characteristics of native and oxidized LDL

LDL	Relative Electrophoretic Mobility	TBARS	Lipid Peroxides	Conjugated Dienes	7-Ketocholesterol
		nmol/mg protein	*mmol/L*	A234	*μg/ml*
Native	1.0	1.9	0	0.17	3.68
AAPH	1.5	12.0	397	0.62	6.40
SLO	0.8	3.1	199	0.80	0.24
HOCl	2.3	2.3	48	0.21	0.54
Cu	2.6	56.3	436	0.89	29.46

between apoE mRNA expression and cellular UC content ($r = 0.91$; $P < 0.001$; Fig. 4A), EC ($r = 0.60$; $P < 0.05$; Fig. 4B), and TC ($r = 0.91$; $P < 0.001$; Fig. 4C) after exposure of cells to Cu-oxidized LDL. However, the extent of stimulation of apoE mRNA was much greater after exposure of cells to Cu-oxidized versus HOCl-oxidized LDL. Statistical analysis revealed that the regression lines between apoE mRNA and UC, EC, and TC were different for Cu-oxidized and HOCl-oxidized LDL ($P < 0.01$).

Changes in apoE transgene expression in response to native and oxidized LDL

Having found that oxidized LDL stimulated apoE mRNA and protein synthesis, we next examined whether this effect was accompanied by stimulation of the apoE promoter. THP-1 cells were transiently transfected with a construct containing the apoE promoter linked to a luciferase reporter gene. Subsequently, the transfected cells were exposed to 30 μg/ml LDL for 24 and 48 h. At 48 h, Cu-oxidized LDL significantly increased luciferase activity with respect to native LDL ($P < 0.05$; **Fig. 5A**). Normalization of the relative luciferase units using β-galactosidase (RLU/β-gal) gave results similar to RLU/mg protein (at 48 h, RLU/β-gal for native LDL was 0.30 vs. 0.94 for Cu-oxidized LDL). Various forms of oxidized LDL were tested for their ability to stimulate transgene expression by exposing transfected cells to 30 μg/ml of the various modified forms of LDL for 48 h (Fig. 5B). Aside from Cu-oxidized LDL, other forms of oxidized LDL did not significantly alter apoE transgene expression (Fig. 5B).

Effect of 7-ketocholesterol on apoE mRNA expression

Several reports indicate that 7-ketocholesterol is the major oxysterol present in Cu-oxidized LDL (28, 34, 35). Therefore, in an effort to investigate which compo-

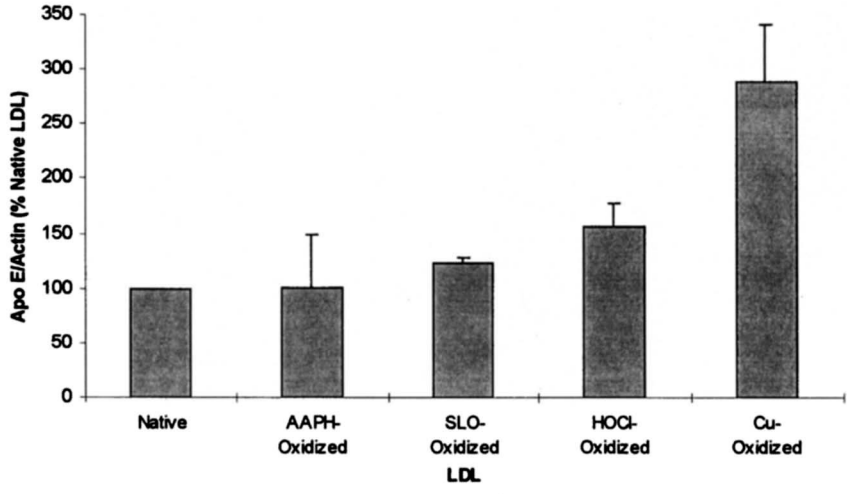

Fig. 2. Effect of native and different forms of oxidized LDL on apoE mRNA. Differentiated THP-1 cells were cultured for 72 h in medium containing 10% FBS then exposed to 25 μg/ml native and LDL oxidized by different methods for 48 h in serum-free medium. These methods of oxidation modify different components of LDL, i.e., lipid and/or protein. THP-1 cellular RNA was extracted, electrophoresed, and transferred to a nylon filter membrane as described in Materials and Methods. The membrane then was hybridized with a cDNA riboprobe. The data are expressed as apoE to actin mRNA content. The data for the different forms of oxidized LDL are compared to native LDL as 100%.

