Multiple lipid oxidation products in low density lipoproteins induce interleukin-1 beta release from human blood mononuclear cells

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Abstract Oxidized low density lipoproteins (LDL) induce the release of interleukin-1 beta (IL-1β) from human peripheral blood mononuclear cells, a process that may contribute to atherogenesis. While 9-hydroxyoctadecadienoic acid (9-HODE) is a constituent of oxidized LDL and can by itself induce IL-1β release, its potency relative to oxidized LDL suggested that other components of modified LDL may also contribute to this phenomenon. In this study, LDL of varying oxidation states were prepared by altering the Cu^{2+} to LDL ratio and/or the length of oxidation. The oxidation status of LDL was measured as thiobarbituric acid reactive substances (TBARS), electrophoretic mobility in agarose gels, and the content of 9- and 13-HODE. High Cu^{2+} to LDL ratios promoted extensive TBARS formation and these LDL were the most potent activators of IL-1β release, although LDL with TBARS greater than 50 nmol/mg protein were cytotoxic and IL-1β release was diminished. An inverse correlation between HODE content and TBARS was found indicating lipid-derived aldehydes also contribute to IL-1β release by oxidized LDL. Accordingly, dialysis of oxidized LDL removed nearly all aldehydes and rendered the LDL unable to induce IL-1β release. The alkenals 2,4-decadienal and 2-octenal were tested and shown to induce IL-1β release while their saturated homologues had no effect. The predominant aldehyde in Cu^{2+}-oxidized LDL was hexanal, with the unsaturated aldehydes 2,4-heptadienal, 2-octenal, and 2,4-decadienal also being present. These data indicate that multiple, lipid-derived species exist in oxidized LDL that can contribute to the release of IL-1β.—**Thomas, C. E., R. L. Jackson, D. F. Ohlweiler, and G. Ku.** Multiple lipid oxidation products in low density lipoproteins induce interleukin-1 beta release from human blood mononuclear cells. *J. Lipid Res.* 1994. **35:** 417–427.

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Numerous reports suggest a causal role for the oxidation of low density lipoproteins (LDL) in the etiology of atherosclerosis (1–5). Oxidized LDL have been shown to affect a number of potentially atherogenic events including cytotoxicity (6), increased expression of adhesion molecules with enhanced monocyte (7) and leukocyte (8) binding to endothelial cells, and foam cell formation (9).

In most in vitro studies, LDL oxidation is achieved by incubation with Cu^{2+} ions or cells including endothelial cells, smooth muscle cells, and monocyte/macrophages (reviewed in ref. 10). In the latter case oxidation requires the presence of transition metal ions such as Fe^{3+} or Cu^{2+} which propagate oxidative reactions via the cleavage of lipid hydroperoxides (LOOH):

$$M^{n-} + LOOH \rightarrow LOO^{\cdot} + H^{+} + M^{n-1+} \quad \textit{Reaction 1)}$$
$$M^{n-1+} + LOOH \rightarrow LO^{\cdot} + {}^{-}OH + M^{n+} \quad \textit{Reaction 2)}$$

The lipid-derived, oxygen-centered radicals produced in reactions 1 and 2 undergo additional rearrangements with the β-scission of alkoxyl radicals ($LO^{\cdot}$), producing a variety of aldehydes. Unsaturated aldehydes have been shown to affect a variety of cellular processes (11) and, in regard to atherosclerosis, their reactivity with apolipoprotein B (apoB) of LDL leads to conformational changes resulting in recognition by the macrophage scavenger receptor (12, 13). The lack of cholesterol-dependent down-regulation of the scavenger receptor causes lipid accumulation and the formation of macrophage-derived foam cells (14).

The extent of oxidation in most in vitro studies is determined by the quantitation of aldehydes using thiobarbituric acid. Generally, the level of thiobarbituric acid reactive substances (TBARS) is upwards of 50 nmol/mg LDL, indicating significant loss of polyunsaturated fatty acids with resultant modification of apoB as determined by macrophage degradation of ^{125}I-labeled LDL (15). How-

Abbreviations: DNPH, dinitrophenylhydrazine; 4-HNE, 4-hydroxynonenal; LDL, low density lipoproteins; HODE, hydroxyoctadecadienoic acid; TBARS, thiobarbituric acid reactive substances; IL-1β, interleukin-1 beta; ALDL, acetylated LDL; Cu^{2+}-LDL, copper-oxidized LDL; M-LDL, mononuclear cell-modified LDL; MM-LDL, minimally modified LDL; LPS, lipopolysaccharide; TNFα, tumor necrosis factor alpha; LDH, lactate dehydrogenase.

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ever, it is reasonable to assume that, in vivo, plasma antioxidants would prevent such extensive oxidation, and only once in the arterial wall would such major modifications occur. If true, then examination of less highly oxidized LDL may be more appropriate for studies directed toward developing a mechanistic understanding of the early stages of atherosclerosis. Accordingly, recent work by Liao et al. (7), Drake et al. (16), and Navab et al. (17) has demonstrated that minimally modified LDL (MM-LDL), which contain less than 5 nmol/mg TBARS and are not recognized by the scavenger receptor, induce a number of biochemical changes both in vitro (16, 17) and in vivo (7) which could promote atherogenesis.

It must also be considered, as emphasized by Lenz et al. (18), that aldehydes may constitute only a small percentage of the total oxidized polyunsaturated fatty acids of modified LDL. They demonstrated the formation of the 9- and 13-isomers of hydroperoxyoctadecadienoic acid (HPODE) and hydroxyoctadecadienoic acid (HODE) in Cu^{2+}-modified LDL, findings confirmed for LDL oxidized with Cu^{2+} (Cu^{2+}-LDL) or mononuclear cells (M-LDL) (19). We have recently demonstrated (20) that oxidized LDL, or 9-HODE and 13-HODE, can stimulate interleukin-1 beta (IL-1β) release from mononuclear cells. As IL-β has been shown to induce smooth muscle cell proliferation (21, 22) and is found in atherosclerotic lesions (23, 24), factors that influence its expression and/or release and may be important in atherogenesis. We previously observed differences among preparations of oxidized LDL with regard to their ability to induce IL-1β and this, in conjunction with varied reports regarding the composition and biologic activity of modified LDL, led us to examine in more detail the heterogeneity of oxidized LDL and its effect on the release of IL-1β from human peripheral blood mononuclear cells. In this report, we show that the oxidation state of LDL markedly influences the product profile of lipid oxidation with multiple products capable of inducing IL-1β release.

