Nonenzymatic oxidative cleavage of peptide bonds in apoprotein B-100

Loren G. Fong, Sampath Parthasarathy, Joseph L. Witztum,[1] and Daniel Steinberg

Department of Medicine, Division of Endocrinology and Metabolism, University of California, San Diego, La Jolla, CA 92093

Abstract Incubation of low density lipoprotein (LDL) with endothelial cells converts it to a form that is avidly degraded by macrophages via the acetyl LDL receptor. This modification has previously been shown to be accompanied by extensive breakdown of the major LDL protein (apoB-100) to smaller peptides. ApoB-100 is known to undergo partial degradation during isolation and purification which is commonly attributed to proteolytic enzymes derived from plasma or to contaminant bacteria. In the present studies addition of any of ten different inhibitors of proteolytic enzymes failed to inhibit the endothelial cell-induced degradation of LDL apoB-100 or its subsequent enhanced rate of degradation by macrophages (termed *biological modification*). Conversely, deliberate digestion of LDL with any of five well-characterized proteolytic enzymes degraded apoB-100 extensively but did not cause *biological modification*. The disappearance of intact apoB-100 during incubation with endothelial cells paralleled the formation of thiobarbituric acid (TBA)-reactive substances and the breakdown could be completely prevented by the addition of antioxidants or metal chelators. Finally, the incubation of LDL with a free radical-generating system (dihydroxyfumaric acid and Fe^{3+}-ADP) in the absence of cells resulted in the breakdown of apoB-100. These results suggest that the breakdown of apoB-100 during oxidative modification of LDL, whether cell-induced or catalyzed by transition metals, is not mediated by proteolytic enzymes but rather is linked to oxidative attack on the polypeptide chain, either directly or secondary to peroxidation of closely associated LDL lipids.—**Fong, L. G., S. Parthasarathy, J. L. Witztum, and D. Steinberg.** Nonenzymatic oxidative cleavage of peptide bonds in apoprotein B-100. *J. Lipid Res.* 1987. **28:** 1466—1477.

Supplementary key words atherosclerosis • lipid peroxidation

The atherosclerotic lesion is marked by the presence of lipid-laden 'foam cells', currently believed to be largely, although not exclusively, of monocyte-macrophage origin (1–3). Resident macrophages possess few receptors for native LDL and take up native LDL poorly compared to the very high rate at which they degrade certain chemically modified forms of LDL (e.g., acetyl LDL (4) and malondialdehyde-conjugated LDL (5)). These modified forms of LDL are degraded by way of a receptor termed the 'scavenger receptor' or acetyl LDL receptor. Recent studies have established that LDL incubated under appropriate conditions with cultured endothelial cells (6, 7), smooth muscle cells (7–9), or phagocytes (10–12) is converted to a modified form that is then toxic to cultured cells (7, 10) and which is degraded 3–10 times more rapidly by macrophages than is native LDL (6, 8, 9, 11). Such degradation is inhibited competitively by ligands that bind to the scavenger receptor (e.g., acetyl LDL, fucoidin, and polyinosinic acid).

The cell-mediated modification of LDL has been shown to involve lipid peroxidation and to be accompanied by extensive breakdown of LDL phospholipids (13) and also of apoB-100 (14). The generation of fragments of apoB-100 during the isolation of LDL has been recognized for some time (15–18) and has generally been assumed to reflect proteolytic degradation due to contaminant enzymes, intrinsic to plasma, such as kallikrein or thrombin (18), or deriving from bacterial contaminants (15). The possibility that oxidative conditions were necessary has been suggested (16, 17) but this issue remains unresolved and the mechanism remains uncertain. Direct damage to the amino acid side chains of proteins has been studied extensively (19–31) but until recently oxidation was not believed to lead to scission of the polypeptide chain. There are now a few examples of limited peptide chain breakage associated with protein oxidation (23–31) but even some of these could also involve the cooperative action of proteolytic enzymes (24). The present study was undertaken to determine whether the extensive degradation of apoB that occurs following incubation of LDL with

Abbreviations: LDL, low density lipoprotein; EC, endothelial cell; apoB-100, apoprotein B-100; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBA, thiobarbituric acid; MDA, malondialdehyde; SBTI, soybean trypsin inhibitor; LBTI, lima bean trypsin inhibitor; PMSF, phenylmethylsulfonyl fluoride; PPACK, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; DHF, dihydroxyfumaric acid; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

[1]Established Investigator of the American Heart Association.

endothelial cells (14) is brought about by a proteolytic enzyme(s) or whether nonenzymatic oxidative damage mediates the breakdown. The results strongly support the latter conclusion, i.e., that apoB breakdown is obligatorily linked to oxidation of LDL lipids and probably to direct or indirect oxidation of the polypeptide chain as well.