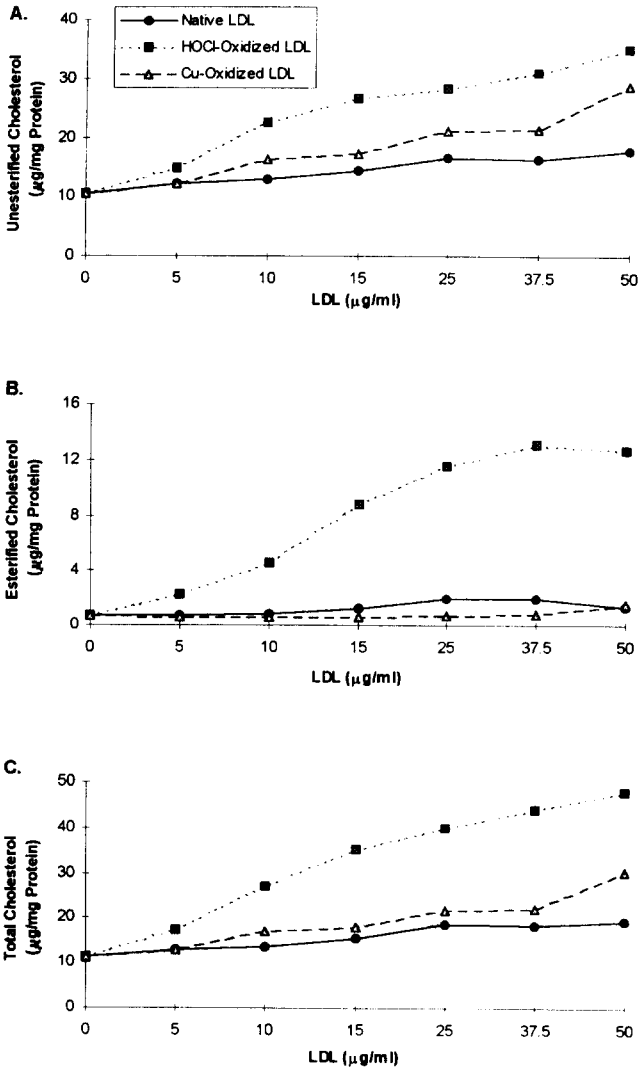

Fig. 3. Effect of native and oxidized LDL on cellular cholesterol accumulation. Differentiated THP-1 cells were cultured for 72 h in medium containing 10% FBS then exposed to native (circles), HOCl-oxidized (squares), and Cu-oxidized (triangles) LDL for 48 h at increasing concentrations. Cellular lipids were extracted with hexane–isopropanol. Cholesterol mass was determined by reaction with cholesterol oxidase. The greatest extent of cholesterol accumulation occurred in the cells exposed to HOCl-oxidized LDL. Cu-oxidized LDL had an intermediate effect on unesterified cholesterol (A) and virtually no effect on esterified cholesterol (B) accumulation.

