Activation of NADPH Oxidase Is Required for Macrophage-Mediated Oxidation of Low-Density Lipoprotein

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Low-density lipoprotein (LDL) oxidation by arterial wall cells, a key event during early atherogenesis, was suggested to involve the activation of 15-lipoxygenase and/or nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. We sought to analyze the role of these oxygenases in macrophage-mediated oxidation of LDL under oxidative stress. Upon incubation of LDL with the J-774 A.1 macrophage-like cell line or with human monocyte-derived macrophages (HMDM) in the presence of 1 μmol/L $CuSO_4$, the release of superoxide anions to the medium was demonstrated. Under these conditions, the cytosolic protein components of the NADPH oxidase complex, P-47 and P-67, translocated to the plasma membrane, indicating LDL-mediated activation of the NADPH oxidase complex. Under the above-mentioned experimental conditions, the macrophage 15-lipoxygenase was also activated, as determined by the release of 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE) and 13-hydroxyoctadecadienoic acid (13-HODE) to the medium. Inhibition of the macrophage NADPH oxidase with apocynin or dismutation of superoxide anions, the product of NADPH oxidase activation, with superoxide dismutase (SOD) significantly inhibited macrophage-mediated oxidation of LDL (by 61% to 89%) under these conditions. Phorbol myristate acetate (PMA), which causes NADPH oxidase activation in J-774 A.1 macrophages, had no significant effect on 15-lipoxygenase activity, but still resulted in cell-mediated oxidation of LDL. Finally, HMDM from two patients with chronic granulomatous disease (CGD) that were shown to lack active NADPH oxidase, but to possess almost normal 15-lipoxygenase activity failed to oxidize LDL. We thus conclude that LDL-induced NADPH oxidase activation (under oxidative stress) is required for macrophage-mediated oxidation of LDL, whereas activation of 15-lipoxygenase may not be sufficient for LDL oxidation under these conditions.

LOW-DENSITY LIPOPROTEIN (LDL) oxidation by cells of the arterial wall has been suggested to be a key event during early atherosclerosis.[1-5]

Arterial wall macrophages were shown to accumulate oxidized lipids in areas of the atherosclerotic lesion,[6] and these cells were shown to be capable of oxidizing LDL.[7-9] The presence of transition metal ions is required for macrophage-mediated oxidation of LDL in vitro, and in most studies transition metal ions are either present or supplemented to the medium.[7-9] Of interest is that copper and iron ions were found in mature atherosclerotic lesions.[10] Both 15-lipoxygenase, which introduces oxygen to the polyunsaturated fatty acid molecule,[11] and NADPH oxidase, which produces superoxide anion radicals, were shown to be involved in macrophage-mediated oxidation of LDL.[12-15] In two recent studies,[16,17] a superoxide-independent mechanism for cell-mediated oxidation of LDL was also shown. In another study,[18] NADPH oxidase was suggested to be nonessential for macrophage-mediated oxidation of LDL, since monocyte-derived macrophages (MDM) from four chronic granulomatous disease (CGD) patients were able to oxidize LDL as suggested by increased lipoprotein uptake by mouse macrophages. Macrophages can generate a significant amount of superoxide anion radicals, secondary to the activation of their NADPH oxidase. This enzyme is a multicomponent electron transport complex that includes a membrane-bound b-type cytochrome (flavocytochrome b_{558}) and three cytosolic proteins that have been characterized as 47-kd (P-47), 67-kd (P-67), and *ras*-related GTP-binding proteins. Upon activation, these cytosolic factors translocate to the plasma membrane and form the assembled activated enzyme.[19,20] Although charged superoxide anion cannot oxidize lipids directly, it can inactivate antioxidants such as catalase or α-tocopherol,[21,22] and thus it can permit a subsequent lipid peroxidation. Recently, superoxide anions were shown to be involved in cell-mediated oxidation of LDL, which was initiated by thiols, in the presence of copper ions.[17,23]

Superoxide can also be converted by protonation (in the presence of transition metal ions) into reactive oxygen species (ROS) such as perhydroxyl radicals that penetrate into membrane lipid regions, where they can initiate lipid peroxidation.[24,25]

The enzyme, 15-lipoxygenase, in monocytes and macrophages was also suggested[14,15] to be involved in cell-mediated oxidation of LDL, but in some of these studies lipoxygenase inhibitors were used at concentrations much higher than required for complete inhibition of cellular lipoxygenase.[26] Recently, we demonstrated that binding of LDL to the LDL receptor under oxidative stress (in the presence of copper ions) is required for macrophage-mediated oxidation of the lipoprotein.[27] Under these experimental conditions, we questioned the involvement of macrophage 15-lipoxygenase and NADPH oxidase in cell-mediated oxidation of LDL. For this purpose, we have used both the J-774 A.1 macrophage-like cell line and human monocyte-derived macrophages (HMDM) from normal subjects, as well as from patients who are lacking active NADPH oxidase. The present study shows for the first time

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that LDL activates both macrophage 15-lipoxygenase and NADPH oxidase. However, our results suggest that activation of NADPH oxidase but not of 15-lipoxygenase was sufficient for macrophage-mediated oxidation of the lipoprotein.

SUBJECTS AND METHODS

Materials

Ficoll-Paque was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Dulbecco's modified Eagle's medium (DMEM), Ham's F-10 medium, fetal calf serum, penicillin, streptomycin, and glutamine were obtained from Gibco Laboratories (Grand Island, NY). Arachidonic acid, linoleic acid, phenidone, apocynin, oxypurinol, indomethacin, butylated hydroxytoluene (BHT), phorbol myristate acetate (PMA), mannitol, cytochrome C, and catalase were obtained from Sigma (St Louis, MO). Cu/Zn-superoxide dismutase (SOD) was a generous gift from General Biotechnology (Rehovot, Israel). [^{14}C]-arachidonic acid (55 mCi/mmol) and [^{14}C]-linoleic acid (50 to 60 mCi/mmol) were obtained from Amersham (Buckinghamshire, UK). 2,2′-Azobis (2-amidinopropane) hydrochloride was obtained from Polysciences (Warington, PA).

Patients

MDM from two patients with proven CGD[28,29] were studied.

One patient is a 15-year-old boy who suffered his first episode of liver abscess at the age of 2.5 years. During the next 4 years, he had several more episodes including lung abscess and was diagnosed as suffering from CGD. In 1991, he had another episode of lung abscess due to *Staphylococcus aureus*. He was referred to the pediatric immunology clinic at the Rambam Medical Center, where the diagnosis was confirmed by both the Nitro Blue Tetrazolium (NBT) reduction test and the chemiluminescence (CL) assay.[28] Since 1991, he has been treated with prophylactic resprim and interferon alfa. Although his neutrophil function test did not change, he did not have any subsequent infections. NBT and CL tests in the father were completely normal. Since no other siblings exist and the mother was not tested, the mode of inheritance in this case could not be determined.

The other patient is an 8-year-old boy who at the age of 1 year suffered from recurrent suppurative lymphadenitis mainly in the cervical area. He was referred to us at the age of 3 years because of a lung abscess. He was diagnosed with CGD by the NBT reduction test and CL assay. His mother, aunt and one sister had intermediate levels of NBT reduction, and thus the diagnosis of X-linked CGD was made. During the following years, he had several episodes of lymphadenitis and pneumonia, together with recurrent skin rash. Currently, he is on interferon alfa, and a marked improvement in his condition was observed with no change in CL and NBT results.