MATERIALS AND METHODS

Materials

Carrier-free Na ^{125}I was purchased from Amersham (Arlington, IL). Ham's F-10 medium, Dulbecco's modified Eagle's (DME) medium, and alpha-minimal essential medium (α-MEM) were purchased from Gibco Laboratories (Grand Island, NY). RPMI 1640 medium was from the cell culture facility at the University of California, San Diego. Fetal bovine serum was supplied by Hyclone Laboratories (Logan, UT). Butylated hydroxytoluene (BHT) and ferric chloride were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). The following chemicals were from Bio-Rad Laboratories (Richmond, CA): acrylamide, N,N'-methylene-bis-acrylamide, and sodium dodecyl sulfate (SDS). Porcine pancreas elastase, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK), and benzamidine were purchased from Calbiochem Behring Corp. (La Jolla, CA). Sigma Chemical Co. (St. Louis, MO) supplied soybean trypsin inhibitor (SBTI), lima bean trypsin inhibitor (LBTI), phenylmethylsulfonyl fluoride (PMSF), aprotinin, bestatin, chymostatin, elastatinal, leupeptin, diazoacetylnorleucine methyl ester, epsilon-amino-n-caproic acid, bacitracin, hydrogen peroxide, dihydroxyfumaric acid (DHF), adenosine diphosphate (ADP), human plasma kallikrein, human plasma thrombin, human plasma plasmin, and bovine pancreatic trypsin. Bovine serum albumin (BSA) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN) and radioiodinated using ^{125}I.

Lipoproteins

Human LDL (d 1.019–1.063 g/ml) was isolated from EDTA-treated plasma by ultracentrifugation (32) and was radioiodinated using Na ^{125}I as described (33). All lipoprotein samples were dialyzed against phosphate-buffered saline (PBS) containing 0.01% EDTA. Lipoprotein amounts are given as μg of protein.

Cells

An established line of endothelial cells from rabbit thoracic aorta was a gift from Dr. V. Buonassissi (34). These cells were grown in Ham's F-10 medium containing 15% fetal bovine serum in 60-mm plastic culture dishes and were used at confluency. Resident peritoneal macrophages were isolated from female Swiss Webster mice (25–35 g) by peritoneal lavage with phosphate-buffered saline. They were suspended in α-MEM and 10% fetal bovine serum and plated at 2×10^6 cells in a 12-well dish. The adherent macrophages were used on the following day.

Oxidative modification of LDL

Cell-induced modification of LDL was carried out by incubation with endothelial cells as described earlier (13). Briefly, ^{125}I-labeled LDL (100 μg/ml) was incubated with washed endothelial cells in a volume of 2 ml in Ham's F-10 at 37°C for 24 hr. In other experiments, oxidative modification of LDL was achieved in the absence of cells by incubating ^{125}I-labeled LDL (100 μg/ml) in Ham's F-10 for 24 hr in the presence of 5 μM cupric sulfate (13).

Degradation studies

Macrophage degradation of ^{125}I-labeled lipoprotein was carried out by incubating washed mouse peritoneal macrophages in 1 ml of DME medium containing 10 μg of lipoprotein at 37°C for 5 hr, following which the trichloroacetic acid-soluble non-iodide radioactivity was determined (13). The macrophages were then dissolved in 0.2 N NaOH for the determination of protein by the method of Lowry et al. (35) using BSA as a standard.

Peroxidation assay

The extent of peroxidation was determined by measuring the thiobarbituric acid (TBA)-reactive materials (36) at 532 nm. Freshly diluted samples of tetramethoxypropane, which yields malondialdehyde (MDA), were used as standard.

SDS-polyacrylamide gradient gel electrophoresis

Lipids from iodinated lipoprotein samples were extracted using chloroform and methanol by the method of Bligh and Dyer (37) prior to analysis. The chloroform and aqueous phases were removed without disturbing the protein interface. The tubes were centrifuged briefly at 1500 rpm for 10 min in a Sorvall centrifuge (model RC-3B) with a H2000 rotor to densely pack the protein interfaces. The protein band was then dissolved in sample buffer containing 3% SDS, 10% glycerol, and 5% 2-mercaptoethanol by incubating in boiling water for 3.5 min. Vertical gel electrophoresis was performed according to the method of Laemmli (38) using a 3–14% gradient acrylamide gel at a constant current of 7 mA for 14 hr in an LKB electrophoresis unit with tap water cooling. The gels were fixed in 50% trichloroacetic acid, stained with Coomassie Blue R-250, and then subjected to autoradiography. Proteins of known molecular weights were used as standards (myosin, 205,000; β-galactosidase, 116,000; phosphorylase b, 97,400; bovine serum albumin, 66,000; egg albumin, 45,000; carbonic anhydrase, 29,000; and α-lactalbumin, 14,200).

Agarose gel electrophoresis

The electrophoretic mobility of LDL was measured on 0.7% agarose gels at pH 8.6 in 0.05 M barbital buffer (39).