MATERIALS AND METHODS

Isolation of mononuclear cells

Human peripheral blood was collected in 10 mM sodium citrate from healthy volunteers. Erythrocytes and neutrophils were removed by low speed centrifugation in Leucoprep tubes (Becton Dickinson, Oxnard, CA) according to the manufacturer's suggested protocol. The resulting mixture of platelets and mononuclear cells was incubated in tissue culture dishes (3×10^6 cells per well in a 24-well plate, Corning, Corning, NY) for 1 h at 37°C followed by removal of nonadherent platelets and other cells. Fresh medium RPMI-1640 (Gibco, Grand Island, NY) was then added to the adherent cells. Greater than 98% of the adherent cells stained positive for nonspecific esterase. Furthermore, flow cytometric analyses showed that 99.5% of the adherent cells were capable of internalizing a fluorescent acetylated LDL (ALDL) probe, suggesting a macrophage-like phenotype bearing the scavenger receptor (data not shown). All experiments were performed with freshly adhered cells in the absence of serum.

Preparation and oxidation of LDL

Human plasma was obtained from a local blood center. To each pool of plasma (18–20 normolipidemic fasting donors) was added 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 50 units/ml aprotinin, and 0.01% sodium azide prior to LDL fractionation. LDL were isolated by ultracentrifugal flotation in KBr (d 1.019–1.063 g/ml) as previously described (25). Prior to oxidation, EDTA was removed from the LDL by dialysis against phosphate-buffered saline (PBS), pH 7.4, at 4°C in the dark. Acetylated LDL were prepared according to Fraenkel-Conrat (26).

Copper-dependent oxidation of LDL was carried out in PBS at 37°C in 12 × 75 mm loosely capped, polypropylene test tubes. To vary the extent of oxidation, the LDL concentration ranged from 0.25 to 4.0 mg LDL protein/ml. In some instances the Cu^{2+} concentration also varied, generally in the 6–20 μM range. By altering the concentration of LDL and Cu^{2+}, the Cu^{2+} to polyunsaturated fatty acid ratio was varied which greatly affects the kinetics and extent of LDL oxidation. The length of oxidation varied from 2 to 24 h as described in the figure legends. Oxidation was terminated by the addition of EDTA in excess of the Cu^{2+} concentration. Prior to addition to mononuclear cells, the oxidized LDL were passed over a pre-packed endotoxin affinity column (Detoxigel, Pierce, Rockford, IL).

Mononuclear cell-modified LDL were prepared by incubating LDL (2 mg LDL/ml) with human peripheral blood monocytes (adherent cells) for 24 h at 37°C. The modified LDL were re-isolated from the culture medium by preparative ultracentrifugation in KBr (d 1.006–1.15 g/ml).

Determination of LDL oxidation

The extent of LDL oxidation was determined by TBARS (27). Aliquots of the incubation mixture containing 100 μg of LDL were removed and added to tubes containing 0.05 ml of 2% butylated hydroxytoluene and 2 ml of 0.67% TBA and 10% trichloroacetic acid (2:1) in 0.25 N HCl. After heating at 100°C for 20 min, the tubes were cooled and centrifuged at 2,500 rpm for 10 min. The absorbance of the supernatant fraction was read at 532 nm and quantitation was achieved by comparison to a standard curve of malondialdehyde equivalents generated by acid-catalyzed hydrolysis of 1,1,3,3-tetraethoxypropane. The yield of TBARS was approximately 10–12 nmol/mg protein for M-LDL and varied for Cu^{2+}-LDL as described in Results.

Lipid peroxidation products were also determined by HPLC analysis of saponified lipids. For LDL and modified-LDL, the lipids were extracted from the sample (0.25 mg protein) with chloroform–methanol–acetic acid 2:1:0.01. After centrifugation at 3,500 rpm for 10 min, the chloroform fraction was removed and dried under nitrogen. The dried lipids were reconstituted in 0.5 ml of ethanol to which was added 0.05 ml of 10 N NaOH, followed by heating at 60°C for 20 min to generate the free acids. The solution was neutralized by the addition of 0.03 ml of glacial acetic acid and dried under nitrogen. To the lipid was added 1 ml of water and then 1 ml of heptane. After mixing and centrifugation at 1,800 rpm for 5 min, 0.8 ml of the heptane layer was removed, dried under nitrogen, and resolubilized into 0.15 ml of heptane. Fatty acid oxidation products were separated using a normal-phase silica column (Zorbax Sil, 25 cm × 4.6 mm, 5 μm) according to Teng and Smith (28) with minor modification. The mobile phase was a quaternary mixture of heptane–diethylether–isopropanol–acetic acid 100:10:0.9:0.1 run at a flow rate of 2 ml/min; conjugated dienes were monitored at 234 nm. Standards of linoleate hydroperoxides, i.e., 9(S)- and 13(S)-HPODE, and linoleate hydroxides, i.e., 9(S)- and 13(S)-HODE (*cis, trans* isomers, Cayman Chemical, Ann Arbor, MI) in ethanol were dried under nitrogen, resolubilized in heptane, and subjected to chromatography as described for the saponified lipid fractions. Quantitation was achieved by comparison to a standard curve (0–1 μg) generated from the standards.

Separation and quantitation of aldehydes in Cu^{2+}-LDL were determined using TLC and HPLC essentially as described by Esterbauer and Cheeseman (29). LDL (0.5 mg protein/ml) were oxidized for 18 h with 6 μM $CuSO_4$. A 50-μg aliquot was removed for TBARS determination and to the remainder was added 2 ml of dinitrophenylhydrazine (DNPH; 0.35 mg/ml in 1 N HCl). The samples were sonicated for 30 sec and allowed to sit in the dark for 2 h at room temperature, followed by 1 h at 4°C. The samples were centrifuged at 3,000 rpm for 10 min and the resultant pellet was extracted with 3 ml of CH_2Cl_2–H_2O 2:1. The CH_2Cl_2 layer was removed and the sample was again extracted with 2 ml CH_2Cl_2. A 100-μl aliquot of the combined CH_2Cl_2 phases was diluted to 1 ml with CH_2Cl_2 and the absorbance was recorded from 350–450 nm for use in determining total aldehyde content using ϵ 27,000 $M^{-1}cm^{-1}$ at 378 nm.

