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J K Liao, S L Clark

Research Article

Oxidized low-density lipoprotein (LDL) inhibits signalling pathways mediated by pertussis toxin-sensitive guanine nucleotide-binding proteins (Gi proteins). To determine whether this inhibition is due to altered G protein alpha i subunit expression, mRNA and protein levels of alpha i isoforms were assessed in bovine aortic endothelial cells treated with oxidized LDL (0-100 micrograms/ml, 0-72 h). Oxidized LDL did not affect the expression of alpha i3, but did cause time- and concentration-dependent decrease in alpha i2 mRNA and protein resulting in a 3.2- and 3.5-fold reduction, respectively, after 72 h. This decrease in alpha i2 coincided with a 86% decrease in alpha i2 GTPase activity. Nuclear run-off studies did not show any significant effect of oxidized LDL on alpha i2 or alpha i3 transcription. In the presence of actinomycin D, oxidized LDL shortened the t1/2 of alpha i2 mRNA from 16 h to 8 h which was attenuated by cycloheximide. In addition, pulse-chase labelling with [35S]methionine revealed that oxidized LDL reduced the t1/2 of alpha i2 protein from 27 to 14 h. Our results indicate that oxidized LDL can modulate receptor-Gi coupling by downregulating the expression of alpha i2, but not alpha i3. The mechanism involves both mRNA destabilization and protein degradation.

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Regulation of G-Protein α_{i2} Subunit Expression by Oxidized Low-Density Lipoprotein

James K. Liao and Stephen L. Clark

Cardiovascular Division, Brigham & Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115

Abstract

Oxidized low-density lipoprotein (LDL) inhibits signalling pathways mediated by pertussis toxin-sensitive guanine nucleotide-binding proteins (G_i proteins). To determine whether this inhibition is due to altered G protein α_i subunit expression, mRNA and protein levels of α_i isoforms were assessed in bovine aortic endothelial cells treated with oxidized LDL (0–100 $\mu g/ml$, 0–72 h). Oxidized LDL did not affect the expression of α_{i3}, but did cause time- and concentration-dependent decrease in α_{i2} mRNA and protein resulting in a 3.2- and 3.5-fold reduction, respectively, after 72 h. This decrease in α_{i2} coincided with a 86% decrease in α_{i2} GTPase activity. Nuclear run-off studies did not show any significant effect of oxidized LDL on α_{i2} or α_{i3} transcription. In the presence of actinomycin D, oxidized LDL shortened the $t_{1/2}$ of α_{i2} mRNA from 16 h to 8 h which was attenuated by cycloheximide. In addition, pulse-chase labelling with [^{35}S]methionine revealed that oxidized LDL reduced the $t_{1/2}$ of α_{i2} protein from 27 to 14 h. Our results indicate that oxidized LDL can modulate receptor–G_i coupling by downregulating the expression of α_{i2}, but not α_{i3}. The mechanism involves both mRNA destabilization and protein degradation. (*J. Clin. Invest.* 1995. 95:1457–1463.) Key words: atherosclerosis • gene expression • transcription • Northern blotting • Western blotting

Introduction

Endothelial dysfunction is perhaps one of the earliest manifestation of atherosclerosis (1, 2). Previous studies have suggested that this abnormality is, in part, due to altered membrane signal transduction in endothelial cells (3). For example, coronary arteries from hypercholesterolemic animals demonstrate impaired endothelial-dependent vasodilation to receptor-mediated agonists such as acetylcholine and serotonin, but not to the non-receptor-mediated calcium ionophore A23187 (4–6). This impairment probably occurs at the level of guanine nucleotide-binding proteins (G_i proteins)[1] since the release of endothelial-

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1. *Abbreviations used in this paper:* G_i protein, guanine nucleotide-binding protein; TBARS, thiobarbituric acid reactive substances.

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derived nitric oxide via pertussis toxin-sensitive pathway is inhibited by lipoproteins, early atherosclerosis, and trauma, while its release via the pertussis toxin-insensitive pathway is relatively unaffected (7, 8). The mechanism(s) by which signal transduction is altered in atherogenesis is not known, but may, in part, be due to the effects of low-density lipoprotein (LDL).

We have recently shown that short-term exposure to native, or unmodified LDL causes a functional inhibition of G_i proteins in endothelial cells (9). This could account for the observed rapid inhibitory effects of native LDL on endothelial-dependent relaxtion in response to acetylcholine, serotonin, and thrombin (10, 11). However, atherosclerosis is a chronic process involving oxidative modification of the LDL particle (12). Oxidized LDL is atherogenic and its presence has been documented in atherosclerotic vessels (13). Thus, its role in atherosclerosis may be clinically more relevant than native LDL. Indeed, many studies have shown that the inhibitory effects of oxidized LDL differ from that of native LDL in being relatively more profound and irreversible (14, 15). We hypothesized that in contrast to native LDL, oxidized LDL produces further inhibitory effects on endothelial signal transduction by altering G_i protein expression.