Human Monocytes

Human monocytes were isolated by density gradient centrifugation[30] of blood derived from fasting normolipidemic subjects. Twenty milliliters of blood (anticoagulated with heparin 10 U/mL) was layered over 15 mL Ficoll-Paque and centrifuged at 500 xg for 30 minutes at 23°C. The mixed mononuclear cell band was removed by aspiration, and the cells were washed twice in DMEM containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamine. The cells were plated at 2×10^6 monocytes/35-mm dish (Primaria Falcon Labware; Becton Dickinson, Oxnard, CA) in the same medium and further incubated in the presence of 20% autologous serum. HMDM were used within 7 to 10 days of plating. After 7 days in culture, the cell population consisted of over 98% pure macrophages as analyzed by alpha naphthyl esterase staining in the absence or presence of sodium fluoride (which blocks the staining of macrophages).

J-774 A.1 Murine Macrophage-Like Cell Line

The J-774 A.1 cell line was purchased from the American Type Culture Collection (Rockville, MD). J-774 A.1 macrophages were plated at 1×10^6 cells/35-mm dish in DMEM supplemented with 10% fetal calf serum. The cells were fed every 3 days and used for experiments within 7 days of plating.[31]

Lipoproteins

LDL was prepared from human plasma derived from fasted normolipidemic volunteers. LDL was prepared by discontinuous density gradient ultracentrifugation as described previously.[32] The lipoprotein was washed at a density of 1.063 g/mL and dialyzed against 150 mmol/L NaCl and 1 mmol/L EDTA (pH 7.4) at 4°C. LDL was then sterilized by filtration (0.45 μm), kept at 3 to 6 mg protein/mL under nitrogen in the dark at 4°C, and used within 2 weeks. Two days before the oxidation studies, LDL was dialyzed against 150 mmol/L NaCl, EDTA-free solution (pH 7.4) under nitrogen at 4°C. The lipoprotein was found to be free of lipopolysaccharide contamination as analyzed by the Limutus Amebocyte Lysate assay (Associates of Cape Cod, Woods Hole, MA).

LDL Oxidation by Macrophages

Cells (1×10^6/35-mm dish) were incubated with LDL (0.2 mg protein/mL) in DMEM supplemented with 1 μmol/L $CuSO_4$ for 18 hours at 37°C. This incubation system was used because Ham's F-10 (which contains ~0.01 μmol/L $CuSO_4$ and 2.5 μmol/L $FeSO_4$), commonly used in LDL oxidation studies, was cytotoxic to macrophages. LDL oxidation by J-774 A.1 macrophages in Ham's F-10 medium resulted in a 15% to 25% increase of cell-mediated LDL oxidation in comparison to LDL oxidation in DMEM + 1 μmol/L $CuSO_4$. However, using Ham's F-10 medium, 20% to 33% of the cells died, whereas no cell cytotoxicity could be found when using DMEM + 1 μmol/L $CuSO_4$. Oxidation was terminated by addition of 1 mmol/L EDTA and refrigeration at 4°C. The extent of LDL oxidation was measured directly in the medium by the thiobarbituric acid–reactive substances (TBARS) assay, in which the standard curve was shown to involve malondialdehyde (MDA) generation.[33] In addition, LDL oxidation was determined by a lipid peroxidation test that analyzed lipid peroxides by their capacity to convert iodide to iodine (which can then be measured photometrically at 365 nm),[34] and also by assay of conjugated dienes in the LDL lipid extracts at 234 nm.[35] Control LDL was always incubated under the same conditions (1 μmol/L $CuSO_4$) in the absence of cells.

Analysis of Macrophage Lipoxygenase Activity

J-774 A.1 macrophages (1×10^6 cells/35-mm dish) were incubated for 15 minutes at 37°C with 5 μmol/L [^{14}C]-labeled arachidonic acid or 5 μmol/L [^{14}C]-labeled linoleic acid (in ethanol) followed by cell wash and addition of LDL (0.2 mg protein/mL) in the presence of 1 μmol/L $CuSO_4$ for 1 hour at 37°C. At the end of the incubation, 25 μmol/L of the antioxidant, BHT, were added to the medium to block any further lipid peroxidation. The medium content of [^{14}C]-labeled 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE) and 13-hydroxyoctadecadienoic acid (13-HODE) was then determined. Thin-layer chromatography (TLC) separation of oxygenated lipids in macrophage-conditioned medium (MCM) was performed using MCM (1 mL) acidified to pH 3.0 with 20 μL

2-mol/L citric acid.[26,36] MCM lipids were extracted with chloroform: methanol (1:2 vol/vol) followed by addition of 0.6 mL chloroform. This is a modification of the method described by Bligh and Dyer.[37] All MCM lipids were extracted from the medium by this method, since all the radioactivity was found in the chloroform phase and the methanolic phase contained no radioactivity ([^{14}C]-15-HETE or [^{14}C]-13-HODE). The chloroform phase was evaporated under nitrogen and redissolved in a minimal volume of chloroform. TLC was performed on silica gel plates (F_{254}, 0.25 mm; Merck, Darmstadt, Germany). The solvent system used for fatty acid analyses consisted of petroleum ether: diethyl ether: acetic acid (50:50:1 vol/vol/vol). At the end of the analysis, the plates were exposed to iodine vapors, 15-HETE, and 13-HODE, identified (using the appropriate standards), scraped, and analyzed for radioactivity in [^{14}C]-15-HETE (in the study with [^{14}C]-arachidonic acid) or [^{14}C]-13-HODE (in the study with [^{14}C]-linoleic acid). In addition to 15-lipoxygenase, macrophages contain 5-lipoxygenase.[38] However, on TLC analysis, the products of 5-lipoxygenase action on fatty acids migrated much lower than those of 15-lipoxygenase.

Analysis of Macrophage NADPH Oxidase Activity

The production of superoxide anion (O_2^-) by intact cells was measured by the SOD-inhibitable reduction of acetyl ferricytochrome C.[39] Hanks balanced Salt solution ([HBSS] 100 μL) containing 150 μmol/L acetyl ferricytochrome C in the presence or absence of 60 μg/mL SOD was added to intact cells (2×10^5 well). The production of superoxides by adherent cells (for NADPH oxidase activity) or by cells in suspension (for the translocation assay) was similar. Cells were stimulated with LDL + $CuSO_4$ or with PMA, and the reduction of acetyl ferricytochrome C was kinetically monitored by analysis of the change in absorbance at 550 nm using a Microplate Reader (MR 600; Dynatech Laboratories, Chantilly, VA). The maximal rate of superoxide generation was determined using an extinction coefficient (E_{550}) of 21 $mmol/L^{-1} \cdot cm^{-1}$.[39]