The remaining CH_2Cl_2 extract was dried under N_2 gas and reconstituted in 100 μl of CH_2Cl_2. The samples were applied to a silica gel TLC plate (E. Merck, Darmstadt, Germany) and developed in CH_2Cl_2 to a distance of 5 cm and in toluene to a height of 15 cm. The strongly colored yellow bands comprising Class I and Class III aldehydes were scraped from the plates and the DNPH derivatives were extracted with 1 ml of methanol followed by centrifugation at 14,000 rpm for 10 min. Following an additional extraction, the methanol extracts were dried under N_2 gas and resolubilized in 200 μl of methanol. For use as standards, 200 μmol of each aldehyde was incubated with 200 μmol DNPH in 30% perchloric acid for 2 h at room temperature. The mixture was centrifuged and the pellet was resolubilized in CH_2Cl_2. The standards prepared were hexanal, 2,4-heptadienal, 2,4-decadienal, octanal, and 2-octenal from Aldrich (Milwaukee, WI); 2-hexenal and decanal from Sigma (St. Louis, MO); 2-nonenal (Alfa Products, Danvers, MA) and 4-hydroxynonenal (4-HNE; Cayman Chemical, Ann Arbor, MI). The DNPH standards were subjected to TLC and extraction as described for Cu^{2+}-LDL.

The Cu^{2+}-LDL and standards were subsequently analyzed by HPLC using a Waters 990 System equipped with a diode array detector. The mobile phase was methanol–water 31:9 run at 1 ml/min using a Spherisorb ODS-2 column obtained from Alltech (5 micron, 4.6 mm × 25 cm). The absorbance was monitored over the range 300–400 nm with peak assignment based on comparison of retention times to standards, as well as spectral characteristics. For confirmation of peak identity, aliquots of the Cu^{2+}-LDL derivatives were spiked with derivatized standards. Quantitation was achieved using extinction coefficients determined for each of the derivatized standards.

The electrophoretic mobility of LDL in 1% agarose gels was determined by staining with Fat Red 7B.

Determination of IL-1β release by modified lipoproteins

To control for possible endotoxin contamination of LDL incurred during oxidation, dialysis, or re-isolation, modified LDL were routinely passed over a Detoxigel column. As determined by the *Limulus polyphemus* amebocyte lysate test (LAL, Sigma, St. Louis, MO; sensitivity 1 ng endotoxin), the column has the capacity to remove 2 mg endotoxin; the affinity of endotoxin for the column is unaffected by the presence of LDL (data not shown). As endotoxin is equally potent in inducing IL-1β and tumor necrosis factor alpha (TNFα), and as oxidized-LDL stimulate IL-1β but not TNFα release (see below), a TNFα enzyme-linked immunosorbent assay (ELISA, sensitivity 20 pg) was also used routinely for oxidized LDL-stimulated culture supernatants to monitor for possible endotoxin contamination. Human IL-1β and TNFα ELISA kits were from Cistron (Pinebrook, NJ) and both were used according to the manufacturer's suggested protocol. Mononuclear cells were incubated with modified lipoproteins or 9-HODE for 24 h prior to IL-1β determination. The incubation time with aldehydes was as described in the figure legends. Lactate dehydrogenase (LDH) levels were routinely measured by a clinical analyzer (Coulter, Hialeah, FL).

RESULTS

The data in **Fig. 1** demonstrate that LDL oxidatively modified by incubation with mononuclear cells (M) or Cu^{2+} induced a significant release of IL-1β from human peripheral blood monocyte-derived macrophages. Neither LDL nor ALDL, which were not subjected to oxidation, induced IL-1β release. As previously reported (19, 20), the major conjugated diene-containing products in these modified LDL are 9- and 13-HODE with the 9-isomer identified as the most potent stimulator of IL-1β release. However, 300 μg of M-LDL, for example, contains approximately 2.7 μg of 9- plus 13-HODE and yet M-LDL induced significantly more IL-1β than did 9.5 μg of 9-HODE (33 μM) (20). Furthermore, Cu^{2+}-LDL, while containing less 9-HODE than M-LDL (20), are a more potent activator of IL-1β release. These findings suggested to us that modified LDL contain other constituents, derived from either oxidized lipid or protein, that induce IL-1β.

In an effort to examine the apparent heterogeneity of oxidized LDL with regard to its effect on IL-1β release, we prepared LDL that were modified to varying extents. This was accomplished initially by varying the Cu^{2+} to LDL ratio with a fixed, 24-h incubation time. The Cu^{2+}-LDL preparations were evaluated for oxidation status by determining TBARS and electrophoretic mobility in agarose gels. As shown in **Fig. 2A,** oxidation was inversely correlated with protein concentration irrespective of Cu^{2+} concentration. At a concentration of 0.25 mg LDL protein/ml, TBARS formation was near maximal with 2 μM Cu^{2+} and increased slightly to 36.6 nmol TBARS/mg protein with 20 μM Cu^{2+}. In contrast, LDL oxidation at 4 mg/ml had very little TBARS which reached a maximum of 9.4 nmol/mg protein with 20 μM Cu^{2+}. The increase in electrophoretic mobility induced by Cu^{2+} oxidation is shown in Fig. 2B. It is apparent that the effect of varying Cu^{2+} and protein concentration on electrophoretic mobility correlated well with TBARS (r^2 = 0.91). The degree of TBARS formation and the increase in electrophoretic mobility indicated the formation of aldehydes in highly modified LDL. As unsaturated aldehydes can be cytotoxic (11) the viability of mononuclear cells exposed to the various preparations of oxidized LDL

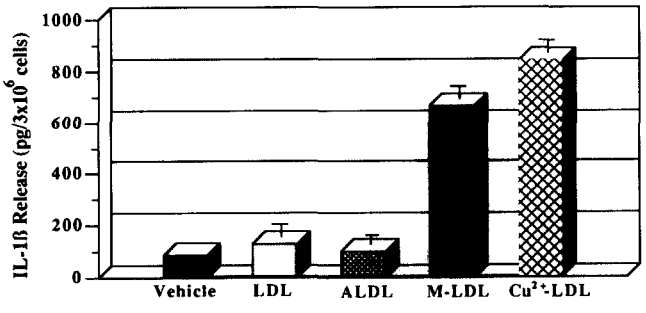

Fig. 1. Induction of IL-1β release from mononuclear cells by modified lipoproteins. Human peripheral blood mononuclear cells (3 × 10^6 cells/well) were incubated in 24-multiwell plates with medium alone or LDL, ALDL, M-LDL, or Cu^{2+}-LDL at 300 μg/ml for 24 h. Cu^{2+}-modified LDL (2 mg/ml, 10 μM Cu^{2+} for 18 h) had an average TBARS value of 35–40 nmol/mg protein while macrophage-modified LDL were typically 15–20 nmol/mg protein. Secreted IL-1β was measured with an IL-1β ELISA. Data represent means ± SEM for triplicate wells from a single cell preparation.