Methods

Materials. All standard culture reagents were obtained from JRH Bioscience (Lenexa, KS). Lipoprotein-deficient serum (lot no. 83H9478), Hepes, ascorbic acid, creatinine phosphate, phosphocreatine kinase, phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, bacitracin, 1,10-phenanthroline, triethanolamine HCl, dithiothreitol (DTT), bovine serum albumin (BSA), cupric sulfate (CuSO_4), polymyxin B, butylated hydroxytoluene (BHT), thiobarbituric acid, and 1,1,3,3-tetramethoxypropane were purchased from Sigma Chemical Co. (St. Louis, MO). UK14304 was a gift from Pfizer (Kent, UK). Rauwolscine was obtained from Roth (West Germany). [α-^{32}P]CTP (3,000 Ci/mmol), [γ-^{32}P]-GTP (10 Ci/mmol), [α-^{32}P]UTP (800 Ci/mmol), and L-[^{35}S]-methionine (1175 Ci/mmol) were supplied by New England Nuclear. Purified human low-density lipoprotein (LDL, lot no. 832293, 730793, B08850, 665893), actinomycin D, and cycloheximide were obtained from Calbiochem Corp. (San Diego, CA). The Limulus amebocyte lysate kinetic assay was performed by BioWhittaker (Walkersville, MD). The polyclonal rabbit antisera P4 and JL14 were raised against purified decapeptides corresponding to the COOH-terminal regions of α_{i2} (P4 peptide, KENLKDCGLF) and α_{i3} (JL14 peptide, KENLKECGLY) (Research Genetics, Inc., Huntsville, AL). Protein A-sepharose was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Nucleic acid and protein molecular weight markers were purchased from Bethesda Research Laboratories, Inc. (Bethesda, MD). The Western Blotting kit (Enhanced Chemiluminescence) using horseradish peroxidase and luminol was obtained from Amersham Corp. (Arlington Heights, IL). Nylon transfer membranes were purchased from Schleicher and Schuell (W. Germany). The full-length rat cDNA probes for α_{io}, α_{i1}, α_{i2}, and α_{i3} were generously provided by Randall R. Reed (Johns Hopkins School of Medicine, Baltimore, MD).

Cell culture. Bovine aortic endothelial cells of less than three passages were cultured in a growth medium containing DME, 5 mM L-

glutamine (GIBCO BRL, Gaithersburg, MD), 10% fetal calf serum (Hyclone lot no. 1114577), and antibiotic mixture of 100 U/ml penicillin/ 100 μg/ml streptomycin/250 ng/ml Fungizone. They were characterized by Nomarski optical microscopy (Zeiss ICM 405, 40X objective) and staining for Factor VIII-related antigens (16). For all experiments, the endothelial cells were placed in 5% lipoprotein-deficient serum for 48 h before treatment with the indicated concentrations of LDL. In some experiments, cells were pretreated with either actinomycin D (5 μg/ml) or cycloheximide (10 μg/ml) for 1 h before LDL treatment. Cellular confluence was maintained for all treatment conditions. Cellular viability was determined by Trypan blue exclusion.

Characterization of LDL. Four separate preparations of native LDL (density 1.02–1.06 g/ml) were obtained from Calbiochem using sequential ultracentrifugation of plasma samples from a single donor (17, 28). Oxidized LDL (80% lipid, 20% protein) was prepared by exposing samples of native LDL to CuSO$_4$ (5 μM) at 37°C for 2 to 24 h. Both native and oxidized LDL were dialyzed with three changes of sterile buffer (150 mM NaCl, 0.01% EDTA, and 100 μg/ml polymyxin B, pH 7.4) prior to filtering through 0.2 μm membrane. Its purity was confirmed by SDS/polyacrylamide and cellulose acetate gel electrophoresis. Cholesterol and triglyceride content were determined as previously described (9). The protein content was determined by the method of Lowry (18). The degree of LDL oxidation was estimated by measuring the amounts of thiobarbituric acid reactive substances (TBARS) produced using a colorimetric assay for malondialdehyde (MDA) as previously described (19). The TBARS value is expressed as nmol of MDA per mg of LDL protein.

Northern blotting. Equal amounts of total RNA (20 μg/lane) were separated by 1% formaldehyde-agarose gel electrophoresis, transfered overnight onto nitrocellulose membrane by capillary action, and baked (72°C) for 2 h before hybridization. Radiolabeling of α_i cDNA probes was performed using random hexamer priming, [α-^{32}P]-CTP, and Klenow (Pharmacia Five Chemicals). The membranes were hybridized overnight at 42°C with the appropriate α_i cDNA probes in a solution containing 50% formamide, 5X SSC, 2.5X Denhardt's Solution, 25 mM sodium phosphate buffer (pH 6.5), 0.1% SDS, and 250 ng/ml salmon sperm DNA (Sigma Chemical Co.). All Northern blots were subjected to stringent washing conditions (0.2X SSC/0.1% SDS at 68°C) before autoradiography with intensifying screen at −80°C. Loading conditions were determined by subsequent hybridization to β-tubulin cDNA.

In vitro transcription studies. Transcription was measured according to the method previously described by Kavanaugh et al. with some modifications (20). Confluent endothelial cells (7 × 10^6 cells) were placed in lipoprotein-deficient serum for 48 h to achieve quiescence prior to treatment with oxidized LDL for the indicated durations. Cells were washed twice with PBS, trypsinized, and centrifuged at 300 g for 5 min at 4°C. The cellular pellet was gently resuspended in a buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl$_2$, and 0.5% Nonidet P-40, allowed to swell on ice for 15 min, and lysed by a Dounce homogenizer (30–35 strokes) with intermittent inspection of nuclei. The lysate was recentrifuged at 300 g and the resulting nuclear pellet was resuspended in 100 μl of buffer containing 20 mM Tris-HCl (pH 8.1), 75 mM NaCl, 0.5 mM EDTA, 1 mM DTT, and 50% glycerol.