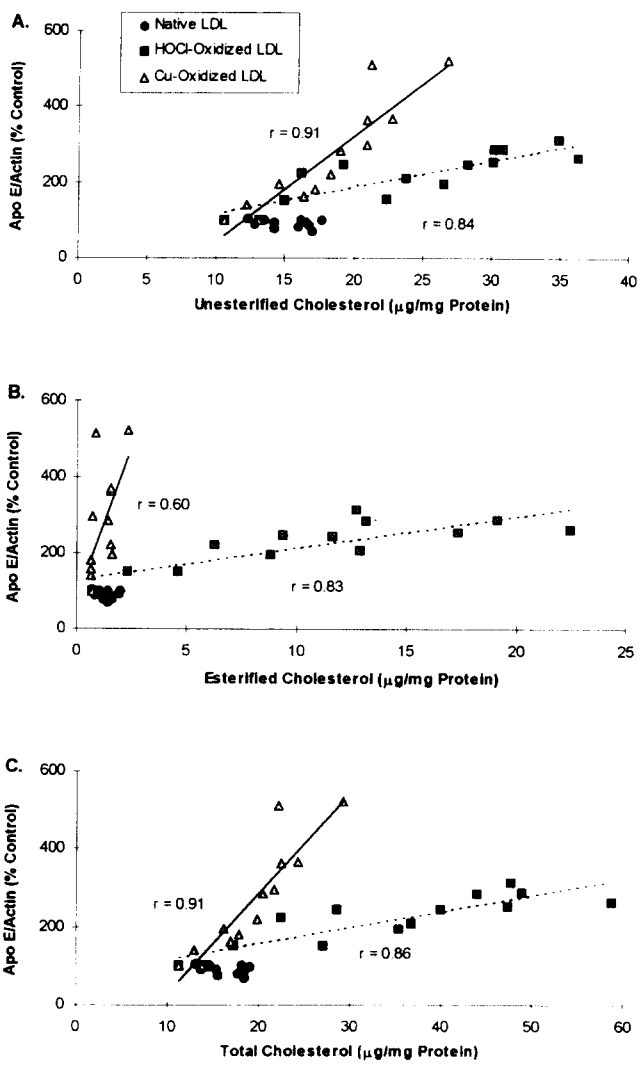

Fig. 4. The relationship between apoE mRNA expression and cholesterol accumulation. THP-1 cells were exposed to increasing concentrations of native (circles), HOCl-oxidized (squares), and Cu-oxidized (triangles) LDL for 24 and 48 h. The scatter plots shown relating apoE mRNA to unesterified cholesterol (A), esterified cholesterol (B), and total cholesterol (C) are a compilation of the data obtained at 24 and 48 h. The correlation coefficient and its significance are indicated for each regression line. Statistical comparison of the regression lines for HOCl- and Cu-oxidized LDL indicated that the lines are significantly different at a level of $P < 0.01$ for unesterified, esterified, and total cholesterol.

nent of oxidized LDL may be responsible for the increase in apoE synthesis and secretion, HPLC analysis was performed on the various forms of oxidized LDL (Table 1). Cu-oxidized LDL contained 8-fold greater 7-ketocholesterol than native LDL. AAPH-oxidized LDL contained approximately 1.7-fold more 7-ketocholesterol than native LDL. However, SLO- and HOCl-oxidized LDL both contained less 7-ketocholesterol than native LDL. To directly test the effect of 7-ketocholesterol on apoE mRNA expression, THP-1 cells were incubated with increasing concentrations of 7-ketocholesterol for 24 and 48 h. A linear trend with increasing concentration was observed at 48 h ($P < 0.01$; **Fig. 6**).

Alterations in apoE mRNA expression in human monocyte-derived macrophages

Several key experiments were repeated in human monocyte-derived macrophages (MDM) to verify the results obtained with THP-1 cells. MDM were incubated with 50 μg/ml lipoprotein for 24 h. Northern blot analysis revealed a 2-fold increase in apoE mRNA in response to Cu-oxidized LDL with respect to native LDL