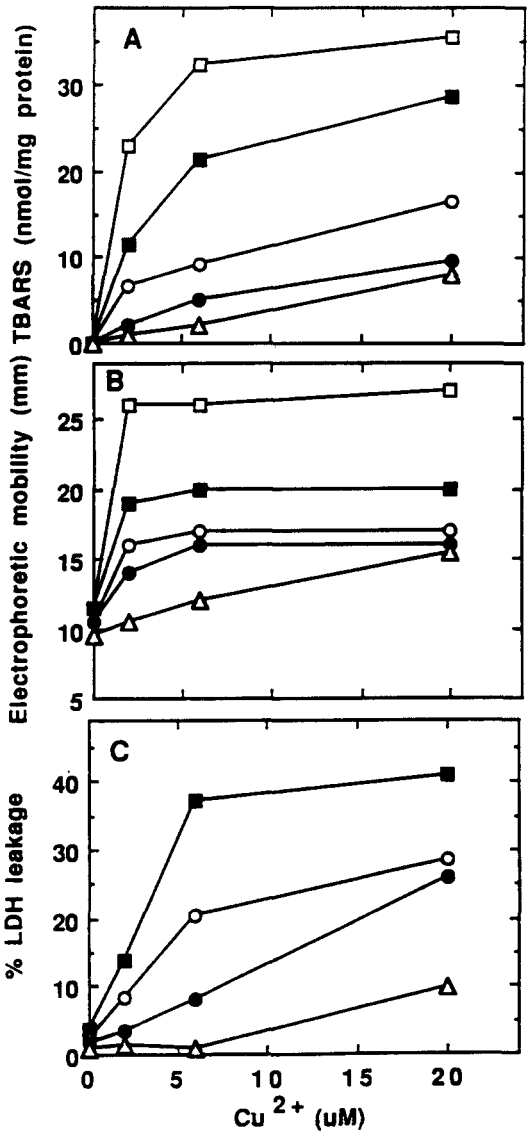

Fig. 2. Effect of Cu^{2+} and LDL concentration on LDL oxidation and cytotoxicity. LDL were incubated with 2, 6, or 20 μM Cu^{2+} for 24 h in PBS at concentrations of 0.25 mg/ml (□), 0.5 mg/ml (■), 1 mg/ml (○), 2 mg/ml (●), or 4 mg/ml (△). Oxidation was assessed by TBARS (A) and electrophoretic mobility in agarose gels (B). Cytotoxicity, except for LDL at 0.25 mg/ml, was measured as LDH release from mononuclear cells incubated with the modified lipoproteins for 24 h (C). LDH leakage (%) represents the amount in the supernatant relative to the total amount in the supernatant plus the cells as determined following cell lysis.

was assessed by LDH leakage as shown in Fig. 2C. The most highly modified preparations of LDL induced substantial LDH leakage with the overall profile comparing favorably with both TBARS and electrophoretic mobility.

The above results demonstrated that altering the Cu^{2+}/protein ratio produced LDL with widely varying degrees of oxidation. This approach was exploited to evaluate the efficacy of LDL modified to differing extents to induce the release of IL-1β. In **Fig. 3** is shown the effect on IL-1β release of LDL incubated for 24 h with 3, 10 or 30 μM Cu^{2+}, at LDL concentrations of 0.2, 1.1, or 2 mg/ml. LDL oxidized at 0.2 mg/ml with 3 μM Cu^{2+} induced a significant release of IL-1β with no further induction observed with 10 or 30 μM Cu^{2+}. These results agree with the data in Fig. 2 that demonstrated that, at a low LDL concentration, near maximal TBARS formation and electrophoretic mobility occurred with the lowest tested Cu^{2+} concentration. On the other hand, at the higher LDL concentrations, increasing amounts of Cu^{2+} were required to generate LDL capable of inducing IL-1β release. The correlation between IL-1β inducing activity and TBARS suggested that aldehydes produced from Cu^{2+}-dependent cleavage of lipid hydroperoxides may activate IL-1β release.

To further corroborate the heterogeneity of LDL oxidation and its effect on IL-1β, LDL were oxidized by varying the length of oxidation at a fixed Cu^{2+} concentration of 6 μM (**Fig. 4**). As was observed with increasing Cu^{2+} concentration (Fig. 2), the most extensive and rapid oxidation occurred at the lowest LDL concentration. After a 2-h incubation, the TBARS content at 0.25 mg LDL protein/ml was 100-fold greater than that of incubations conducted at 2 mg LDL/ml. The extent of oxidation at 1 mg/ml increased with time such that, by 18 h, the TBARS level was approximately 50% that of LDL oxidized at 0.25 or 0.5 mg/ml.

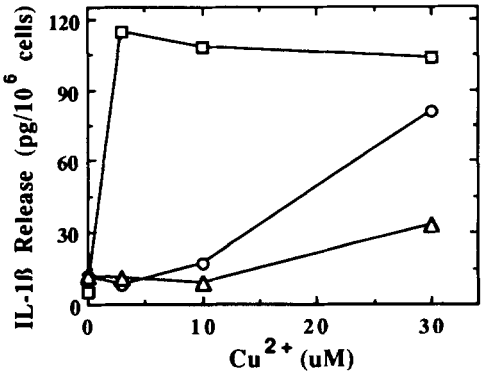

Fig. 3. Induction of IL-1β release from mononuclear cells by LDL of varying degrees of oxidation. LDL were oxidized by incubation with 3, 10, or 30 μM Cu^{2+} at 0.2 mg/ml (□), 1.1 mg/ml (○), or 2 mg/ml (△). The modified LDL were then incubated (400 μg/ml) with mononuclear cells (3 × 10^6 cells/well) for 24 h and IL-1β release was determined by ELISA. Data represent the average of duplicate wells from one cell preparation.