In vitro transcription using the nuclear pellet (100 μl) was carried out in a shaking waterbath at 30°C for 30 min in a buffer containing 10 mM Tris-HCl (pH 8.0), 5 mM MgCl$_2$, 300 mM KCl, 50 μM EDTA, 1 mM DTT, 0.5 U RNAsin, 0.5 mM CTP, ATP, GTP, and 250 μCi [α-^{32}P]UTP. The reaction was terminated by incubating the assay with 40 U of DNAse I (Ambion, Austin, TX) for 20 min at 30°C. Proteins in the mixture were degraded by a solution containing 0.4% SDS, 40 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 400 μg/ml of proteinase K (E. Merck, Darmstadt, W. Germany), and extracted by phenol/chloroform. Ammonium acetate (2 M) and equal volume of EtOH were added to the radiolabeled RNA transcripts. The mixture was kept in dry-ice for 20 min before precipitation by centrifugation at 12,000 g for 10 min.

Equal amounts (1 μg) of purified, denatured full-length cDNA inserts of α_{i2}, α_{i3}, β-tubulin, b-actin, and constitutive nitric oxide synthase

(GenBank™/EMBL Data Bank accession number L26914) and linearized, denatured pGEM-3z vector cDNA (Promega, Madison, WI) were vacuum-transferred onto nylon membranes using a slot blot apparatus (Schleicher & Schuell, Keene, NH). The membranes were baked and prehybridized as described for Northern blots. The precipitated radiolabelled transcripts ($\sim$ 5 × 10^7 cpm) were resuspended in 2 ml of hybridization buffer containing 50% formamide, 5X SSC, 2.5X Denhardt's Solution, 25 mM sodium phosphate buffer (pH 6.5), 0.1% SDS, and 250 μg/ml salmon sperm DNA. Hybridization of radiolabelled transcripts to the nylon membranes was carried out at 45°C for 48 h. The membranes were then washed with 1X SSC/0.1% SDS for 1 h at 65°C before autoradiography for 72 h at −80°C.

Protein biosynthetic labeling. Confluent endothelial cells (5 × 10^6) were placed in methionine-deficient DME medium for 1 h at 37°C. Pulse-labeling of proteins was accomplished by replacing the medium with methionine-deficient DME medium supplemented with 0.1 μM [^{35}S]methionine, 10% LPDS and antibiotics, and incubating for 24 h at 37°C. The endothelial cells were washed three times with PBS and then placed in DME with 1 mM of unlabelled methionine and 10% LPDS with and without lipoproteins. The labelled proteins were chased by harvesting endothelial cells at various time points and preparing partially-purified membranes as previously described (16).

Membrane proteins (200 μg) were resuspended in 0.1 ml of immunoprecipitation buffer containing NaCl (150 mM), Tris-HCl (50 mM, pH 7.4), SDS (0.2%), and Triton X-100 (1%). The P4 antisera (specific for α_{i2}) was added to give a final dilution of 1:50 and the samples were allowed to incubate for 16 h at 4°C. The antibody–G protein complexes were incubated with 50 μl of protein A–Sepharose (1 mg/ml) for 2 h at 4°C, and the precipitate was collected by centrifugation at 12,000 g. Preliminary studies indicated that all α_{i2} were completely precipitated by this procedure since Western blot analysis of the supernatant with the P4 antisera did not reveal the presence of 40-kD proteins. The pellets were washed twice with immunoprecipitation buffer (pH 8.3) and once with NaCl (150 mM), Tris-HCl (50 mM, pH 7.4), and EDTA (5 mM). The immunoprecipitates were then suspended in denaturing buffer containing Tris-HCl (125 mM, pH 6.8), SDS (4%), glycerol (20%), and 2-mercaptoethanol (10%) and placed in boiling water for 5 minutes. The samples were centrifuged at 12,000 g for 10 min. The supernatants and known molecular weight markers (Bethesda Research Lab) were separated by SDS–PAGE (10% running, 4% stacking gel). The gels were then fixed with Coomassie Blue (0.4%), methanol (20%), and glacial acetic acid (10%), and dried by a gel dryer before autoradiography at −70°C for 72 h.

Western blotting. Membrane proteins (25 μg) and molecular weight markers were separated by SDS/PAGE (10% running, 4% stacking gel) as previously described (16). We have previously shown that bovine aortic endothelial cell membranes contain α_{i2} and α_{i3}, but little or no α_{i1} and α_o (16). The membrane proteins were electrophoretically transferred onto Westran and incubated overnight at 4°C with blocking solution (5% dried skim-milk in PBS) prior to the addition of the following dilutions of specific antisera: P4 (1:500) and JL14 (1:400). The blots were washed twice with PBS buffer containing 0.1% Tween-20 and then treated with donkey anti-rabbit horseradish peroxidase antibody (1:2000). Immunodetection was accomplished using the Enhance Chemiluminescence Kit (ECL, Amersham Corp.) (21). Autoradiography was performed several times at 23°C and the appropriate exposures were subjected to densitometric analysis (NIH Image Program).