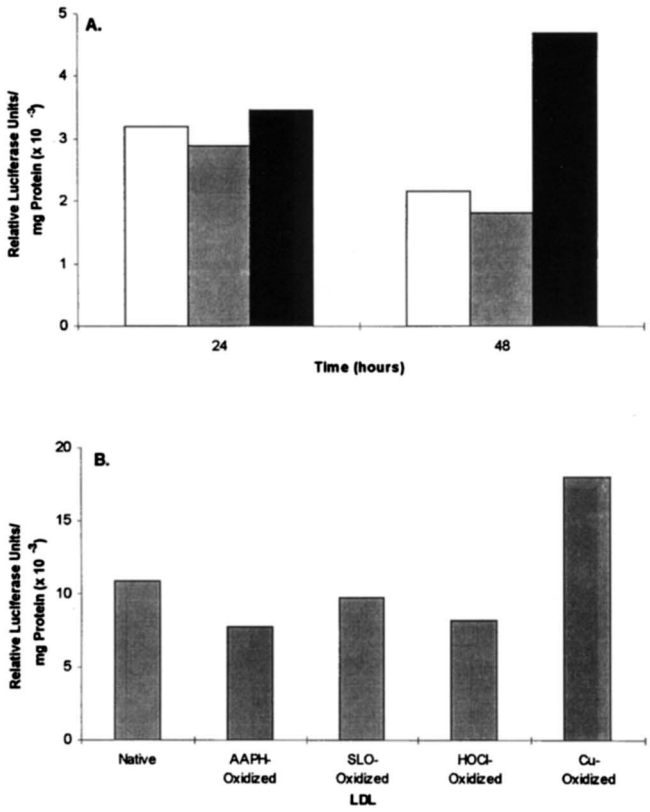

Fig. 5. A: Effect of native and Cu-oxidized LDL on apoE transgene expression. THP-1 cells were acutely transfected with the apoE promoter linked to a luciferase reporter gene as described in Materials and Methods. Subsequently, the transfected cells were exposed to no addition (open bars), 30 μg/ml native (gray bars), or Cu-oxidized (black bars) LDL for 24 and 48 h in 0.2% bovine serum albumin. Cu-oxidized LDL increased luciferase activity 2.6-fold at 48 h. B: Effect of native and different forms of oxidized LDL on apoE transgene expression. THP-1 cells were acutely transfected with the apoE promoter linked to a luciferase reporter gene as described in Materials and Methods. Subsequently, the transfected cells were exposed to 30 μg/ml LDL for 48 h in 0.2% bovine serum albumin. Only Cu-oxidized LDL increased apoE transgene expression with respect to native LDL.

(**Fig. 7A**). MDM also were exposed to increasing concentrations of 7-ketocholesterol for 24 and 48 h (Fig. 7B). As with THP-1 cells, 7-ketocholesterol stimulated apoE mRNA expression in a time- and concentration-dependent manner. A linear trend with increasing concentration was observed at 48 h ($P < 0.01$). The relationship between apoE mRNA and cholesterol accumulation in MDM also was similar to that seen in THP-1 cells (data not shown).

DISCUSSION

ApoE is widely present in human atherosclerotic plaques, but not in areas of diffuse intimal thickening (18). This protein is found in association with extracellular matrix and adorning macrophages, particularly macrophage foam cells, and some smooth muscle cells. However, it appears to be synthesized primarily by macrophages in atherosclerotic lesions (17). Macrophages become foam cells during atherosclerotic lesion development as a result of unregulated cholesterol uptake via scavenger receptors and possibly other cell surface binding sites. ApoE synthesis by macrophages is regulated by increasing cellular cholesterol content (15, 21, 36), which can be achieved by incubating macrophages with acetyl LDL in vitro. An increase in unesterified cholesterol appears to be the major determinant of increased apoE production (21). The present study was performed to evaluate the effect of oxLDL on apoE expression by macrophages. Indeed, findings from the present study indicate that oxLDL stimulates apoE mRNA and protein expression in macrophages. Zhang and Lin (37) also have examined the issue of apoE secretion from macrophages in response to modified forms of LDL. They compared the effects of Cu-oxidized LDL to acetyl LDL and phospholipase C-treated LDL. Using rat peritoneal macrophages, they observed that modified LDL increased apoE mRNA, protein, and cellular cholesterol. As Cu-oxidized LDL affected apoE mRNA and cholesteryl ester formation in a manner different from acetyl LDL and phospholipase C-LDL, the authors concluded that Cu-oxidized LDL stimulated apoE by a different mechanism (37).

In the present study, we show that Cu-oxidized LDL also stimulates apoE mRNA and protein synthesis in human macrophages. Further, the data in transfected cells shows that Cu-oxidized LDL enhances expression of an apoE reporter transgene by 2- to 3-fold. These results, however, do not rule out an additional role for enhanced mRNA stability for the apoE mRNA response to Cu-oxidized LDL. In addition, our data indicate that 7-ketocholesterol may be an important constituent of Cu-oxidized LDL that enhances steady state apoE mRNA levels, either as a result of transcriptional or post-transcriptional regulation.

OxLDL can be taken up by the classical scavenger receptor (11) or by one of several putative oxLDL receptors such as CD36 (38), the 94–97 kDa protein recently described by Ottnad et al. (39), or the Fc receptor, FcγRII-B2 (40). However, intracellular cholesterol that accumulates in response to Cu-oxidized LDL is primarily free cholesterol and not esterified cholesterol (34, 41), as opposed to the uptake of acetyl LDL by the scavenger receptor, which leads to the accumulation of both free and esterified cholesterol (21). Our results are in accordance with these earlier observations in that Cu-oxidized LDL led to the accumulation primarily of unesterified and not esterified cholesterol. The failure of oxLDL to stimulate cholesteryl ester formation in macrophages may be the result of a direct inactivation