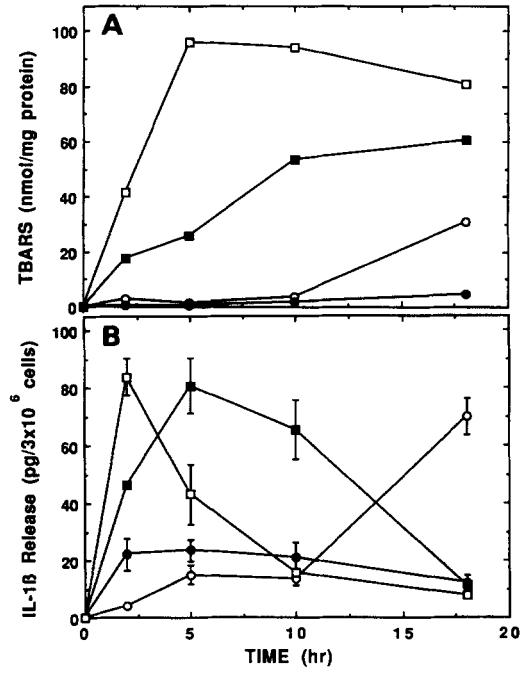

Fig. 4. Effect of oxidation time and LDL concentration on oxidation of LDL and their ability to induce mononuclear cell IL-1β release. LDL were incubated with 6 μM Cu^{2+} for 2, 5, 10, or 18 h at LDL concentrations of 0.25 mg/ml (□), 0.5 mg/ml (■), 1 mg/ml (○), or 2 mg/ml (●). The modified LDL (350 μg) were then incubated for 24 h with mononuclear cells (3 × 10^6 cells/well) and the secreted IL-1β was determined by ELISA. TBARS values represent the average of duplicate determinations with less than 10% variance (A). IL-1β data points represent means ± SEM for triplicate wells from a single cell preparation (B).

The ability of these LDL preparations to induce IL-1β was also determined (Fig. 4, bottom). At 0.25 mg/ml LDL, there was a marked increase in IL-1β release with 2 h of oxidation. Beyond this time, IL-1β release declined, presumably due to cytotoxicity. With LDL oxidized at 0.5 mg/ml, a similar curve was obtained that was shifted to the right. At 1 mg/ml no significant IL-1β release was observed until 18 h of oxidation, which was the only timepoint at which significant TBARS was observed. With LDL at 2 mg/ml, little oxidation took place and IL-1β release was low.

Our previous demonstration (19, 20) that 9-HODE was a major component of modified LDL and could, by itself, induce IL-1β release from mononuclear cells led us to determine the content of HPODE and HODE in LDL subjected to varying conditions of oxidation. As determined by normal phase HPLC, oxidation of LDL at 0.2 mg protein/ml produced preparations with virtually no detectable conjugated dienes (**Fig. 5**). At 2 mg LDL protein/ml, numerous conjugated dienes were detected with the predominant species being 9- and 13-HPODE and 9- and 13-HODE. Apparently, at low LDL concentrations the HPODEs, which serve as precursor to HODEs, undergo rapid, Cu^{2+}-dependent cleavage to aldehydes as a

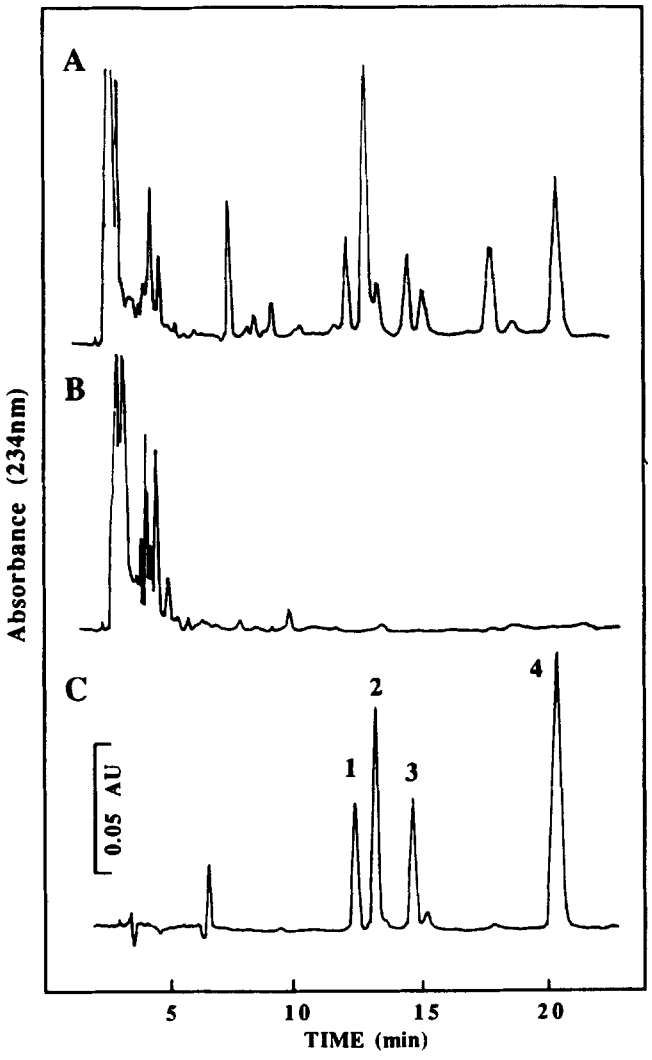

Fig. 5. HPLC analysis of conjugated dienes in oxidized LDL. The lipid fraction was extracted from LDL oxidized at a concentration of 2 (A) or 0.2 mg/ml (B) with 10 μM Cu^{2+} for 24 h. After saponification, the lipids were analyzed by normal phase HPLC and monitored by absorbance at 234 nm. (C) Peaks 1,2,3, and 4 represent 1 μg each of standards of 13-HPODE, 9-HPODE, 13-HODE, and 9-HODE, respectively.

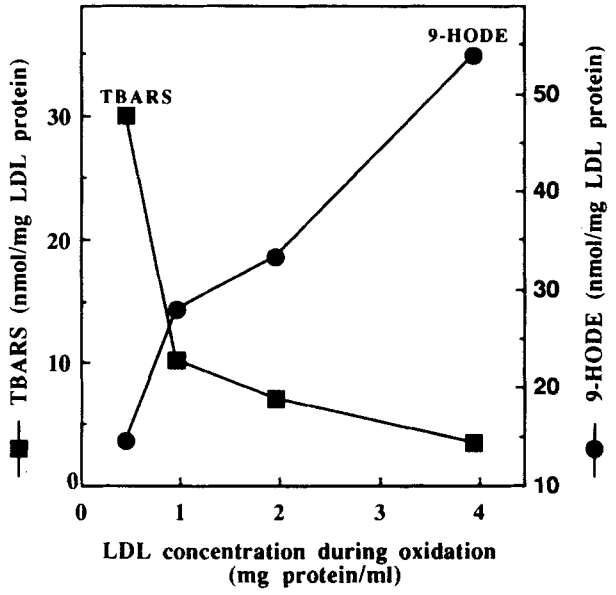

Fig. 6. Effect of LDL concentration on 9-HODE and TBARS content of Cu^{2+}-modified LDL. LDL were oxidized with 20 μM Cu^{2+} for 18 h at concentrations of 0.5, 1, 2, or 4 mg LDL protein/ml. Aliquots were taken for TBARS (■) determination and the lipid fraction was extracted and analyzed for 9-HODE content (●) by HPLC.

result of the high Cu^{2+} to polyunsaturated fatty acid ratio. This is supported by TBARS of 12 and 66 nmol/mg LDL protein at 2 and 0.2 mg/ml LDL, respectively. The inverse relationship between aldehyde formation (TBARS) and HPODE or HODE content was examined in more detail by incubating LDL at 0.5, 1, 2, or 4 mg/ml with 20 μM Cu^{2+} for 18 h. It was seen (**Fig. 6**) that as the ratio of Cu^{2+} to LDL decreases a corresponding decrease in TBARS occurs concomitant with an increase in 9-HODE.