GTPase assay. The assay was performed as previously described (9, 21). The reaction was initiated by the addition of UK14304 (10 nM) to endothelial membranes (30 μg) in a mixture consisting of 0.5 μM of [γ-^{32}P]GTP, 5 mM MgCl$_2$, 0.1 mM EGTA, 50 mM NaCl, 4 mM creatine phosphate, 5 U of phosphocreatine kinase, 0.1 mM ATP, 1 mM DTT, pH 7.4 in a total volume of 0.1 ml. The reaction was allowed to proceed for 15 min at 22°C and terminated with 500 μl of ice-cold 10% activated charcoal in 50 mM phosphoric acid. The mixture was then centrifuged for 10 min at 12,000 g at 4°C, and 300 μl of the supernatant containing the liberated ^{32}P$_i$ was counted in a liquid scintillation counter. Nonspecific activity was determined in the pres-
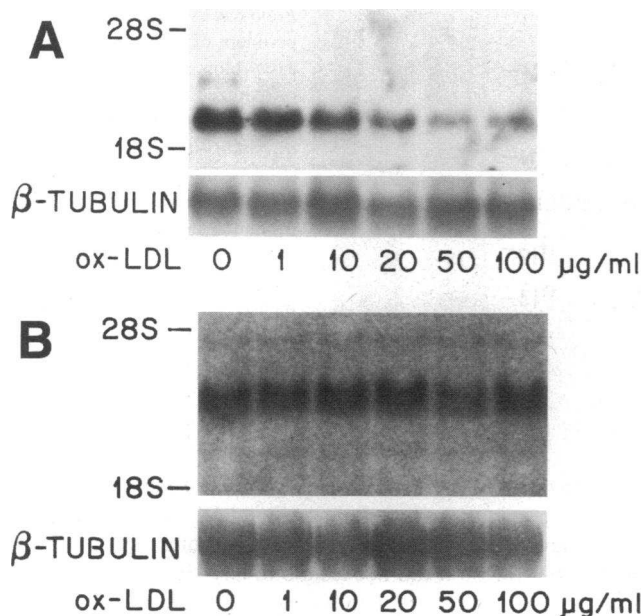
Figure 1. Northern blots (20 μg total RNA/lane) showing the effects of increasing concentrations of oxidized LDL (TBARS 23.4±2.6 nmol/mg) at 72 h, hybridized with cDNA probes for (A) α_{i2} and (B) α_{i3}. RNA loading was determined by hybridization to β-tubulin and was nearly equal. This is representative of three separate experiments.

ence of 0.1 μM rauwolscine and represented between 10 and 15% of total activity. Agonist-stimulated GTPase activity was calculated as the difference between total and nonspecific activity and expressed as pmol/min per mg of membrane protein. Assays were performed in duplicate with < 10% variation.

Data analysis. Band intensities from Northern and nuclear run-off assay blots were analyzed densitometrically by the National Institutes of Health IMAGE program (22). All values are expressed as mean±SEM compared with controls and among separate experiments. Paired and unpaired Student's t tests were employed to determine any significant changes in densitometric values and GTPase activities. A significant difference was taken for P values < 0.05.

Results

Cell culture. Relatively pure (> 95%) bovine aortic endothelial cell cultures were confirmed by their morphological features (i.e., cuboidal, cobble-stone, contact inhibited) using phase-contrast microscopy and by immunofluorescent staining with Factor VIII antibodies. There were no observable adverse effects of LDL on cellular morphology, cellular number, immunofluorescent staining, and Trypan blue exclusion (> 95%) for all treatment conditions.

Characterization of lipoproteins. SDS-PAGE of native or unmodified LDL revealed a single band ($\sim$ 510 kD) corresponding to ApoB100 protein which became degraded upon oxidative modification (data not shown). Similarly, cellulose acetate electrophoresis revealed only one band corresponding to the presence of a single class of low-density lipids. In contrast, lipoprotein-deficient serum was devoid of both ApoB100 protein and low-density lipid bands, and had non-detectable levels of cholesterol. The LDL had a protein, cholesterol and triglyceride concentration of 5.75±0.14 mg/ml, 21.3±1.1 mg/ml, and 1.8±0.1 mg/ml, respectively. There was no detectable level of
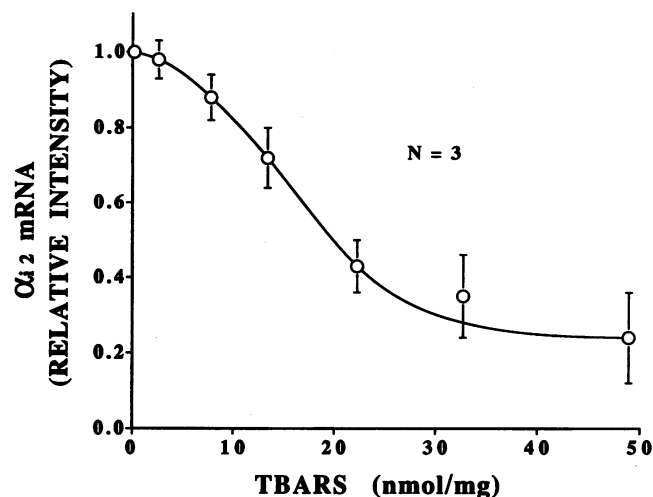

Figure 2. The effects of increasing degrees of LDL (50 μg/ml) oxidation as expressed in TBARS (nmol MDA/mg of LDL protein) on steady-state α_{i2} mRNA levels after 72 h. The band intensities of α_{i2} mRNA were normalized to the band intensities of corresponding β-tubulin mRNA (Relative Intensity).

endotoxin (< 0.10 EU/ml) in the lipoprotein-deficient serum or oxidized LDL samples. Native LDL had a TBARS value of 0.4±0.2 nmol/mg, but after exposure to bovine aortic endothelial cells in LPDS/DME media for 72 h, this value increased to 4.2±0.6 nmol/mg. Copper-oxidized LDL had TBARS values ranging from 2.6±0.6 to 48.9±5.6 nmol/mg. Unless specified otherwise, the TBARS value of oxidized LDL used in this study is 23.4±2.6 nmol/mg.