Analysis of P-47 and P-67 Translocation

Membrane and cytosol fractions from HMDM were prepared as described previously.[40] Monocytes were treated with 5 mmol/L diisopropyl fluorophosphate for 1 hour at 18°C, washed and resuspended at 10^8 cells/mL in relaxation buffer (100 mmol/L KCl, 3 mmol/L NaCl, 3.5 mmol/L $MgCl_2$, 1.25 mmol/L EGTA, 1 mmol/L ATP, and 10 mmol/L HEPES, pH 7.4) containing 1 mmol/L phenylmethylsulfonyl fluoride and 100 mmol/L leupeptin (at 4°C), and sonicated three times for 10 seconds (which resulted in ~95% cell breakage). After centrifugation (5 minutes at 15,000 × *g*) to remove granules, nuclei, and unbroken cells, the supernatant was centrifuged in a Beckman (Irvine, CA) airfuge (30 minutes at 134,000 × *g*) to obtain a cell membrane pellet and a cytosol supernatant. Membranes were suspended at 10^9 cell equivalents/mL in 0.34 mol/L sucrose in half-strength relaxation buffer containing 1 mmol/L dithiothreitol. Immunoblot detection of cytosolic NADPH oxidase components was performed as previously described.[40] Samples were solubilized in sample buffer (12% sodium dodecyl sulfate, 8 mol/L urea, 250 mmol/L Tris, 8 mmol/L EDTA, 0.2 mmol/L leupeptin, and 2 mmol/L phenylmethylsulfonyl fluoride, pH 6.9). The amount of protein in each sample was quantified with the Pierce protein assay (Pierce Chemical, Rockford, IL) using bovine serum albumin standards. Cytosols or membranes (10 μg protein) were analyzed by electrophoresis on 10% polyacrylamide gels. The resolved proteins were electrophoretically transferred to nitrocellulose stained with fast green to detect protein banding, and then blocked in 5% nonfat dry milk in Tris-buffered saline (TBS). The blots were incubated in TBS/1% gelatin containing goat antiserum to either human recombinant P-47 or P-67 (a gift from Dr T.L. Leto). Since the antibodies were raised against human recombinant P-47 and P-67 and have low cross-reactivity with other species, translocation of the cytosolic factors to the membranes during activation of NADPH oxidase was analyzed in HMDM cells.

Immunoblots were incubated with 1 μg/mL peroxidase-conjugated rabbit antigoat IgG (Biomakor, Rehovot, Israel) and developed with 4-chloro-1-naphthol and H_2O_2. The relative levels of each oxidase component were measured using densitometry in a reflectance mode (Hoefer Scientific Instruments, San Francisco, CA). These measurements are adequate to determine the changes of each individual NADPH oxidase component under different conditions, but not to compare changes between the various components. Detection of P-47 and P-67 was linear in the range of 0.25 to 30 μg membrane protein, which corresponds to 10^5 to 1.2×10^7 cell equivalents.[40]

Statistical Analysis

Statistical analysis was performed using either the nonpaired Student's *t* test or ANOVA (for comparing more than two conditions). Results are presented as the mean ± SD.

RESULTS

Oxidants and Oxygenases Involved in Macrophage-Mediated Oxidation of LDL: Inhibitor Studies

J-774 A.1 macrophage-like cells (1×10^6/35-mm dish) were incubated with LDL (0.2 mg protein/mL) for 18 hours at 37°C in medium containing 1 μmol/L $CuSO_4$ in the absence (control) or presence of antioxidants (Fig 1). Preliminary studies using increased concentrations of the various antioxidants were performed to determine the inhibitor concentration required for inhibition of over 80% of macrophage-mediated oxidation of LDL. Using these concentrations, both SOD (Cu/Zn-SOD 5 μg/mL) and catalase (5 μg/mL), which inactivate superoxides and hydrogen peroxides, respectively, inhibited cell-mediated oxidation of LDL by 89% and 97% (analyzed by the TBARS assay and expressed as nanomoles of TBARS per milligram LDL protein), whereas mannitol (25 μmol/L), the hydroxyl radical scavenger,[41] had no effect on cell-mediated oxidation of LDL (Fig 1A). Similarly, heat inactivation of the enzymes SOD or catalase (100°C for 30 minutes) completely blocked the inhibitory effects of these antioxidants on macrophage-mediated oxidation of LDL (data not shown). BHT (25 μmol/L), the general free-radical scavenger, completely inhibited macrophage-mediated oxidation of LDL (Fig 1A).

NADPH oxidase activity in the presence of these antioxidants was measured by the release of superoxide anions from the cells to the medium (Fig 1B). In the presence of SOD or BHT, the amount of superoxides released was decreased by 80% and 89%, respectively, in comparison to control cells (Fig 1B). However, catalase and mannitol had no effect on superoxide anion release from the cells (Fig 1B). All of these antioxidants had no effect on 15-lipoxygenase activity as measured by the release of [^{14}C]-15-HETE from the cells to the medium (Fig 1C).

We next analyzed the involvement of macrophage oxygen-

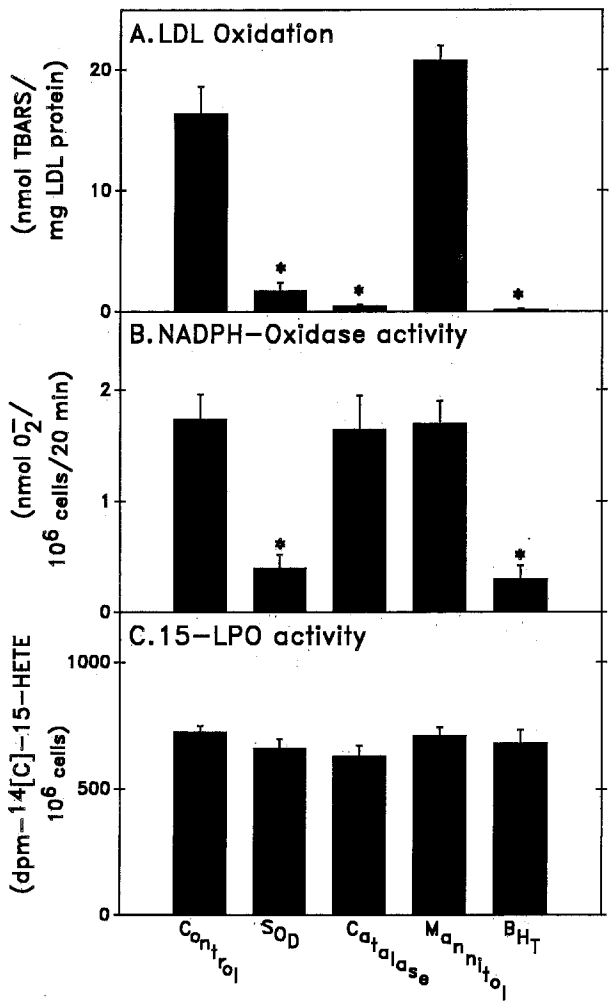

Fig 1. Effect of antioxidants on macrophage-mediated oxidation of LDL and on NADPH oxidase and 15-lipoxygenase (15-LPO) activities. (A) J-774 A.1 macrophages (1 × 10^6/35-mm dish) were incubated for 30 minutes at 37°C with no additions (control) or with several antioxidants before addition of LDL (0.2 mg protein/mL) and 1 μmol/L $CuSO_4$ and a further cell incubation for 18 hours at 37°C. The antioxidants include 5 μg/mL Cu/Zn-SOD, 5 μg/mL catalase, 25 mmol/L mannitol, or 25 μmol/L BHT. (B) J-774 A.1 cells (2 × 10^5/100-μL well) were incubated for 20 minutes with LDL (0.2 mg protein/mL) and 1 μmol/L $CuSO_4$ in the absence (control) or presence of the above antioxidants. At the end of the incubation, the amount of superoxide anions released to the medium was measured. (C) J-774 A.1 cells (1 × 10^6/35-mm dish) were incubated with 5 μmol/L [^{14}C]-arachidonic acid for 15 minutes. The cells were washed and further incubated for 1 hour with LDL (0.2 mg protein/mL) and 1 μmol/L $CuSO_4$ in the absence (control) or presence of the different antioxidants. At the end of the incubation, the amount of [^{14}C]-15-HETE released to the medium was measured. Results are the mean ± SD (n = 3). *P < .01 v control.