The negative correlation (Fig. 6) between TBARS and HODE indicates that, in highly modified LDL, products other than HODEs which are derived from hydroperoxides are the major contributors to IL-1β release. Likely candidates for this activity include the various aldehydes that result from Cu^{2+}-dependent cleavage of linoleate hydroperoxides (10). Quantitation of aldehydes in highly modified Cu^{2+}-LDL indicated that the major products were hexanal and malondialdehyde, although 4-HNE, 2,4-heptadienal, 2,4-decadienal, and 2-octenal were also present (**Table 1**). Other DNPH derivatives were also found in minor amounts and not subjected to further study.

The potential for aldehydes to contribute to the induction of IL-1β by oxidized LDL was further examined by dialysis experiments. LDL (0.5 mg/ml) were oxidized for 18 h with 6 μM Cu^{2+}. After termination with EDTA, one-half of the LDL was dialyzed overnight against PBS. Upon analysis of aldehydes in two separate experiments, an average of 67% of the aldehydes was removed from the

TABLE 1. Aldehydes in highly modified Cu^{2+}-oxidized LDL

Aldehyde	LDL Protein[a]	% of Total
	nmol/mg	
Hexanal	135.0 ± 19.5	75
4-Hydroxynonenal	2.7 ± 0.4	1.5
2,4-Heptadienal	2.1 ± 0.3	1.2
Octenal	2.3 ± 0.2	1.3
2,4-Decadienal	2.6 ± 1.0	1.4
Malondialdehyde[b]	34.9 ± 6.9	19.6
Total	179.5 ± 13.4	100

LDL (0.5 mg/ml) were incubated with 6 μM $CuSO_4$ for 18 h. Aldehydes were derivatized with DNPH and quantitated as described in Methods.

[a]Values represent means ± SEM for four separate incubations.

[b]Determined by TBARS, which has been shown to measure predominantly malondialdehyde in Cu^{2+}-oxidized LDL (32).

LDL by dialysis (data not shown). Subsequently, we determined whether simply passing the LDL over the Detoxigel column affected the aldehyde content. The column removed, on average, only 8% of the total aldehydes, while the combination of dialysis and Detoxigel resulted in a 61% loss of aldehydes. These various preparations of LDL were tested for their ability to induce IL-1β with mononuclear cells obtained from three donors. The oxidized LDL that were subjected only to Detoxigel induced IL-1β release, while the preparation that was also dialyzed did not stimulate IL-1β release (**Fig. 7**).

The ability of the aldehydes contained in highly modified Cu^{2+}-LDL to directly induce IL-1β release was next examined. As shown in **Fig. 8,** treatment of mononuclear cells for 30 min with 2,4-decadienal and, to a lesser extent, 2-octenal and 2-hexenal caused IL-1β release as determined at 24 h. Under these conditions no LDH release was observed. Interestingly, their saturated homologues were without effect. A dose–response study (not shown) confirmed the ability of 2,4-decadienal and 2-octenal to induce IL-1β release, and that decanal and octanal were without effect up to 100 μM.

The kinetics of IL-1β release by the aldehydes was further studied using 2,4-decadienal. Mononuclear cells were incubated with the aldehyde (5–80 μM) for time periods of 5 min to 24 h. At the times indicated in **Fig. 9,** the aldehydes were removed, fresh medium was added, and IL-1β in the medium was determined at both 24 h and in the medium that had been removed at the specified time. The release of LDH was also determined such that any cytotoxic effect could be determined. At 5 μM of 2,4-decadienal, IL-1β release was comparable to vehicle-treated controls at all time points (Fig. 9). At 20 μM, there was a time-dependent increase in IL-1β release beginning with a 1-h incubation, while 80 μM 2,4 decadienal induced IL-1β in a similar fashion but to a greater extent. Only at 24 h with 20 and 80 μM 2,4-decadienal was any LDH release observed (27 and 30%, respectively).

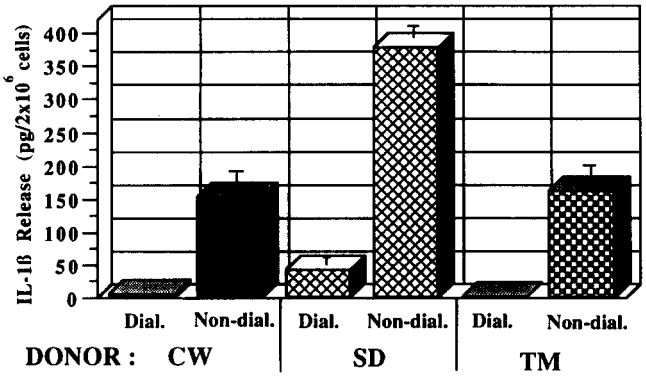

Fig. 7. Effect of dialysis on the ability of Cu^{2+}-modified LDL to induce IL-1β release. LDL were oxidized (0.5 mg/ml) with 6 μM Cu^{2+} for 18 h. A portion of the LDL was then dialyzed overnight against 4 l of PBS. All preparations were then passed over Detoxigel prior to incubation with mononuclear cells (2 × 10^6 cells/well) from three donors. IL-1β release was determined at 24 h and the data represent means ± SEM for triplicate wells. The TBARS values following Detoxigel were 4 and 32 nmol/mg protein for dialyzed and nondialyzed LDL, respectively.