Effect of oxidized LDL on α_{i2} and α_{i3} mRNA expression. Under stringent hybridization and washing conditions, α_{i2} and α_{i3} subunit probes detected major bands on Northern blotting corresponding to 2.3 and 3.5 kb size mRNA messages, respectively. The α_{i2} subunit probe also identified a minor band at 3.0 kb probably representing alternative splicing of α_{i2} mRNA. Little or no bands were detected using cDNA probes for α_{io} and α_{i1}. Neither pretreatment with LPDS for 48 h nor treatment with native LDL or conditioned medium from endothelial-modified LDL for 72 h significantly affect the mRNA expression of α_{i2} and α_{i3} (data not shown). In contrast, copper-oxidized LDL (TBARS value 23.4±2.6 nmol/mg) produced a concentration-dependent decrease in the steady-state mRNA levels of α_{i2}, but not α_{i3} (Fig. 1, A and B). After 72 h of treatment, there was a 3.2-fold reduction in α_{i2} mRNA with respect to β-tubulin mRNA. The calculated IC$_{50}$ value for oxidized LDL was 18.7±2.1 μg/ml. Additional studies using oxidized LDL preparations with comparable TBARS values from a different donor yielded similar results (data not shown). In addition, decreases in α_{i2} mRNA was dependent upon the degree of LDL oxidation (Fig. 2). At a LDL concentration of 50 μg/ml, the calculated TBARS IC$_{50}$ value is approximately 19.6±2.2 nmol/mg.

Similarly, oxidized LDL (50 μg/ml) also caused a time-dependent decrease in steady-state α_{i2} mRNA levels resulting in a 46% decrease after 24 h (Fig. 3 A). The half-life of α_{i2} mRNA was determined in the presence of actinomycin D (5 μg/ml). Assuming first-order kinetics, oxidized LDL (50 μg/ml) shortened the half-life of α_{i2} mRNA from 16±3 h to 8±2 h (Fig. 3 B). Pretreatment with cycloheximide (10 μg/ml),

A ox-LDL ACT CHX

+	−	−
−	+	−
+	+	−
−	−	+
+	−	+

0 6° 12° 18° 24°

B

α_{i2} mRNA (RELATIVE INTENSITY)

1.0 — 0.5 — 0.2 — 0.1

N = 3

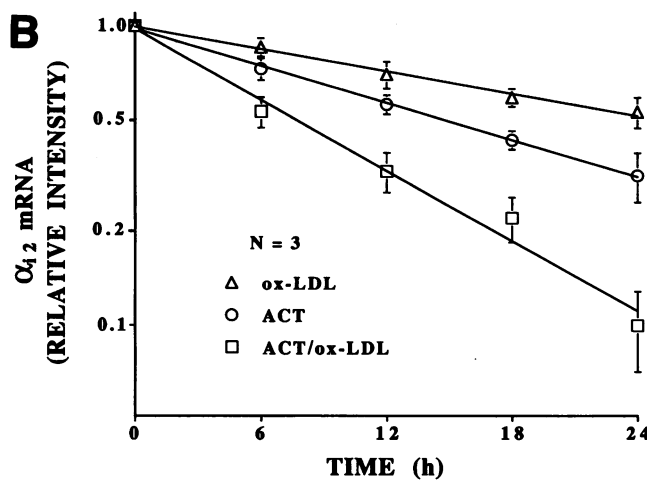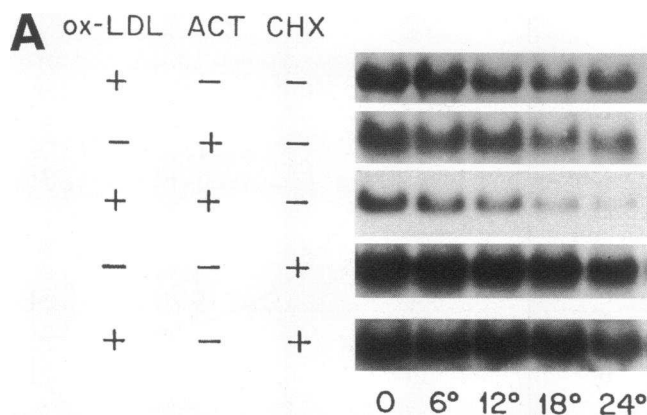△ ox-LDL
○ ACT
□ ACT/ox-LDL

0 6 12 18 24

TIME (h)

Figure 3. (A) Northern blots (20 μg total RNA/lane) showing the time-course of α_{i2} mRNA expression in presence of oxidized (ox)-LDL (50 μg/ml), actinomycin D (ACT, 5 μg/ml), cycloheximide (CHX, 10 μg/ml), alone or in combination. (B) Effects of actinomycin D and/or oxidized LDL on α_{i2} mRNA levels. Band intensities were analyzed by densitometry, normalized to β-tubulin mRNA, and plotted logarithmically as a function of time (relative intensity).

pGEM—
NOS—
β-ACTIN—
β-TUBULIN—
α_{i2}—
α_{i3}—

0   12
Time (h)

Figure 4. A representative experiment of nuclear run-off assay blots showing the effects of oxidized LDL (50 μg/ml) on transcriptional activity of α_{i2}, α_{i3}, and β-tubulin, β-actin, constitutive nitric oxide synthase (NOS) gene. Band intensities of α_{i2} and α_{i3} were normalized to β-tubulin at the indicated time points for three separate experiments. Specificity was determined by lack of hybridization to the vector cDNA, pGEM-3z (pGEM).

however, attenuated the decline in α_{i2} steady-state mRNA in the presence of oxidized LDL and prolonged its half-life to 21±3 h. Preliminary studies with cycloheximide under these conditions showed that it inhibited [^{35}S]methionine incorporation by > 95%. Since treatment with cycloheximide alone produced minimal changes in α_{i2} mRNA, these findings suggest that translational induction of protein(s) by oxidized LDL is required for α_{i2} mRNA destabilization.