Enzyme digestion of LDL

All incubations were carried out at 37°C with 200 μg of LDL (1 mg/ml) in the presence of 20 μM butylated hydroxytoluene (BHT). Kallikrein digestion of LDL was performed by the method of Yamamoto, Ranganathan and Kottke (40). Human plasma kallikrein (0.002 units; 9.5 units/mg of protein) was added to LDL in 10 mM Tris-HCl (pH 7.4) and incubated for 24 hr. A second sample of kallikrein (0.002 units) was then added and incubation was continued for another 24 hr. Trypsin and plasmin digestion of LDL were carried out by the method of Coetzee, Gevers, and van der Westhuyzen (41) with slight modifications. Bovine plasma trypsin (2.0 μg) or human plasma plasmin (20 μg) was added to LDL in 20 mM Tris-HCl (pH 7.6) and incubated with trypsin for 1.0 hr or with plasmin for 2.0 hr. Thrombin digestion of LDL was accomplished by the method of Cardin et al. (18). Human plasma thrombin (2.5 μg) was added to LDL in 10 mM Tris-HCl (pH 8.0) and incubated for 20 hr. Elastase digestion was carried out by adding 4.0 μg of porcine pancreas elastase to LDL in 20 mM Tris-HCl (pH 7.4) and incubating for 1.0 hr. Control samples of LDL incubated in the same manner but in the absence of enzymes were always included. Immediately following enzyme digestion, 0.03-ml aliquots (30 μg of LDL protein) of each preparation were removed to assess the completeness of LDL digestion by SDS-PAGE as described above. Lipoprotein-deficient serum was added to the remaining mixture to yield a final concentration of 5 mg/ml and this was then dialyzed overnight against DME medium at 4°C.

Hydrogen peroxide treatment of bovine serum albumin

BSA (100 μg/ml) was incubated with 0.18 M H_2O_2 in F-10 medium (2 ml) at 37°C for 1.0 hr. The solution was then dialyzed overnight against F-10 medium at 4°C. BSA treated in the same manner but in the absence of H_2O_2 was also included as a control.

Free radical-induced lipid peroxidation of LDL

The free radical-generating system described by Mak, Kramer, and Weglicki (42) was used. Briefly, ^{125}I-labeled LDL (100 μg/ml) was incubated with 0.83 mM dihydroxyfumaric acid (DHF) and Fe^{3+}-ADP (0.025 mM $FeCl_3$ chelated by 0.25 mM ADP) in 0.15 M NaCl buffered with 10 mM phosphate (pH 7.2) at 37°C for 20 hr. LDL incubated with either DHF, Fe^{3+}-ADP, or buffer alone were included as controls.

Amino acid analysis

Native LDL or copper-oxidized LDL were delipidated by the method of Bligh and Dyer (37). The protein was then hydrolyzed at 108°C for 24 hr with constant boiling HCl under reduced pressure in sealed tubes. The hydrolyzate was then analyzed on a Spinco model 121 automatic amino acid analyzer.

RESULTS

Endothelial cell-induced modification

We recently reported that incubation of LDL with rabbit aortic endothelial cells in Ham's F-10 medium at 37°C for 24 hr resulted in the complete loss of intact apoB-100 and the appearance of many smaller polypeptides (14). As noted previously by Tal, Silberstein, and Nusser (43), we found that the Coomassie blue staining intensity of the product peptides appeared to be less than expected from the intensity of the original, unoxidized apoB band. Silver staining of the gels also confirmed the complete disappearance of intact apoB-100. An inability of the apoB to enter the resolving gel due to aggregation or loss of low molecular weight fragments off the end of the gel have now been excluded since more than 90% of the radioactivity of ^{125}I-labeled LDL applied can be accounted for by slicing and counting the gel **(Fig. 1)**. There were no discrete peaks of radioactivity that might correspond to specific fragments of apoB. However, autoradiography demonstrated the appearance of a series of lower mole-

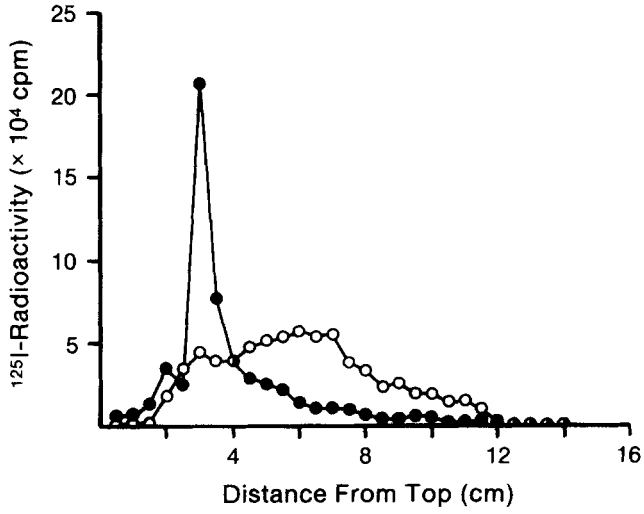

Fig. 1. Distribution of ^{125}I radioactivity associated with native and EC-modified LDL following SDS-PAGE. Samples (30 μg of protein) of ^{125}I-radiolabeled control LDL (●) or EC-modified LDL (○) were subjected to gel electrophoresis. After the gel was fixed, stained, and dried, slices of 0.5 cm in length were cut and counted for ^{125}I radioactivity. The first three sections correspond to the stacking gel.

cular weight proteins with molecular weights ranging from 14,000 to 200,000 **(Fig. 2)**. All autoradiograms of oxidized LDL samples showed a diffuse, dark background plus these several broad protein bands. Five such bands were generally observed whether the LDL was oxidized in the presence of endothelial cells or in the presence of copper in the absence of cells. It should be noted that these fragments did not lose their ability to associate with lipids. They were all isolated together with the modified LDL as an intact lipoprotein complex on density gradient ultracentrifugation. In the absence of inhibitors of proteolytic enzymes, there were additional peptide fragments generated either by enzymes associated with the endothelial cells or adsorbed to LDL.