ases in cell-mediated oxidation of LDL. J-774 A.1 macrophages (1 × 10^6/35-mm dish) were incubated with LDL (0.2 mg protein/mL) for 18 hours at 37°C in medium containing 1 μmol/L $CuSO_4$ in the absence (control) or presence of the oxygenase inhibitors (Fig 2). Analysis of the effect of several oxygenase inhibitors on macrophage-mediated oxidation of LDL showed that oxipurinol (100 μg/mL) and indomethacin (20 μmol/L), inhibitors of xanthine oxidase and cyclooxygenase, respectively, had no significant effect on cell-mediated oxidation of LDL (Fig 2A). The specific inhibitor of NADPH oxidase (apocynin 100 μg/mL) and the nonspecific inhibitor of several lipoxy-

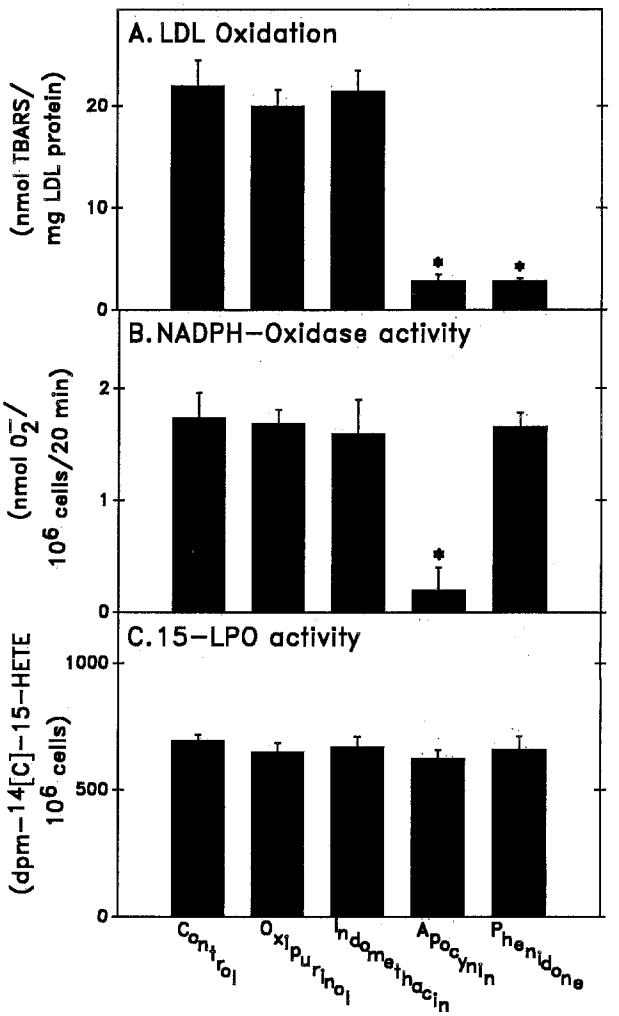

Fig 2. Effect of oxygenase inhibitors on macrophage-mediated oxidation of LDL and on NADPH oxidase and 15-lipoxygenase (15-LPO) activities. J-774 A.1 macrophages (1 × 10^6/35-mm dish) were incubated for 30 minutes at 37°C with no additions (control) or with several oxygenase inhibitors (A) before addition of LDL (0.2 mg protein/mL) and 1 μmol/L $CuSO_4$ and a further cell incubation for 18 hours. The oxygenase inhibitors include 100 μg/mL oxypurinol, 20 μmol/L indomethacin, 100 μg/mL apocynin, or 25 μmol/L phenidone. At the end of the incubation, LDL oxidation was measured by TBARS assay. (B) J-774 A.1 macrophages (2 × 10^5/100-μL well) were incubated for 20 minutes with LDL (0.2 mg protein/mL) and 1 μmol/L $CuSO_4$ in the absence (control), or presence of the oxygenase inhibitors. At the end of the incubation, the amount of superoxide anions released to the medium was measured. (C) J-774 A.1 (1 × 10^6/35-mm dish) were incubated with 5 μmol/L [^{14}C]-arachidonic acid for 15 minutes. The cells were washed and further incubated for 1 hour with LDL (0.2 mg protein/mL) and 1 μmol/L $CuSO_4$ in the absence (control) or presence of the different oxygenase inhibitors. At the end of the incubation, the amount of [^{14}C]-15-HETE released to the medium was measured. Results are the mean ± SD (n = 3) *P < .01 v control.

genases (phenidone 25 μmol/L) inhibited cell-mediated oxidation of LDL by 87% and 89%, respectively (Fig 2A). Phenidone was found to affect LDL oxidation by its ability to chelate copper ions. Concentrations of copper ions greater than 1 μmol/L overcome the inhibitory effect of phenidone on $CuSO_4$-induced LDL oxidation (data not shown), and phenidone therefore could not be used as a specific inhibitor of 15-lipoxygenase.

In a cell-free system, LDL oxidation was found to be only a maximum of 20% of the oxidation obtained upon LDL incubation with macrophages. None of the inhibitors demonstrated any significant effect on LDL oxidation in a cell-free system.

Apocynin inhibited NADPH oxidase activity by 89% compared with levels in control cells, as measured by the release of superoxide anions from these cells (Fig 2B). Oxypurinol, indomethacin, or phenidone had no effect on NADPH oxidase activity (Fig 2B). Macrophage 15-lipoxygenase activity was not significantly affected by any of the different oxygenase inhibitors (Fig 2C).

In addition, no significant changes in cellular protein content or in the release of lactate dehydrogenase could be found, suggesting that these inhibitors were not cytotoxic to the cells.

NADPH Oxidase Involvement in Macrophage-Mediated Oxidation of LDL

The effect of LDL on macrophage NADPH oxidase activity (in the presence of copper ions) was studied by analysis of superoxide anion release from the cells to the medium (Fig 3).

Activation of J-774 A.1 macrophage NADPH oxidase with PMA (50 ng/mL) resulted in the release of 1.30 ± 0.20 nmol superoxides/10^6 cells after 20 minutes of incubation (control cells released only 0.20 ± 0.01 nmol superoxides/10^6 cells/20 min). Addition of LDL (0.2 mg protein/mL) to J-774 A.1 macrophages in the presence of 1 μmol/L $CuSO_4$ resulted in a time-dependent increment in the release of superoxides to 1.80 ± 0.20 nmol superoxides/10^6 cells/20 min (Fig 3). Addition of LDL or $CuSO_4$ alone to the incubation medium under similar experimental conditions had no effect on the extent of macrophage-released superoxides (Fig 3). Addition of apocynin (100 μg/mL) to the incubation system (cells + LDL + Cu^{2+} or cells + PMA) completely blocked the release of superoxides to the medium, suggesting that LDL-induced macrophage release of superoxides under oxidative stress is indeed associated with lipoprotein stimulation of cellular NADPH oxidase