Effects of oxidized LDL on α_{i2} and α_{i3} transcription. Nuclear run-off studies demonstrate that oxidized LDL (50 μg/ml) did not cause any significant change in transcriptional activity of α_{i2} and α_{i3} gene with respect to β-tubulin or β-actin gene (Fig. 4). Oxidized LDL, however, did produce a 2.8-fold increase in transcriptional activity of the constitutive NO synthase gene. Preliminary studies using different amounts of radiolabelled RNA transcripts demonstrate that under our experimental conditions, hybridization was linear and nonsaturable. The specificity of each band was determined by the lack of hybridization to the insertless pGEM-3z vector cDNA. Nuclear run-off studies were performed three times and band intensities were then analyzed by densitometry.
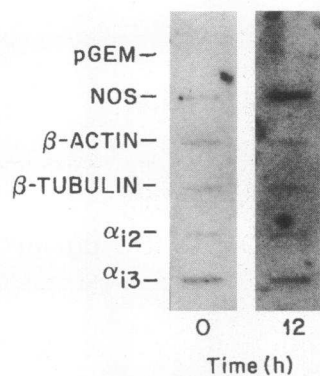
Effects of oxidized LDL on α_{i2} and α_{i3} protein expression. We have previously reported that native LDL does not alter the protein levels of α_{i2} and α_{i3} (9). Incubation of endothelial cells with LPDS for 72 h did not results in any significant changes in α_{i2} and α_{i3}. Treatment with increasing concentrations of oxidized LDL for 72 h, however, caused a significant decrease in the steady-state protein levels of α_{i2} as determined by Western blotting using the P4 antisera (Fig. 5 A). However, the protein level of α_{i3} as determined by the JL14 antisera was unaffected by oxidized LDL (Fig. 5 B). At an oxidized LDL concentration of 50 μg/ml, there was a 3.5-fold reduction in α_{i2} protein. The calculated IC$_{50}$ value for oxidized LDL was 10.1±2.3 μg/ml.

Pulse-chase biosynthetic labelling studies with [^{35}S]-methionine showed that α_{i2} has a protein half-life of 27 ±3 h (Figure 6A and 6B). Treatment with native LDL (100 μg/ml) did not significantly affect the degradation of α_{i2} (half-life 24±3 h). However, treatment with oxidized LDL (50 μg/ml) caused an increase degradation of α_{i2} as evidenced by a reduction in protein half-life to 14±2 h. Subsequent Western blots of the supernatant did not reveal any detectable α_{i2} indicating that all of the radiolabelled α_{i2} had been immunoprecipitated by the P4 antisera. The calculated half-life of α_{i2} assumes first-order kinetics although higher order kinetics may actually be involved in the degradation process (Fig. 6 B).

The P4 and JL14 antisera were quite specific since recognition of their respective α_i subunits could be blocked only in the presence of excess decapeptides from which they were derived (9, 34). Corresponding studies using commercially-available antisera (New England Nuclear, Dupont) to α_{i2} (AS/7) and α_{i3} (EC/2) yielded similar results and additional studies using antisera to α_s (RM/1) and common β (SW/1) did not show any effect of oxidized LDL on α_s and β subunits protein expression.

Effect of oxidized LDL on α_{i2}-stimulated GTPase activity. We have previously shown that the α_2-adrenergic receptor is specifically coupled to α_{i2}, and that specific α_{i2} GTP hydrolysis represented 94% of UK14304 (α_2-adrenergic receptor agonist)-stimulated GTPase activity (21). Treatment with native LDL (100 μg/ml) and oxidized LDL (50 μg/ml) reduced basal GTPase activity from 4.8±0.3 pmol/min/mg to 3.8±0.5 pmol/min/mg and 3.2±0.4 pmol/min per mg, respectively (P < 0.05). There was a small, but significant reduction in basal (4.2±0.3 pmol/min per mg, P < 0.05) and UK14304-stimulated GTPase activity (8.2±0.8 pmol/min per mg, P < 0.05) of endothelial cells grown in 10% fetal-calf serum compared with LPDS. Stimulation of untreated endothelial cell membranes by UK14304 produced a agonist-stimulated GTPase ac-
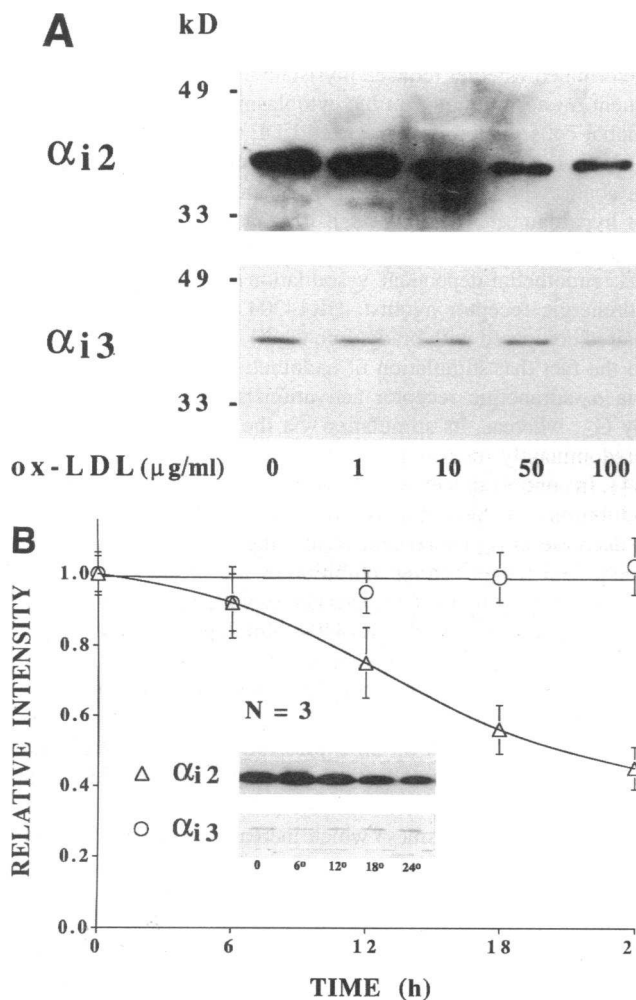
Figure 5. (A) Western blot (30 μg/lane) showing the effects of increasing concentrations of oxidized LDL on α_{i2} and α_{i3} at 72 h. This is representative of three separate experiments. (B) The effects of oxidized LDL (50 μg/ml) on α_{i2} and α_{i3} protein level as a function of time (h). Band intensities from Western blots were analyzed by densitometry and normalized to time zero (Relative Intensity). A representative Western blot is shown in the inset.