The amino acid compositions of the delipidated protein from native LDL and from copper ion-oxidized LDL were compared **(Table 1)**. The composition of native LDL apoB-100 was very similar to that reported by Kane, Hardman, and Paulus (44). Oxidation of LDL by copper ion resulted in a consistent decrease in histidine (32%), lysine (15%), and proline (10%) residues as well as a smaller decrease in methionine (6%), while the amount of aspartic acid actually increased (6%). These were the only amino acids whose percentage contribution changed by more than 5% and the differences were statistically significant, except for the change in proline. The decrease in the above amino acids of the oxidized LDL protein is consistent with the reported susceptibility of these amino acids to oxidation (45).

To follow the time course of disappearance of apoB, LDL was incubated with endothelial cells for increasing periods of time **(Fig. 3)**. Loss of intact apoB-100 was noticeable as early as 2 hr and virtually no intact apoB-100 could be detected by 4 hr. With the loss of apoB-100, the appearance of a variety of Coomassie blue-stained bands

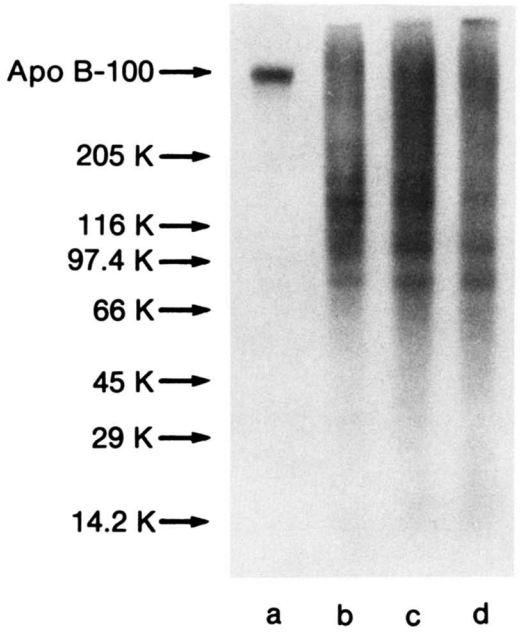

Fig. 2. Autoradiography of native, no cell control, EC- and Cu^{2+}-modified LDL following SDS-PAGE. ^{125}I-labeled LDL (100 μg of protein/ml) was incubated in the presence or absence of endothelial cells or F-10 supplemented with copper (5 μM) in the absence of cells at 37°C for 24 hr. Aliquots of the medium (equivalent to 20 μg of LDL protein) were analyzed by 3–14% gradient SDS-PAGE. The proteins were detected by autoradiography. Lane a, unincubated LDL; lane b, LDL incubated in medium alone; lane c, LDL incubated with EC; lane d, LDL incubated with copper-supplemented F-10 in the absence of cells.

TABLE 1. Amino acid composition of apoB from native and copper ion-oxidized LDL

Amino Acid	Native LDL	Cu-Oxidized LDL	% Difference
Asp	10.8 ± 0.10	11.4 ± 0.08[a]	+5.6
Thr	6.7 ± 0.11	6.7 ± 0.05	0
Ser	8.4 ± 0.25	8.8 ± 0.15	+4.8
Glu	12.4 ± 0.10	12.7 ± 0.11	+2.4
Pro	3.9 ± 0.23	3.5 ± 0.17	−10.2
Gly	4.9 ± 0.14	4.9 ± 0.05	0
Ala	6.0 ± 0.34	6.0 ± 0.20	0
Val	5.2 ± 0.26	5.0 ± 0.12	−3.8
Met	1.7 ± 0.02	1.6 ± 0.02[b]	−5.9
Ileu	5.6 ± 0.07	5.8 ± 0.06[b]	+3.6
Leu	12.4 ± 0.06	12.4 ± 0.20	0
Tyr	3.8 ± 0.14	3.7 ± 0.14	−2.6
Phe	5.4 ± 0.06	5.3 ± 0.11	−1.8
His	2.5 ± 0.05	1.7 ± 0.06[a]	−32.0
Lys	8.0 ± 0.08	6.8 ± 0.16[a]	−15.0
Arg	3.6 ± 0.06	3.7 ± 0.02	+2.8

Unincubated LDL or LDL incubated in F-10 medium supplemented with copper ion (5 μM) at 37°C for 24 hr were delipidated with chloroform and methanol. The amino acid composition of the protein residues was then analyzed as described in Materials and Methods. All values are expressed as nmol of amino acid/100 nmol of total amino acids. Each value is the mean ± SEM of four different determinations done in duplicate.

[a,b] Indicate the mean values that are significantly different at $P < 0.01$ and $P < 0.05$, respectively, from the native LDL group using a two-tailed t-test.