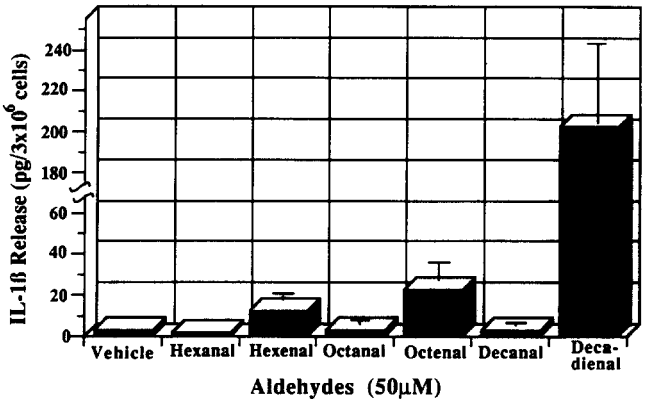

Fig. 8. Effect of linoleate-derived aldehydes on IL-1β release from mononuclear cells. Human peripheral blood mononuclear cells (3 × 10^6 cells/well) were incubated for 30 min with 50 μM of the indicated aldehydes. The cells were washed, the medium was replaced, and incubation was continued for an additional 24 h at which time IL-1β in the culture medium was determined by ELISA. Values represent means ± SEM for triplicate cultures.

DISCUSSION

These findings demonstrate that the term "oxidatively modified LDL" can refer to LDL preparations that vary widely with respect to composition of lipid oxidation

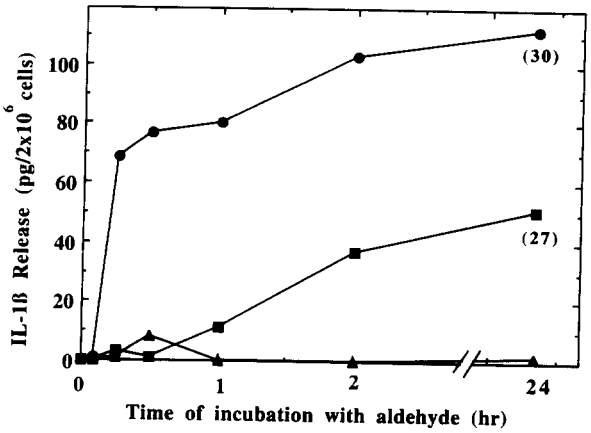

Fig. 9. Dose- and time-dependent effects of 2,4-decadienal on IL-1β release from mononuclear cells. Human peripheral blood mononuclear cells (2 × 10^6 cells/well) were incubated with 5 (▲), 20 (■), or 80 (●) μM 2,4-decadienal for 5, 15, 30, 60, and 120 min or 24 h. At those times, the medium was removed, fresh medium was added, and the cells were incubated for a total time of 24 h. IL-1β was determined in the medium at the time it was removed and at 24 h. Data points represent total IL-1β and are the average of two separate wells, as the requirements to do the entire experiment from a single donor limited the number of replicates. After 24 h, the cell was lysed and LDH in the medium and cell lysate was determined. Only at 24 h, with 20 and 80 μM 2,4-decadienal, was any LDH release observed as indicated in parentheses (% released relative to total LDH).

products and associated biological activity. The heterogeneity among various preparations of LDL modified by incubation with Cu^{2+} or mononuclear cells was apparent to us in our studies on IL-1β release in response to oxidized lipoproteins. While we (19) and others (18) identified 9-HODE as a major component of Cu^{2+}-LDL, and demonstrated its ability to directly induce IL-1β release (20), the degree of induction by 9-HODE or its cholesteryl ester was generally less than that of a lipoprotein containing less 9- and 13-HODE. This finding could be rationalized by multiple factors in modified LDL that contribute to IL-1β release.

The potential for multiple effectors to be present in modified LDL was addressed by preparing LDL of varying degrees of oxidation. Initially, the extent of LDL modification was dictated by varying the concentrations of both Cu^{2+} and LDL in the incubation which, in effect, determines the Cu^{2+} to polyunsaturated fatty acid ratio. It is apparent from the results in Fig. 2 that the extent of oxidation is particularly sensitive to the LDL concentration, with the lowest amount of LDL producing the highest TBARS yield on a per mg LDL protein basis. This can be explained by the ability of the Cu^{2+} to effectively cleave lipid hydroperoxides to aldehydic products. As the LDL concentration is increased, the amount of Cu^{2+}, or perhaps its accessibility to hydrophobic regions of the LDL (30), becomes limiting as evidenced by a much lower TBARS value and less of an increase in mobility in agarose gels.

When the LDL preparations oxidized in this fashion were tested for their ability to induce IL-1β, the most highly modified preparations were the most potent activators. To ensure that this phenomenon was not related to unique oxidation products generated by increasing the Cu^{2+} concentration beyond that generally used for in vitro oxidation, we also varied the length of oxidation time while maintaining Cu^{2+} at 6 μM. Again, significant modification of the LDL (TBARS > 15 nmol/mg protein) was required for induction of IL-1β release (Fig. 4). It was apparent, however, that upon prolonged incubation with Cu^{2+} at low LDL concentrations, oxidation products are produced that are cytotoxic as manifested by a decrease in IL-1β release. While 9-HODE, and to a lesser extent 13-HODE, can induce IL-1β (20) we found that there was an inverse relationship between HODEs and TBARS (Figs. 5 and 6). Thus, a large part of the IL-1β inducing activity in highly modified preparations must be attributable to aldehydes generated from linoleate, and perhaps arachidonate.

Examination of the aldehydes present in oxidatively modified LDL indicated that the predominant species was hexanal, in agreement with Esterbauer et al. (31). While in our experiments hexanal comprised a greater percentage of the total aldehydes (75%) than reported by Esterbauer et al. (42–51%), this may be explained by differences in incubation times and the amounts of Cu^{2+} and LDL in the reaction mixtures. Furthermore, there were several minor peaks in both the Class I and Class III fractions that were not identified and would have decreased the percentage of hexanal relative to total aldehydes. In MM-LDL (data not shown) we found primarily hexanal and 4-HNE in a ratio of 3:1, which is in excellent agreement with other work from Esterbauer and colleagues (32) with autoxidized LDL, that is, oxidized in the absence of Cu^{2+} and thus being much less modified.

The contribution of the aldehydes to the release of IL-1β was further demonstrated by dialysis experiments. If the oxidized LDL were extensively dialyzed prior to incubation with the mononuclear cells, they were no longer able to elicit IL-1β release. While the nondialyzed LDL induced IL-1β to different degrees among the three donors (Fig. 7), in all cases the dialyzed preparations were without significant activity. This data is in accordance with the finding that most of the aldehydes were lost upon dialysis. While some of the longer chain aldehydes remained associated with the LDL, it is possible that their effective concentration was insufficient to activate IL-1β release. This threshold effect also occurs with 9-HODE where concentrations below 10 μM have little effect on the release of the cytokine (20), whereas a dose-dependent increase is observed above this concentration.