Figure 6. (A) SDS/PAGE radioblots of [³⁵S]methionine pulse-chase studies showing disappearance of immunoprecipitated [³⁵S]α_{i2} in the presence and absence (Control) of oxidized LDL (50 μg/ml, TBARS 23.4 nmol/mg). (B) Loss of [³⁵S]α_{i2} (Relative Intensity) as a logarithmic function of time (h) during radiolabelled chase. Each set of experiment was performed three times.

tivity of 10.2±1.3 pmol/min per mg. Treatment with native and oxidized LDL (0–100 μg/ml) for 72 h caused a concentration-dependent decrease in UK14304-stimulated GTP hydrolysis (Fig. 7). Maximum reduction in UK14304-stimulated GTPase hydrolysis was achieved with 50 μg/ml of oxidized LDL (1.8±0.4 pmol/min per mg) as compared with 100 μg/ml of native LDL (3.2±0.5 pmol/min/mg). The calculated IC_{50} values for native and oxidized LDL were 36±5 μg/ml and 12±3 μg/ml, respectively. Specific COOH-terminal antisera to α_s (RM/1) had minimal effect on UK14304-stimulated GTPase activity in both native and oxidized LDL-treated endothelial cell membranes.

Discussion

We have shown that oxidized LDL can regulate the expression of G protein α_{i2} subunit. Our findings indicate tha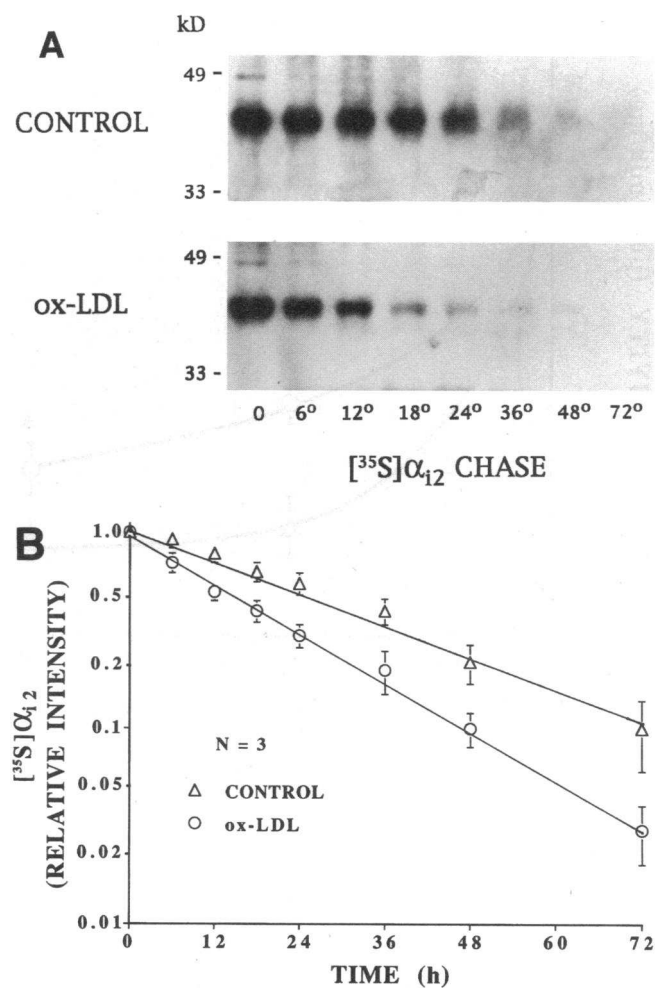t oxidized LDL decreases the steady-state expression of α_{i2} primarily by destabilizing α_{i2} mRNA and protein. This decrease in α_{i2} expression correlated functionally with receptor–G_{i2} uncoupling as evidenced by the marked reduction in agonist-stimulated α_{i2} GTPase activity. The effects of oxidized LDL appear to be posttranscriptional since results from actinomycin D studies demonstrate destabilization of α_{i2} mRNA. This is consistent with our nuclear run-off studies showing little, if any effect of oxidized LDL on α_{i2} transcription. This destabilizing effect of oxidized LDL on α_{i2} mRNA requires the synthesis of new protein(s) as indicated by the studies using the protein synthesis inhibitor, cycloheximide. Furthermore, because of the long half-life of α_{i2} protein as demonstrated by [³⁵S]methionine biosynthetic labeling studies, the decrease in α_{i2} steady-state mRNA does not fully account for the relatively rapid decline in α_{i2} protein. Thus, oxidized LDL must have additional effect(s) on reducing α_{i2} protein levels. It remains to be determined whether α_{i2} degradation also involves the translational induction of other protein(s) and whether this process actually occurs by first-order kinetics.