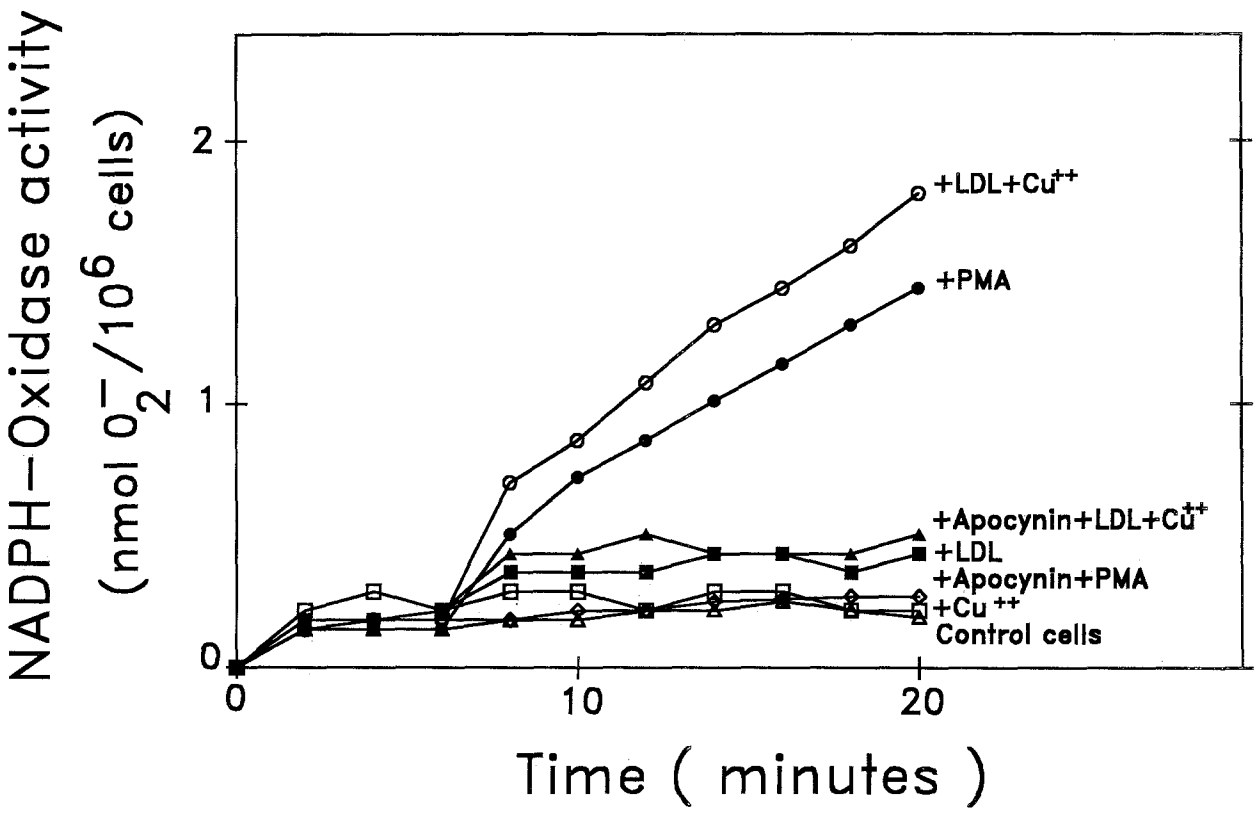

Fig 3. Effect of LDL on cellular NADPH oxidase activity in J-774 A.1 macrophages under oxidative stress. J-774 A.1 macrophages (2×10^5/100-μL well) in a 96-well dish were incubated with no addition (control) or with 1 μmol/L $CuSO_4$ (+Cu^{2+}), LDL 0.2 mg protein/mL (+LDL), LDL + 1 μmol/L $CuSO_4$ (+LDL + Cu^{2+}), 100 μg/mL apocynin + LDL + 1 μmol/L $CuSO_4$ (apocynin + LDL + Cu^{2+}), 50 ng/mL PMA (+PMA), or 100 μg/mL apocynin + 50 ng/mL PMA (+apocynin + PMA). Release of superoxides to the medium was kinetically determined. Results are the mean of duplicate dishes from 1 experiment, representative of 3 different experiments that varied by <10%.

activity. Addition of apocynin alone to the cells had no significant effect on the extent of macrophage-released superoxides (0.20 ± 0.01 nmol superoxides/10^6 cells/20 min).

To assess the role of ROS and other oxidants in macrophage NADPH oxidase–mediated LDL oxidation, we next tested the effect of PMA on cell-mediated oxidation of LDL. The effect of conditioned medium ([CM] 2 mL) derived from PMA-stimulated cells on LDL (0.2 mg protein/mL) susceptibility to lipid peroxidation was determined after 24 hours of incubation in the presence of 1 μmol/L $CuSO_4$ (in the absence of cells). The CM increased LDL oxidation from 3.9 ± 0.2 to 5.9 ± 0.2 nmol TBARS/mg LDL protein ($P < .01$, n = 3). Using medium obtained from nonstimulated macrophages, LDL oxidation was not significantly affected (3.8 ± 0.3 nmol TBARS/mg LDL protein). The CM from the stimulated macrophages was shown by TLC analysis to contain oxidized polyunsaturated fatty acids such as linoleate and arachidonate (data not shown). To further study the involvement of NADPH oxidase in macrophage-mediated oxidation of LDL, PMA-treated J-774 A.1 macrophages (and nontreated control cells) were incubated with LDL (in the absence of copper ions) for 18 hours at 37°C. LDL oxidation by these PMA-treated cells was sevenfold higher than that obtained by control cells (Fig 4A). PMA had no effect on LDL oxidation in a cell-free system (Fig 4B). However, PMA induces macrophage lipid peroxidation (in the absence of LDL), as evident by a ninefold increment in the release of TBARS from PMA-treated cells in comparison to control cells (Fig 4C). In this study, PMA (50 ng/mL) increased macrophage NADPH oxidase activity by sixfold 1.50 ± 0.10 nmol superoxides/10^6 cells/20 min, whereas control cells released only 0.25 ± 0.01 nmol superoxides/10^6 cells/20 min. PMA had no effect on macrophage lipoxygenase activity, since the release of [^{14}C]-15-HETE from cells preincubated with [^{14}C]-arachidonic acid (for 15 minutes at 37°C) was not affected by PMA (374 ± 42 *v* 360 ± 16 dpm of [^{14}C]-15-HETE/dish in the absence or presence of PMA, respectively).

We next analyzed the effect of NADPH oxidase activation on LDL oxidation in another type of macrophage, the HMDM. Addition of LDL (0.2 mg protein/mL) to HMDM (2×10^6/35-mm dish) increased the release of superoxides from 0.4 ± 0.2 to 1.9 ± 0.3 nmol superoxides/10^6 cells/20 min ($P < .01$, n = 4; Fig 5). Addition of LDL and 1 μmol/L $CuSO_4$ (LDL + Cu^{2+}) to the incubation system resulted in a further threefold elevation in the release of superoxides, a value similar to that obtained with 50 ng/mL PMA (Fig 5). NADPH oxidase activation involves the translocation of its cytosolic protein components to the plasma membrane. Immunoblot analysis was used to analyze the translocation of NADPH oxidase cytosolic components (P-47 and P-67) to the HMDM plasma membrane following 6 minutes of cell incubation without (control) or with LDL, LDL + 1 μmol/L $CuSO_4$, or PMA (50 ng/mL).