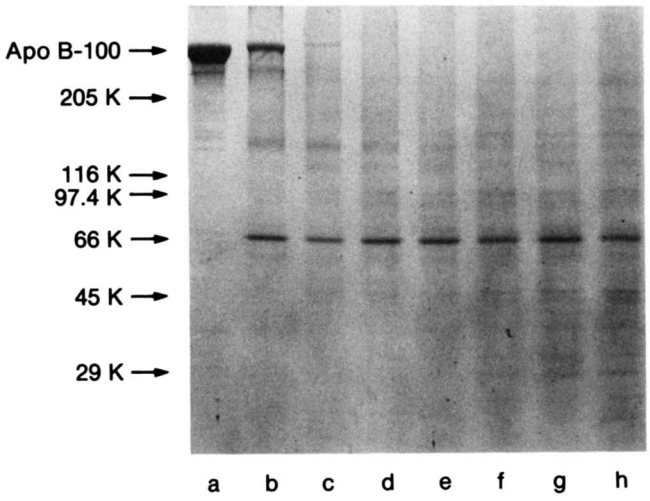

Fig. 3. Time-dependent loss of LDL apoB-100 following incubation with rabbit endothelial cells. Samples (30 μg of LDL protein) of unincubated LDL or LDL incubated with EC from 2 to 24 hr in F-10 were analyzed by SDS-PAGE. Lane a, unincubated LDL; lanes b–h, LDL incubated with cells for 2, 4, 6, 8, 12, 16, and 24 hr, respectively.

of lower molecular weights was evident. Several of these bands, however, were not derived from the LDL as shown by using ^{125}I-labeled LDL and doing autoradiography. These smaller polypeptides originate from the endothelial cells and are present even when LDL is omitted from the medium. Specifically, the prominent 66K band is exclusively derived from endothelial cells; this protein was never detectable on autoradiograms in experiments using ^{125}I-labeled LDL (see Fig. 2) and was equally prominent in medium incubated with EC in the *absence* of LDL (data not shown). This may also be true for the doublet at 45K.

The disappearance of intact apoB-100 closely paralleled the formation of TBA-reactive materials. The formation of TBA-reactive materials (generated as a result of lipid and/or protein peroxidation) could be detected as early as 2 hr, reached a maximun by about 4–8 hr, and then remained constant up to about 24 hr **(Table 2)**. Of the total TBA-reactive materials measured at 24 hr, less than 10% was found to be associated with the delipidated protein of the EC-modified LDL. However, the apparent maximal generation of TBA-reactive material at 4–8 hr must be interpreted cautiously. The TBA assay is not specific for lipid peroxidation products (46) and some of the TBA-reactive materials may be lost to assay due to subsequent reaction with suitable amino groups on the protein or lipid. This could account for the plateauing of the amount of assayable malondialdehyde at later time intervals. Alternatively, peroxides generated initially may go on to yield products with lower yield in the TBA assay. The electrophoretic mobility of the EC-incubated LDL showed the largest increase during the first 6 hr. The LDL migrated as a single discrete band.

Copper ion-induced modification

LDL modified in the absence of cells by cupric ions also showed an increase in TBA-reactive materials and an apparent total loss of intact apoB-100 (Fig. 2, lane d). As in the case of EC-modification of LDL, the generation of TBA-reactive materials during copper-induced modification of LDL correlated well with the loss of intact apoB-100 (data not shown).

Loss of apoB was also observed when LDL was incubated in F-10 medium alone (in the absence of cells and without copper supplementation; see Fig. 2, lane b).

TABLE 2. Modification of LDL during incubation with endothelial cells: correlation between appearance of TBA-reactive materials and increases in electrophoretic mobility

Incubation Condition	Thiobarbituric Acid-Reactive Material	Relative Electrophoretic Mobility
	nmol of MDA/ml	
Unincubated LDL	0.20	1.0
LDL incubated with endothelial cells for:		
2 hr	2.97	1.1
4 hr	5.64	1.6
6 hr	5.98	2.0
8 hr	6.16	2.2
12 hr	6.10	2.3
16 hr	6.10	2.4
24 hr	6.04	2.4

^{125}I-Labeled LDL (100 μg/ml) was incubated in F-10 medium with rabbit endothelial cells at 37°C for the times shown. The generation of TBA-reactive materials in the medium (0.25 ml) and electrophoretic mobility of the LDL (1 μg) were measured as described in Materials and Methods. The results are expressed as the amount of malondialdehyde formed in nmol/ml and the electrophoretic mobility relative to unincubated LDL. The mean values of duplicate samples of a representative experiment are shown.

Although the disappearance of apoB was much slower (intact apoB-100 was still detectable after incubation for 12 hr), it still paralleled the much slower formation of TBA-reactive materials (data not shown). It should be emphasized that the total amount of TBA-reactive materials measured after 24 hr under these mildly oxidative conditions was much lower than the amount generated during endothelial cell-induced LDL modification or during copper-induced LDL modification. In agreement with previous findings (13) this no-cell control LDL was not degraded by macrophages more rapidly than native LDL. The data suggest that limited peroxidation of LDL can result in significant loss of apoB-100 but that a higher level of oxidative damage is required before the modified LDL is recognized by the scavenger receptor.

Tests for LDL-associated proteolytic enzymes

Breakdown of apoB could occur because of the presence of proteolytic activity endogenous to LDL as isolated (47) and possibly activated under oxidative conditions. In an attempt to distinguish this possibility from direct peroxidation-linked protein degradation, heat treatment to inactivate any associated enzymes was tested. LDL was first incubated at 65°C or 70°C for 11 min. Heating the LDL at higher temperatures or for longer periods of time irreversibly denatured it. The heated LDL was then incubated with endothelial cells as previously described and analyzed for TBA-reactive materials and protein breakdown. Such heat treatment had no effect on the subsequent formation of TBA-reactive materials during EC-modification (data not shown) nor did it significantly reduce the breakdown of apoB-100 **(Fig. 4)**.