The unsaturated aldehydes 2,4-decadienal, 2-octenal, and 2-hexenal, in decreasing order of potency, were shown to directly induce IL-1β release. We have also determined that 2,4-heptadienal can elicit a similar response (unpublished results). The total amount of 2,4-heptadienal, 2-octenal, and 2,4-decadienal averaged 6.93 nmol/mg protein (Table 1), which calculates to approximately 5 mM in the LDL. With 300 μg/ml LDL, this represents a concentration of aldehydes of approximately 2 μM in the culture medium. While this concentration of aldehyde is below that at which 2,4-decadienal alone induces IL-1β, it is difficult to directly compare the two phenomena. First, preparations of oxidized LDL that are most active at inducing IL-1β release contain both aldehydes and 9-HODE. Their effects are at least additive; whether they might synergistically induce IL-1β release has not been addressed. Alternatively, being contained within the oxidized LDL may facilitate aldehyde uptake or affect subcellular distribution to increase efficacy. For example, we have determined that incorporating 9-HODE into acetylated LDL significantly enhances the release of IL-1β (33). Thus, the presence of such unsaturated aldehydes may, at least in part, explain the greater ability of Cu^{2+}-LDL to induce IL-1β than equimolar 9-HODE alone. Also, very recent preliminary evidence (34) indicates that the protein component of oxidized LDL can stimulate IL-1α release from lesion-derived macrophage foam cells; IL-1β was not examined. Thus, it is clear that multiple effectors exist in LDL that can all contribute to an overall up-

regulation of IL-1 release from macrophages.

It is of interest that the corresponding saturated homologues of the unsaturated aldehydes were unable to elicit IL-1β release, which suggests that reactivity with cellular constituents is a prerequisite for aldehyde-induced IL-1β release. Unsaturated aldehydes, particularly alkenals and 4-HNE, have been shown to affect a host of cellular functions including mitochondrial respiration (35) and protein synthesis (36) leading to cell cytotoxicity. Unsaturated aldehydes readily undergo Michael addition to -SH groups and thiol-disulfide status affects numerous biochemical processes (37). It was recently reported (38–40) that the binding of the transcription factor NF-$\varkappa$B to its inhibitory protein I-$\varkappa$B is controlled by the sulfhydryl status of NF-$\varkappa$B. Evidence also exists that NF-$\varkappa$B and protein kinases A and/or C may be involved in the transcription of IL-1β (41), thus, activation of IL-1β release by alkenals and highly modified lipoproteins may be mediated via reactivity with cellular -SH groups. Surprisingly, in our studies with several different blood donors, 4-HNE did not directly activate IL-1β release, nor did malondialdehyde (data not shown). Also, there was a trend towards enhanced activity with increasing chain length (Fig. 7) which further suggests some degree of specificity with regard to the activation of signal transduction.

It must be considered that unsaturated aldehydes (11) and oxidized lipoproteins (6) have been shown to be toxic to a variety of cell types. In our studies the most highly modified preparations of LDL induced significant LDH leakage, as did prolonged treatment with 2,4-decadienal or 2-octenal. It has been shown that IL-1β can be released from cells as a result of plasma membrane damage (42). Accordingly, it cannot be ruled out that the stimulation of IL-1β by highly oxidized LDL is partially due to cytotoxicity and, likewise, severe cellular damage likely accounts for the decreased IL-1β response to LDL oxidized at a high Cu^{2+} to LDL ratio (Fig. 4). Our studies with aldehydes, in the absence of LDL, were carefully monitored for cytotoxicity and assessment of effects on IL-1β release done only under conditions that did not overtly damage the cells. Thus, the results obtained indicate a true induction of IL-1β release by unsaturated aldehydes.

The current results and those of others (6–8, 16, 17, 43, 44) indicate that the composition of modified lipoproteins markedly influences the type and degree of biological response they elicit. While we find that oxidized LDL or 9-HODE, at nontoxic doses, induce the release of IL-1β, Fong, Fong, and Cooper (43) very recently reported that oxidized LDL inhibit IL-1β release from macrophages in response to lipopolysaccharide (LPS). Similar findings were described by Hamilton, Ma, and Chisolm (44) for TNFα and IL-1α. However, in each of these studies the macrophages were preincubated with oxidized LDL for a period of time prior to administration of LPS, whereas in our work the enhancement of IL-1β release was observed in the absence of additional stimuli. The difference between those results and ours is not presently known as the exact signal transduction pathway for LPS is incompletely understood and it is possible that 9-HODE and lipid-derived aldehydes activate IL-1β expression by a different mechanism than LPS.

Similar evidence for multiple effects of oxidized LDL in smooth muscle cells was provided by Zhang et al. (45) who reported that LDL containing low TBARS enhanced prostanoid levels in the cells while high TBARS-containing LDL diminished overall prostanoid production but stimulated production of nonspecific lipid oxidation products. We too see such a biphasic response with regard to IL-1β when highly modified LDL are studied. Clearly, extensively oxidized LDL can be cytotoxic and lead to a diminished response. We also find that a TBARS level of 15–20 nmol/mg protein is required for activation of IL-1β release. It is unlikely that, in vivo, LDL are oxidized to the extent that cytotoxicity to mononuclear cells occurs. Whether sufficient oxidation occurs to elicit the response described herein awaits the development of more specific means of quantifying in vivo oxidation.

A contribution of IL-1β to the atherosclerotic process has yet to be demonstrated conclusively, however, a 640-fold increase in IL-1β mRNA level was found in human lesions (23) with a significant increase also reported in cholesterol-fed nonhuman primates (24). Several studies (46–49) have reported on the presence of lipid oxidation products in human atherosclerotic plaques with a major constituent identified as cholesteryl-9-HODE. A recent report demonstrated that human recombinant IL-1 receptor antagonist inhibited proliferation of rat vascular smooth muscle cells in culture (50). Together, these findings indicate a causal role for lipid oxidation and IL-1β in atherosclerosis and suggest that antioxidants may provide a rational approach for the prevention or treatment of the disease. Recent work has demonstrated that α-tocopherol (51, 52), β-carotene (53), and ascorbic acid (54, 55) all increase the resistance of LDL to oxidation. Probucol, a hypocholesterolemic drug with potent antioxidant activity (56), markedly diminishes lesion formation in animal models of atherosclerosis and inhibits IL-1β release from murine peritoneal macrophages (57) and THP-1 cells (58). Prevention or minimization of LDL oxidation by antioxidant therapy could protect against the release of IL-1β, as well as other biologic effects attributed to oxidized LDL. This approach could conceivably provide a therapeutic entity that acts upon multiple processes that may all impact on the initiation and/or progression of atherosclerosis.

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