Relative amounts of the cytosolic oxidase components were quantified by densitometry (Table 1). P-47 and P-67 could hardly be detected in membranes of resting macrophages. LDL alone induced minimal translocation of P-47 and P-67 to the macrophage membrane, whereas addition of LDL in the presence of 1 μmol/L $CuSO_4$ to HMDM caused an extensive translocation of these cytosolic factors, similar to that induced by PMA (Table 1).

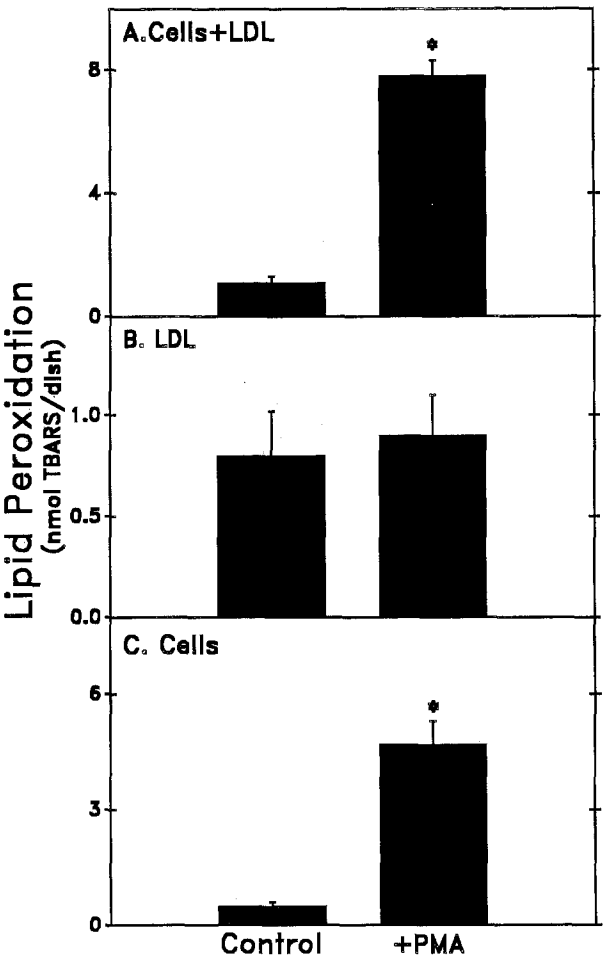

Fig 4. Effect of PMA on macrophage-mediated oxidation of LDL. J-774 A.1 macrophages (1 × 10^6/35-mm dish) were incubated without (control) or with PMA (50 ng/mL) for 30 minutes at 37°C. LDL (0.2 mg protein/mL) was added to the incubation medium, and incubation continued for a further 18 hours at 37°C (A). In control studies, similar systems with no cells (B) or with no LDL (C) were used to analyze the direct effect of PMA on the cells or on LDL. TBARS were assayed in the medium at the end of the incubation. Results are the mean ± SD (n = 3). *$P < .01$ *v* control.

15-Lipoxygenase Involvement in Macrophage-Mediated Oxidation of LDL

Incubation of J-774 A.1 macrophages with 5 μmol/L [^{14}C]-radiolabeled linoleic acid or [^{14}C]-radiolabeled arachidonic acid for 15 minutes at 37°C to label cellular lipids resulted in incorporation of approximately 70% of the labeled fatty acids into the macrophage phospholipids. Addition of 0.2 mg LDL protein/mL in the presence of 1 μmol/L $CuSO_4$ to the cells and a further 1-hour incubation at 37°C resulted in a 33% elevation in the release of the linoleate metabolite, [^{14}C]-13-HODE (Fig 6A), to the

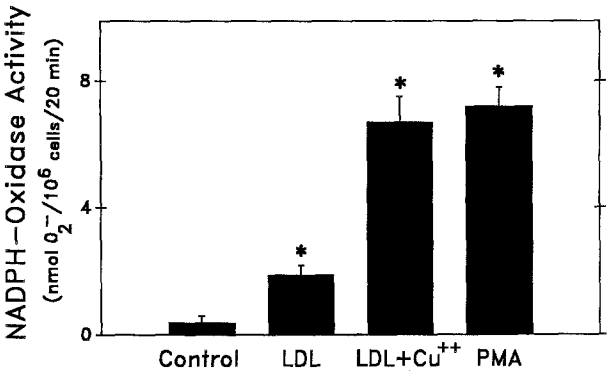

Fig 5. Superoxide release from HMDM following incubation with LDL in the presence of copper ions. Experimental procedure was similar to that described for Fig 3, except that HMDM were used instead of J-774 A.1 cells. Results are the mean ± SD (n = 3). *$P < .01$; LDL and PMA *v* control and LDL + Cu^{2+} *v* LDL.

medium and a 93% elevation in the release of the arachidonate metabolite, [^{14}C]-15-HETE, to the medium (Fig 6B), in comparison to control cells incubated in the absence of LDL (Fig 6). These results show that LDL can induce the activation of macrophage 15-lipoxygenase under oxidative stress, and suggest that 15-lipoxygenase may possess higher specific activity toward arachidonic acid than toward linoleic acid in macrophage phospholipids. Alternatively, differences in specific activities of the linoleic acid and arachidonic acid within cellular phospholipids may also account for the difference in the amount of [^{14}C]-13-HODE and [^{14}C]-15-HETE released from the cells to the medium.

Recently, we have shown[27] that CM from macrophages incubated with LDL in the presence of 1 μmol/L $CuSO_4$ for 5 hours at 37°C, contained oxidized polyunsaturated fatty acids. This CM stimulated LDL oxidation in the presence of copper ions.[27] We thus questioned whether oxidized arachidonic or linoleic acids, which are found in this CM,[27] can enhance LDL oxidation. Upon LDL incubation in the presence of 10 μmol/L $CuSO_4$ without (control LDL) or with 2 μg/mL oxidized arachidonic acid or oxidized linoleic acid (prepared by fatty acid incubation under air atmosphere for 72 hours at 37°C, and analyzed for oxidation by

Table 1. Translocation of Cytosolic P-47 and P-67 to the Cell Membrane in HMDM Upon Stimulation With LDL or PMA

Treatment	P-47	P-67
Control (resting cells)	7 ± 3	5 ± 2
LDL	12 ± 7	13 ± 5
Cu^{2+}	14 ± 6	16 ± 7
LDL + Cu^{2+}	129 ± 12*	115 ± 15*
PMA	135 ± 18*	110 ± 12*

NOTE. HMDM (3 × 10^6/mL) were incubated in suspension in the absence (control) or presence of LDL 0.2 mg protein/mL (LDL) or with 1 μmol/L $CuSO_4$ (Cu^{2+}), LDL + 1 μmol/L $CuSO_4$ (LDL + Cu^{2+}), or PMA 50 ng/mL (PMA) for 6 minutes at 37°C before membrane isolation. Determinations (by densitometry) of the relative amounts of P-47 and P-67 in the macrophage membrane are the mean ± SD from 3 different experiments. Units are arbitrary units of density, with a higher number representing a darker band on the immunoblot.

*$P < .01$: LDL + Cu^{2+} *v* LDL and PMA *v* control.