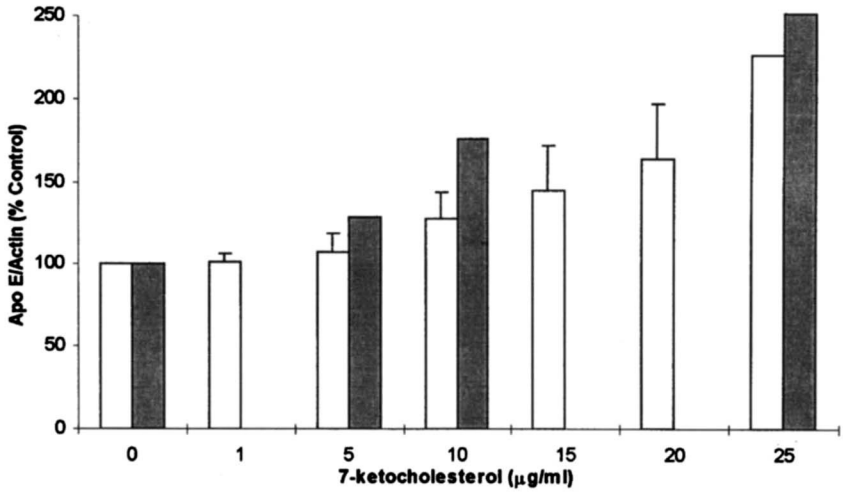

Fig. 6. Effect of 7-ketocholesterol on apoE mRNA. Differentiated THP-1 cells were cultured for 72 h in medium containing 10% FBS. Subsequently, the cells were exposed to increasing concentrations of 7-ketocholesterol without serum for 24 h (open bars) and 48 h (filled bars). Cellular RNA was extracted, electrophoresed on a 1.2% agarose gel, and transferred to a nylon filter membrane as described in Materials and Methods. The filter was hybridized to ^{32}P-labeled cDNA probes for apoE and actin. The data are expressed as the mean and standard deviation of the apoE to actin mRNA content. The 48 h incubation was done once, therefore no variance is shown. Northern blot analysis revealed that at 24 h, a 2-fold increase in apoE mRNA was seen at 25 μg/ml, whereas at 48 h, a concentration-dependent increase in apoE mRNA was observed.

of a lysosomal protease leading to impaired degradation and lysosomal trapping (42). As a result, macrophages exposed to oxLDL accumulate free cholesterol in lysosomes as opposed to cholesteryl ester accumulation as observed with acetyl LDL. Maor and Aviram (43) recently have shown that oxidized and acetyl LDL cholesteryl esters are hydrolyzed at similar rates. However, free cholesterol from oxLDL remains trapped in the lysosomes. In the present work, there was a slight increase in unesterified cholesterol with Cu-oxidized LDL; however, it was a much smaller increase than has been reported with acetyl LDL (21). Therefore, it appears that cellular unesterified cholesterol accumulation does not account for the entire increase in apoE expression in response to Cu-oxidized LDL.

Incubation of cells with HOCl-oxidized LDL, a less extensively modified form of LDL than Cu-oxidized LDL, led to large increases in both esterified and free cholesterol, to a much greater extent than observed with Cu-oxidized LDL. Nonetheless, Cu-oxidized LDL resulted in greater stimulation of apoE expression than HOCl-oxidized LDL, especially in relation to the extent of cholesterol accumulation. These findings suggest that Cu-oxidized LDL increases apoE expression by a mechanism other than cholesterol accumulation, possibly as a result of compounds present in extensively oxidized LDL. The impaired processing of oxLDL is believed to be due, at least in part, to the inhibition of lysosomal sphingomyelinase by 7-ketocholesterol (44). Maor, Mandel, and Aviram (44) concluded that inhibition of sphingomyelinase by oxLDL 7-ketocholesterol resulted in an increase in lysosomal sphingomyelin which then could trap oxLDL-derived unesterified cholesterol in the lysosome, thereby preventing further processing. As 7-ketocholesterol is the major oxysterol in Cu-oxidized LDL, it is likely to play a multifaceted regulatory role in the effects of oxLDL. In fact, several proteins involved in lipid metabolism are sensitive to oxysterols, including the genes encoding hydroxymethylglutaryl Co A reductase (45) and the LDL receptor (46). In addition, macrophage apoE synthesis is stimulated by 25-hydroxycholesterol (21). In the present study we show that 7-ketocholesterol also stimulates steady state apoE mRNA expression. It is possible that the combination of 7-ketocholesterol with the slight increase in unesterified cholesterol in macrophages exposed to Cu-oxidized LDL could account for the rise observed in apoE expression. On the other hand, HOCl-oxidized LDL led to massive cholesterol accumulation and a lesser increase in apoE mRNA than Cu-oxidized LDL while containing virtually no 7-ketocholesterol. Therefore, HOCl- and Cu-oxidized LDL may stimulate apoE expression by different mechanisms.