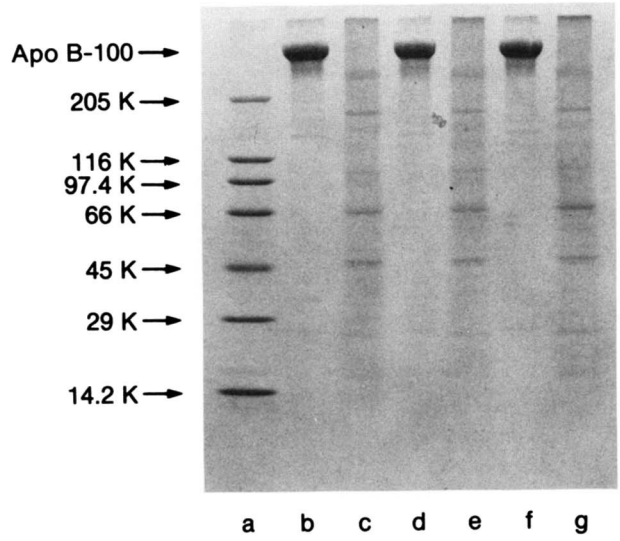

Fig. 4. Effect of preheating LDL on the breakdown of apoB-100 by endothelial cells. LDL (4.8 mg/ml) in PBS containing 0.01% EDTA was incubated at 65°C or 70°C for 11 min and then immediately placed on ice. LDL incubated at 4°C was included as a control. The LDL preparations were then incubated with endothelial cells in F-10 medium and samples (30 μg of LDL protein) were removed and analyzed by SDS-PAGE. Lane a, molecular weight standards; lanes b, d, and f, unincubated 4°C-LDL, 65°C-LDL, and 70°C-LDL, respectively; lanes c, e, and g, endothelial cell-incubated 4°C-LDL, 65°C-LDL, and 70°C-LDL, respectively.

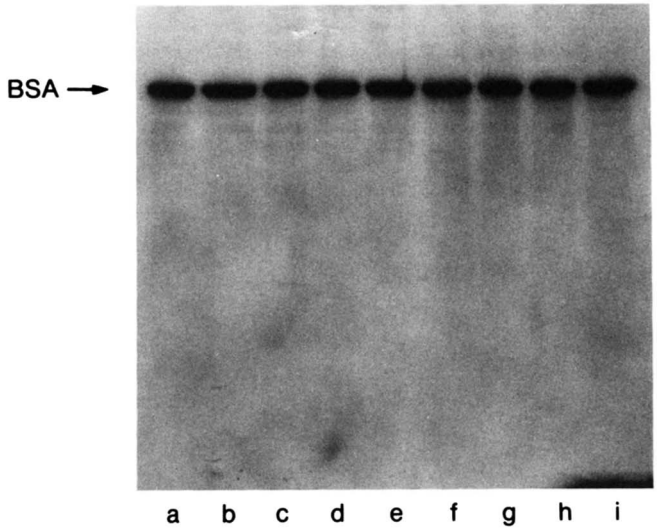

Fig. 5. Inability of LDL to degrade unoxidized or oxidized bovine serum albumin. Samples (50 μg of protein) of unlabeled unincubated LDL, no-cell control LDL, or endothelial cell-incubated LDL were added to 16 μg of ^{125}I-labeled BSA or oxidized BSA (BSA incubated with 0.18 M H_2O_2 at 37°C for 1.0 hr and then dialyzed) and incubated together at 37°C for 2.0 hr. Aliquots of the medium (equivalent to 5 μg of BSA protein) were then subjected to electrophoresis on a 10% polyacrylamide gel. The radiolabeled BSA was then analyzed by autoradiography. Lane a, unincubated native BSA; lanes b–e, native BSA incubated in F-10 medium, or with unincubated LDL, no-cell control incubated LDL, or cell-incubated LDL, respectively; lanes, f–i, oxidized BSA incubated in F-10 medium, or with unincubated LDL, no-cell control incubated LDL, or cell-incubated LDL, respectively.

The possible presence of proteolytic activity was also tested for by using iodinated bovine serum albumin as a substrate. ^{125}I-Labeled native BSA (16 μg) was added to unlabeled native or EC-modified LDL (50 μg) in 0.70 ml of F-10 media and incubated at 37°C for 2.0 hr. Samples of BSA (5 μg) were then subjected to electrophoresis on a 10% polyacrylamide gel and breakdown products were detected by autoradiography. As shown in **Fig. 5**, the co-incubation of native or EC-LDL with BSA did not result in any detectable breakdown of the ^{125}I-labeled BSA. No breakdown was obtained even when the BSA was first oxidized by incubation with 0.18 M H_2O_2 at 37°C for 1.0 hr (Fig. 5) or when native BSA was incubated with LDL during the modification of that LDL by endothelial cells (data not shown).