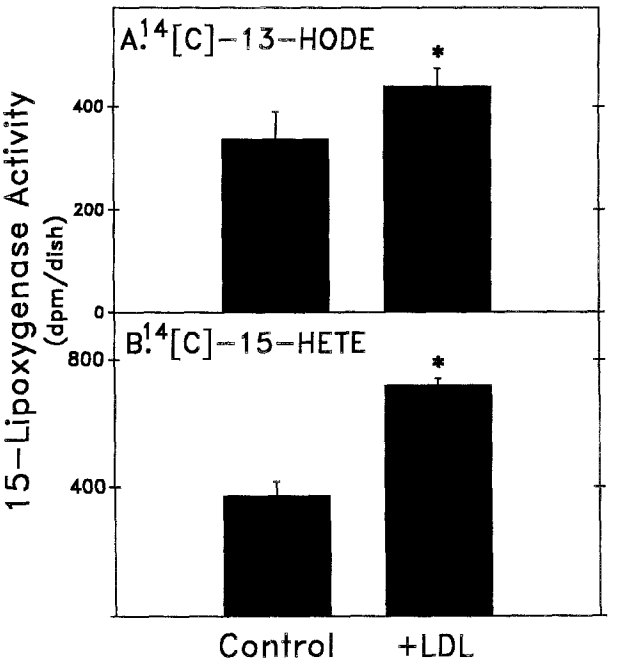

Fig 6. Effect of LDL on macrophage lipoxygenase activity under oxidative stress. J-774 A.1 cells (1 × 10^6/35-mm dish) were incubated with 5 μmol/L [^{14}C]-linoleic acid or 5 μmol/L [^{14}C]-arachidonic acid for 15 minutes at 37°C (to label cellular lipids). The cells were then washed, followed by a further 1-hour cell incubation in the presence of 1 μmol/L $CuSO_4$ in the absence (control) or presence of LDL (0.2 mg protein/mL). At the end of the incubation, the medium was analyzed for radioactive [^{14}C]-13-HODE (A) and [^{14}C]-15-HETE (B) by TLC. Results are the mean ± SD (n = 3). *$P < .01$ *v* control.

determination of conjugated-dienes content,[35] the lag time (monitored at 234 nm[42] required to initiate LDL oxidation was reduced from 18 ± 3 minutes in control LDL to 10 ± 2 minutes in oxidized arachidonate–treated LDL and to 0 minutes in oxidized linoleate–treated LDL ($P < .01$ *v* control LDL, n = 3; Fig 7). Addition of 2 μg/mL nonoxidized arachidonic acid or linoleic acid to LDL in the presence of copper ions did not change the lag time required to initiate LDL oxidation (21 ± 3 minutes for arachidonic acid–treated LDL and 19 ± 2 minutes for linoleic acid–treated LDL). These results demonstrate the ability of peroxidized polyunsaturated fatty acids to participate in cell-mediated oxidation of LDL. 15-HETE, a product of 15-lipoxygenase action on arachidonic acid, did not increase LDL oxidizability, but instead increased the lag time required for LDL oxidation (Fig 7).

LDL Oxidation by Monocyte-Macrophages With NADPH Oxidase Deficiency

Finally, we analyzed HMDM from two patients with CGD lacking NADPH oxidase activity. Upon incubation of HMDM from these patients with LDL (0.2 mg protein/mL) in the presence of 1 μmol/L $CuSO_4$, minimal (4 nmol TBARS/mg LDL protein) cell-mediated oxidation of LDL could be found (Fig 8A). Cell-mediated oxidation of LDL by the HMDM, was only 17% of that obtained by HMDM derived from healthy subjects (4 ± 1 *v* 24 ± 4 nmol

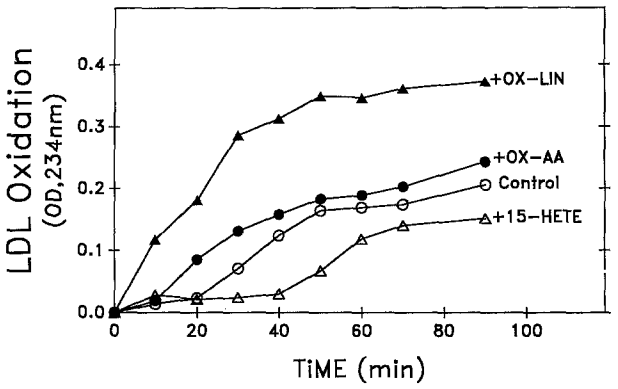

Fig 7. Effect of oxidized polyunsaturated fatty acids on lag time required for initiation of LDL oxidation. LDL (0.2 mg protein/mL) was incubated in the presence of 10 μmol/L $CuSO_4$ with no addition (control) or with addition of 2 μg/mL oxidized arachidonic acid (+0X-AA), oxidized linoleic acid (+0X-LIN), or 2 μg/mL 15-HETE. Oxidized arachidonic and linoleic acids contained 110 ± 2 and 335 ± 25 nmol conjugated dienes (CD)/mL, respectively (n = 3). In nonoxidized arachidonic and linoleic acids and in 15-HETE, 21 to 35 nmol CD/mL were obtained. LDL oxidation was kinetically monitored by analysis of changes in absorbance at 234 nm. Figure is representative of 2 different experiments.

TBARS/mg LDL protein, respectively). Monocyte-macrophages from CGD patients did not release superoxide anions at all (Fig 8B). However, the patients' MDM demonstrated almost normal 15-lipoxygenase activity, since the release of [^{14}C]-15-HETE from the MDM (preincubated with [^{14}C]-labeled arachidonic acid) was similar to that obtained with control MDM (Fig 8C). These results suggest that in the presence of copper ions, NADPH oxidase but not 15-lipoxygenase is required and sufficient for macrophage-mediated oxidation of LDL.

DISCUSSION

The present study demonstrated that both macrophage NADPH oxidase and 15-lipoxygenase were activated by LDL (in the presence of copper ions) as determined by superoxide formation and NADPH oxidase subunit translocation, as well as by the formation of 15-HETE and 13-HODE, respectively. However, activation of NADPH oxidase was required and sufficient, whereas activation of 15-lipoxygenase was not sufficient to induce LDL oxidation. This conclusion was reached because monocyte-macrophages from CGD patients, who lack active NADPH oxidase but possess normal 15-lipoxygenase activity, failed to oxidize LDL. Furthermore, using PMA as a stimulator (rather than LDL + $CuSO_4$), activated monocytes from CGD patients also failed to oxidize LDL.[12]

Although free metal ions are found in vivo at low concentrations, cell-mediated oxidation of LDL in the presence of copper ions is probably still a valid model for LDL oxidation by arterial cells. A close association between the thickness of the carotid medial intima and plasma copper ion concentration was shown, and copper ions were found in mature human atherosclerotic lesions.[10] Even in the absence of copper ions, activation of NADPH oxidase but not of 15-lipoxygenase in PMA-treated J-774 A.1 macrophages resulted in cell-mediated oxidation of LDL (albeit to a lower extent than obtained in the presence of copper ions). These latter results further support the requirement of sufficient active NADPH oxidase for macrophage-mediated oxidation of LDL.

However, there is a possibility that other factors or processes are also needed along with activated NADPH oxidase for LDL to be oxidized.