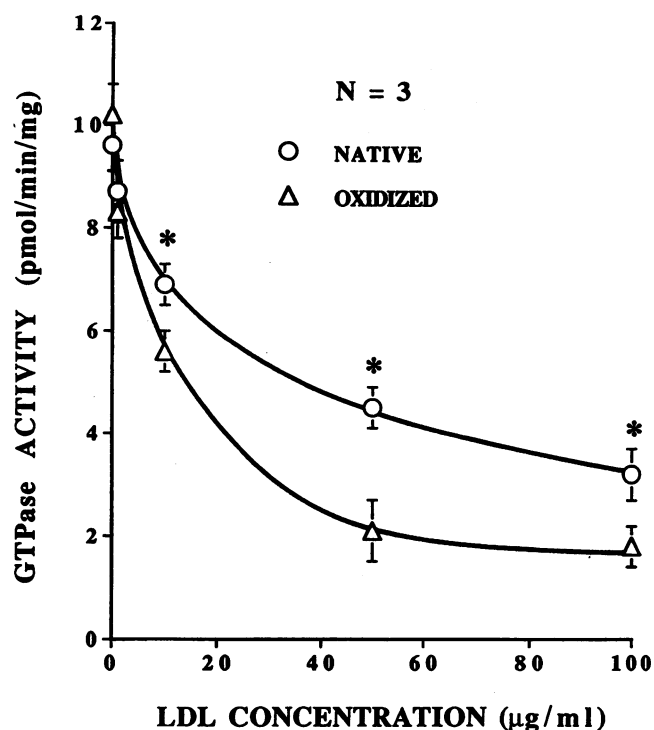Oxidative modification of the LDL particle include lipid

Figure 7. The effects of increasing concentrations of native and oxidized LDL on UK14304 (10 nM)-stimulated GTPase activity in endothelial cell membranes (30 μg). * A significant difference between the effects of native and oxidized LDL ($P < 0.05$).

peroxidation, formation of conjugated dienes, carbonyl modification of apoB-100, and conversion of phosphatidylcholine to lysophosphatidylcholine (12, 24). It is not known which component(s) of oxidized LDL may be responsible for altering α_{i2} expression, although we have found that the degree of LDL oxidation correlate with its inhibitory effect on α_{i2} expression. Oxidation of the LDL particle allows quicker uptake by the endothelial cell scavenger receptor compared with the relatively slower uptake of native LDL by the LDL receptor (25). This may result in a greater access and accumulation of the oxidized LDL particle into endothelial subcellular compartments where it may exert its inhibitory effects. Lysophosphatidylcholine which is present in oxidized LDL has been shown to attenuate bradykinin-stimulated calcium influx and inhibit endothelium-dependent relaxations (26, 27). It is not known whether lyso-phosphatidylcholine does this by altering the function and/or expression of G_i-proteins. In any case, preventing oxidative modification of LDL or minimizing its oxidative effects in the presence of reducing agents such as ascorbic acid, β-carotene, or α-tocopherol has been shown to improve receptor-mediated endothelium-dependent relaxation of vessels from hypercholesterolemic rabbits (28–30).

Several possible mechanisms could account for the observed decrease in α_{i2} protein levels in endothelial cells treated with oxidized LDL. Since α_{i2} is associated with the plasma membrane, any changes in the physical dynamics of the membrane caused by cholesterol or phospholipids could potentially dissociate α_{i2} from the membrane thereby rendering it more susceptible to degradation (31). Furthermore, proper function and attachment of α_{i2} to the plasma membrane requires NH_2 terminal

myristoylation, a process which may be altered by the effects of oxidized, but not native LDL (32, 33). It remains to be determined whether reduced myristate incorporation and subsequent α_{i2} accumulation in the cytoplasm are observed in endothelial cells treated with oxidized LDL.

Previous studies have shown that endothelial dysfunction or abnormal endothelial-dependent responses of atherosclerotic or hypercholesterolemic vessels correlate with their sensitivity to pertussis toxin (3). In atherosclerotic porcine coronary arteries, endothelial-dependent vasodilation in response to the α_2-adrenergic receptor agonist, UK14304, is more severely impaired compared with bradykinin (7, 8). This may be attributed to the fact that stimulation of endothelial nitric oxide synthase via α_2-adrenergic receptor activation is specifically mediated by G_{i2}; whereas, its stimulation via the bradykinin receptor is predominantly mediated by pertussis toxin-insensitive G_q (21, 34). In contrast to native LDL which produces only a functional inhibition of G_i probably as a result of membrane perturbation, a decrease in α_{i2} expression would likely produce a more profound and longer-lasting inhibition of receptor-mediated pathways which utilize G_{i2} (9). This agrees with our findings regarding further decrease in UK14304-stimulated GTPase activity and is consistent with recent reports showing that oxidized LDL produces a greater inhibition of endothelial-dependent vasorelaxation than native LDL (14, 15, 35).

Finally, inhibition of G_{i2} expression by oxidized LDL may affect other signal transduction pathways. For example, minimally modified LDL activates the DNA binding protein NF-κB through mechanism(s) which increases intracellular cAMP levels (i.e., cholera toxin, pertussis toxin) (36). Since α_{i2} can decrease the activity of adenylyl cyclase, oxidized LDL may indirectly activate NF-κB by downregulating the expression and function of α_{i2}. The loss of α_{i2} may also account for the impaired flow-mediated vasodilation frequently observed in vessels from hypercholesterolemic and atherosclerotic humans (37, 38). Recent studies indicate that mechanical shear-stress is coupled to G_i-proteins and potassium channels in releasing endothelial-derived nitric oxide (39, 40). These signal transducing elements mediate increases in intracellular cGMP which is blocked by pertussis toxin. Thus, the findings in this study suggest that decreases in α_{i2} expression caused by oxidized LDL may not only inhibit the response of endothelial cells to α_{i2}-coupled receptor activation, but also to other non-receptor pathways mediated by G_{i2}.

Acknowledgment

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