To further test the possibility that a proteolytic enzyme(s) associated with LDL or released from endothelial cells might account for the breakdown of apoB, we studied

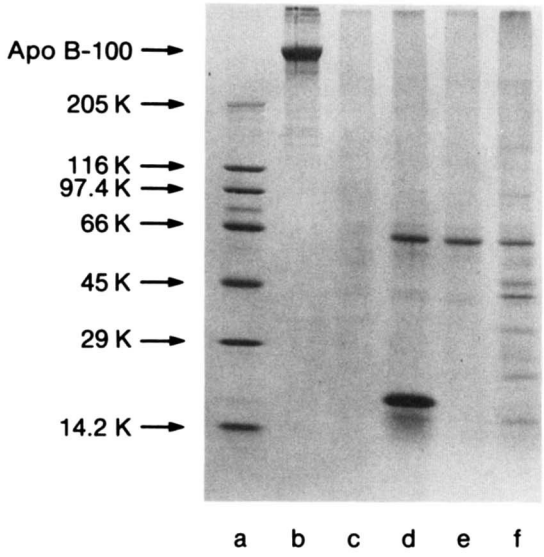

Fig. 6. Effect of the enzyme inhibitors soybean trypsin inhibitor, epsilon-amino-n-caproic acid, or phenylmethylsulfonyl fluoride on the breakdown of apoB by endothelial cells. Soybean trypsin inhibitor (SBTI, 100 μg/ml), epsilon-amino-n-caproic acid (1 mM), or phenylmethylsulfonyl fluoride (PMSF, 1 mM) were incubated together with LDL (100 μg/ml) and cells in F-10 medium at 37°C throughout the 24-hr incubation. Samples of LDL (30 μg of LDL protein) were then analyzed by SDS-PAGE. Lane a, molecular weight standards; lane b, unincubated LDL; lane c, LDL incubated with EC; lane d, LDL incubated with EC and SBTI; lane e, LDL incubated with EC and epsilon-amino-n-caproic acid; lane f, LDL incubated with EC and PMSF.

the effect of a large number of inhibitors of proteolytic enzymes. The addition of soybean trypsin inhibitor (100 μg/ml), epsilon-amino-n-caproic acid (1 mM), or phenylmethylsulfonyl fluoride (1 mM) to the incubation of LDL with endothelial cells did not inhibit either the formation of TBA-reactive materials or the macrophage degradation of the modified LDL (data not shown). Moreover, these inhibitors also were unable to prevent the breakdown of apoB-100 **(Fig. 6)**. Similar negative results were observed during copper-induced modification of LDL (data not shown). Additional inhibitors examined are listed in **Table 3**. Lima bean trypsin inhibitor, elastatinal, benzamidine, aprotinin, chymostatin, bestatin, PPACK, or diazoacetylnorleucine methyl ester had variable effects on the generation of TBA-reactive materials and on the rate of macrophage degradation of the incubated LDL, but none of them significantly inhibited the breakdown of apoB-100. Leupeptin decreased the macrophage degradation of the modified LDL by about 30%. However, it did so without affecting the formation of TBA-reactive materials or apoprotein breakdown. The observed effect was shown to be the result of carry-over of leupeptin to the macrophage degradation system. Bacitracin, a known inhibitor of proteolytic activity (48), actually *increased* the macrophage degradation of the LDL previously incubated with cells. Again, none of the above enzyme inhibitors inhibited the degradation of apoB.

TABLE 3. Lack of effect of proteolytic enzyme inhibitors on the oxidative and biological modification of LDL by endothelial cells

Incubation Condition	Thiobarbituric Acid-Reactive Materials	Macrophage Degradation	Intact ApoB-100
	percent of control		
LDL incubated with endothelial cells alone:	100	100	none
LDL incubated with endothelial cells plus:			
Lima bean trypsin inhibitor (100 μg/ml)	93	103	none
Elastatinal (20 μg/ml)	103	96	none
Benzamidine (2 mM)	88	90	none
Aprotinin (100 μg/ml)	112	104	none
Chymostatin (100 μg/ml)	99	81	none
Bestatin (1 mM)	101	103	none
PPACK (0.1 mM)	100	98	none
Diazoacetylnorleucine methyl ester (1 mM)	124	88	none
Leupeptin (1 mM)[a]	145	70	none
Bacitracin (1 mg/ml)	92	155	none

Lima bean trypsin inhibitor, elastatinal, benzamidine, aprotinin, chymostatin, bestatin, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK), diazoacetylnorleucine methylester, leupeptin, or bacitracin were incubated at the concentrations shown with LDL (100 μg/ml) and endothelial cells in F-10 medium at 37°C for 24 hr. Samples of the medium containing LDL were then analyzed for TBA-reactive products and degradation by macrophages and the results are expressed as a percent of the control incubation (LDL incubated with EC in the absence of any drug). The mean value of duplicate samples of a representative experiment are shown. The values of the control incubations (mean ± SEM) were 5.04 nmol of MDA/ml ± 0.51 (7) and 11.68 μg of ^{125}I-labeled LDL/5 hr per mg cell protein ± 0.65 (7), respectively. The breakdown of apoB-100 was determined by SDS-PAGE.

[a] Leupeptin inhibited the macrophage degradation of EC-modified LDL by 31%.