Lipoxygenase is a dioxygenase that incorporates one molecule of oxygen at a certain position on polyunsaturated fatty acids such as arachidonic or linoleic acids.[11] No specific role can yet be attributed to the macrophage 15-lipoxygenase,[43] but this enzyme is present in macrophage foam cells[44] and is suggested to be involved in macrophage-mediated oxidation of LDL.[14,15] Monocytes activated with zymozan release superoxides that can oxidize LDL by a process involving the action of 15-lipoxygenase, since inhibitors of 15-lipoxygenase blocked cell-mediated oxidation of LDL.[14] However, the use of such inhibitors was

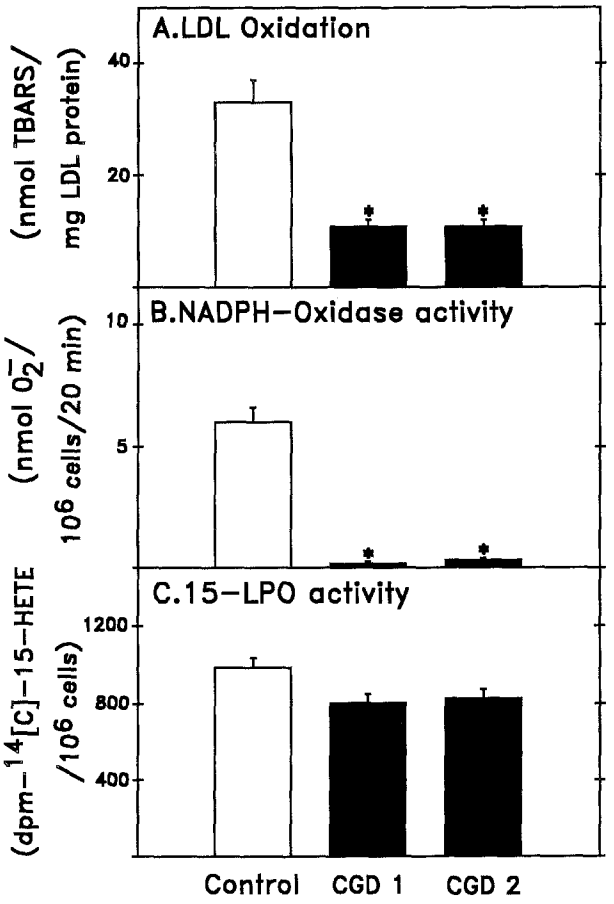

Fig 8. Cell-mediated oxidation of LDL and the activities of NADPH oxidase and 15-lipoxygenase in HMDM from patients with CGD. HMDM from healthy subjects (n = 5) and 2 patients with CGD were incubated (separately, not pooled) with LDL (0.2 mg protein/mL) in the presence of 1 μmol/L $CuSO_4$ for 18 hours at 37°C. Macrophage-mediated oxidation of LDL (A), cellular NADPH oxidase activity (B), and macrophage 15-lipoxygenase activity (C) were analyzed. LDL oxidation in a cell-free system was 7 ± 1 nmol TBARS/mg LDL protein. Results are the mean ± SD (n = 3). *$P < .01$ v control.

shown to affect not only cellular lipoxygenase activity but also other enzymes involved in lipid peroxidation processes.[26] Even in the present study, the use of phenidone could not be specifically related to 15-lipoxygenase activity, since we found that it possesses some copper ion–chelating activity. Although macrophage incubation with LDL in the presence of 1 μmol/L $CuSO_4$ induced cellular activation of both NADPH oxidase and 15-lipoxygenase, the activation of NADPH oxidase alone (in PMA-activated cells) but not of 15-lipoxygenase alone (in HMDM from CGD patients) was required for macrophage-mediated oxidation of LDL. It is thus suggested that activation of macrophage 15-lipoxygenase may not be related to cell-mediated oxidation of LDL under the conditions used in the present study.

The cells used in the present study consisted of both HMDM and the macrophage-like cell line, J-774 A.1. Although NADPH oxidase activity of the J-774 A.1 cell line was about fourfold less than that of HMDM, their cell-mediated oxidation of LDL was similar, suggesting that a certain level of cellular superoxide anions is probably sufficient to induce maximal macrophage-mediated oxidation of LDL. Similarly, in a study using mouse peritoneal macrophages, addition of a 10-fold excess concentration of superoxide anions to the macrophage-LDL incubation system did not further enhance cell-mediated oxidation of LDL.[45] Although the immediate product of NADPH oxidase (superoxide) is not reactive enough to induce LDL oxidation, it can be converted (in the presence of transition metal ions) to reactive oxygen-centered free radicals, such as hydroperoxyl radicals (HO_2)[25] or to some other type of ROS.[46] These ROS can directly oxidize the lipoprotein, or can act on the macrophage phospholipids, resulting in the formation and release of peroxidized fatty acids. The CM obtained after macrophage incubation with LDL in the presence of copper ions was indeed shown to contain peroxidized polyunsaturated fatty acids.[27] These oxidized fatty acids bind to the LDL and can oxidize the lipoprotein extracellularly,[47] as shown in the present study by the action of oxidized arachidonate and oxidized linoleate on LDL. It is of interest that, unlike the effect of these oxidized fatty acids that contain peroxides (which can act as an oxidizing agent toward LDL), the use of 15-HETE, the product of 15-lipoxygenase activity, did not enhance LDL oxidation in the present study. In fact, it was shown[47] that 15-HPETE but not 15-HETE stimulated LDL oxidation in the presence of copper ions. The increment in the lag time required for LDL oxidation by 15-HETE may be explained by a stearic effect of this fatty acid on the LDL surface. It is not possible to measure the oxidation of LDL and the activities of 15-lipoxygenase and NADPH oxidase at the same time point, since the immediate products of these enzymes, 15-HPETE, 13-HPODE, and superoxide anions, are not stable and are rapidly converted to 15-HETE, 13-HODE, and ROS, respectively. Both macrophage NADPH oxidase and 15-lipoxygenase can produce oxidized fatty acids from cell membrane phospholipids, either secondary to the formation of ROS (by the action of NADPH oxidase) or even directly (by 15-lipoxygenase). However, oxidized fatty acids secreted by cells rarely include peroxides that are oxidizing agents, whereas chemically oxidized fatty acids and probably fatty acids that are attacked by ROS contain peroxides that can act as oxidizing agents toward LDL. In contrast, the action of 15-lipoxygenase on fatty acids results in rapid conversion of 15-HPETE and 13-HPODE to their stable derivatives, 15-HETE and 13-HODE, which do not contain peroxides.

During the 18 hours of macrophage incubation with LDL under oxidative stress, lipoxygenase and NADPH oxidase activities were changed. However, the products of these enzymes could be responsible for cell-mediated oxidation of LDL, since 15-HETE and 13-HODE remain elevated during prolonged exposure of cells to LDL and copper ions. In addition, although superoxide anions, the products of NADPH activation, have a short half-life, these oxidants in the presence of copper ions can form lipid peroxides on the macrophage surface, and these peroxides can oxidize the LDL. Another mechanism that possibly contributes to superoxide-mediated oxidation of LDL is the reduction of copper ions to a form capable of decomposing preexisting lipid hydroperoxides in LDL, hence initiating the lipid peroxidation of new LDL molecules.[46]

Oxidized LDL is one of several modified forms of LDL that can contribute to foam cell formation.[48-53] Macrophages are capable of producing such modified forms of LDL, including phospholipase D–modified LDL, which is highly susceptible to oxidation.[52] Since NADPH oxidase was shown to activate cellular phospholipase D,[54] it may be speculated that activation of the macrophage NADPH oxidase can lead to formation of oxidized LDL secondary to phospholipase D activation.

In conclusion, then, the present study shows that macrophage NADPH oxidase plays a role in LDL oxidation by these cells under oxidative stress. Determining the physiological/pathological relevance of this finding awaits development of better methods to assess LDL oxidation in vivo.

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