Increased apoE secretion in response to modified lipoproteins could have important implications in vessel wall cholesterol homeostasis. ApoE may play a role in reverse cholesterol transport, as originally suggested by Basu, Goldstein, and Brown (47). This hypothesis has received additional support by the recent observations

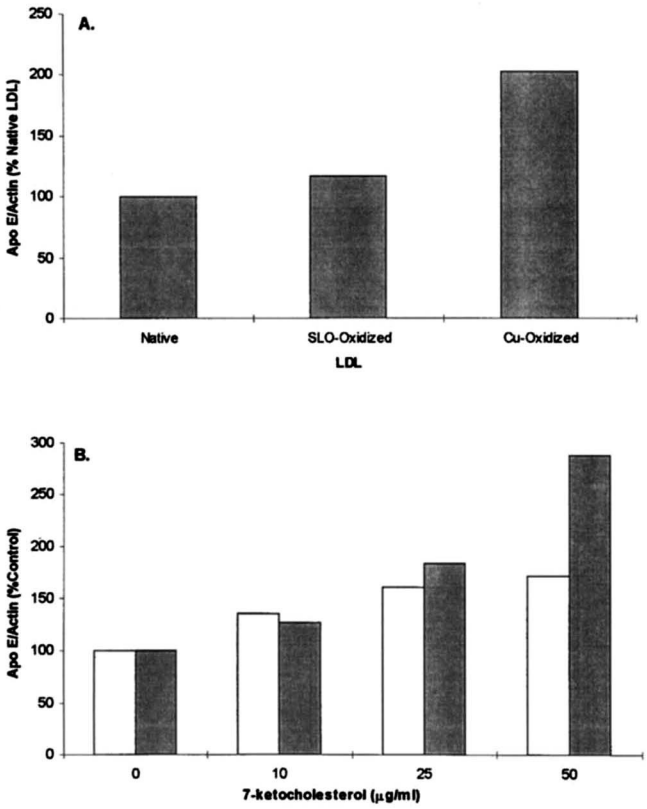

Fig. 7. A: Effect of native and oxidized LDL on apoE mRNA in monocyte-derived macrophages. Human monocyte-derived macrophages were cultured for 10 days in 20% autologous serum after which cells were incubated with 50 μg/ml LDL for 24 h in serum-free medium. Cellular RNA was extracted, electrophoresed on a 1.2% agarose gel, and transferred to a nylon filter membrane as described in Materials and Methods. The filter was hybridized to ^{32}P-labeled cDNA probes for apoE and actin. The data are expressed as apoE to actin mRNA content. Northern blot analysis revealed a 2-fold increase in apoE mRNA in response to Cu-oxidized LDL with respect to native LDL. B: Effect of 7-ketocholesterol on apoE mRNA in monocyte-derived macrophages. Human monocyte-derived macrophages were cultured for 8 days in 20% autologous serum after which cells were incubated with increasing concentrations for 7-ketocholesterol for 24 h (open bars) and 48 h (filled bars) in serum-free medium. Cellular RNA was extracted, electrophoresed, and transferred to a nylon filter membrane as described in Materials and Methods. The membrane then was hybridized with a cDNA riboprobe and the data are expressed as apoE to actin mRNA content. ApoE to actin mRNA ratio increased in a time- and concentration-dependent fashion.

that macrophage-specific expression of apoE resulted in marked amelioration of atherosclerosis in apoE-deficient mice (19), that overexpression of apoE in the mouse vessel wall led to enhanced cholesterol efflux from the vessel wall (48), and by the demonstration of enhanced cholesterol efflux from J774 macrophages after expression of human apoE cDNA (49). Thus, increased macrophage expression of apoE in response to extensively oxidized LDL, or possibly 7-ketocholesterol and other regulatory oxysterols that may form intracellularly, may represent a mechanism to facilitate cholesterol removal during atherogenesis.

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