Effects of purified proteolytic enzymes added to LDL

It has been reported that limited enzymatic digestion of apoB in LDL can in some cases alter its recognition by cells (40, 41, 49, 50). To test whether the enhanced macrophage degradation of oxidatively modified LDL was nonspecifically related to such breakdown of apoB, we examined the effects of limited digestion of LDL with a number of purified proteases (trypsin, kallikrein, plasmin, thrombin, or elastase) on the subsequent degradation of such enzyme-treated LDL by macrophages. Incubation conditions were chosen to insure that very little or no intact apoB-100 remained **(Fig. 7)**. The possible contribution of peroxidation during the incubation with the enzymes was prevented by including 20 μM BHT in the media. As shown in **Table 4**, none of the enzyme-treated LDL preparations were degraded by macrophages any more rapidly than their respective controls.

TABLE 4. Rates of macrophage degradation of EC-modified LDL and of LDL pretreated with purified proteolytic enzymes

LDL Sample	Macrophage Degradation (μg ^{125}I-labeled LDL per 5 hr per mg cell protein)
Unincubated LDL	1.78
LDL incubated with EC	14.50
Kallikrein LDL control	3.34
Kallikrein-treated LDL	2.10
Plasmin LDL control	2.60
Plasmin-treated LDL	3.14
Trypsin LDL control	1.80
Trypsin-treated LDL	2.66
Elastase LDL control	3.74
Elastase-treated LDL	3.32
Thrombin LDL control	1.22
Thrombin-treated LDL	1.42

^{125}I-Labeled LDL (200 μg) was incubated with kallikrein (two additions of 0.002 units), trypsin (2.0 μg), plasmin (20 μg), thrombin (2.5 μg), or elastase (4.0 μg) in Tris-HCl containing 20 μM BHT at 37°C for 48, 1, 2, 20, or 1 hr, respectively. Very little or no intact apoB-100 remained after these treatments (see Fig. 7). LDL incubated under the same conditions but in the absence of any enzyme was also included as a control. Samples of the enzyme-treated LDL (10 μg) were then incubated with macrophages and the amount of trichloroacetic acid-soluble noniodide radioactivity generated was then measured. The results are expressed as the amount of protein degraded in μg after 5 hr per milligram of macrophage cell protein. Unincubated LDL or LDL incubated with endothelial cells for 24 hr were included as controls. The mean values of duplicate samples of a representative experiment are shown.

Linkage between oxidation and apoB breakdown

We have previously reported that oxidation of LDL is very much dependent on the nature of the cell culture medium used. Media such as DME or RPMI do not support the oxidative modification of LDL, probably due to their lower concentrations of metals such as copper and iron (13, 51). The requirement of metals to support cell-mediated oxidation was also indicated by the complete inhibition of modification by low concentrations of EDTA (13). Accordingly, when LDL was incubated in RPMI 1640 instead of F-10 or in F-10 medium in the presence of an antioxidant (BHT), both the formation of TBA-reactive materials **(Table 5)** and the breakdown of apoB **(Fig. 8)** were prevented, confirming the close linkage between oxidation and protein breakdown. The metal chelator EDTA, which considerably inhibited the formation of TBA-reactive materials and the EC-induced biological modification of LDL (i.e., macrophage degradation of the EC-modified LDL (Table 5)), also prevented the breakdown of apoB (Fig. 8). Similar results (i.e., inhibition of all three processes) were also observed when LDL was incubated with endothelial cells in DME medium or in F-10 medium containing vitamin E (data not shown).

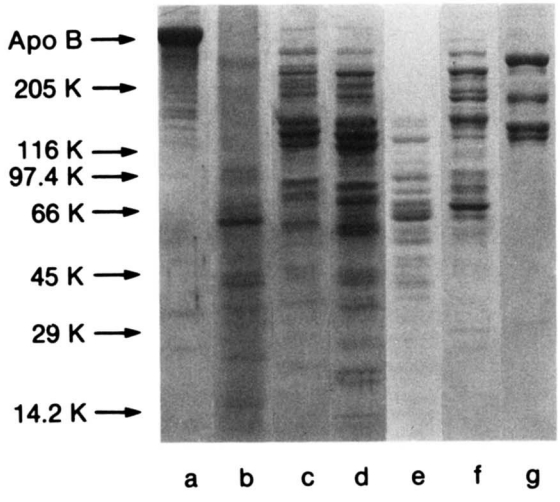

Fig. 7. Limited digestion of apoB-100 by the enzymes kallikrein, plasmin, trypsin, elastase or thrombin. LDL (200 μg) was incubated with kallikrein (2 additions of 0.002 units), plasmin (20 μg), trypsin (2.0 μg), elastase (4.0 μg), or thrombin (2.5 μg) in the presence of 20 μM BHT as described in Materials and Methods. LDL incubated in the absence of any enzyme were also included. Samples of LDL (30 μg of protein) were then analyzed by SDS-PAGE. Lane a, unincubated LDL; lane b, LDL incubated with EC; lanes c–g, LDL incubated with the enzymes kallikrein, plasmin, trypsin, elastase, or thrombin, respectively.

Several lines of indirect evidence, some of which have been described above, make it seem very likely that generation of oxygen free radicals accounts for EC-modification and copper-induced modification of LDL, but this remains an inference. As a direct demonstration that oxygen free radicals can both cause apoB degradation and generate the configuration recognized by the acetyl LDL receptor, we utilized the Fe^{3+}-ADP/di-hydroxyfumaric acid (DHF) system (42). This system provides a rich source of superoxide anion, as the primary product, with subsequent generation of other radicals, including hydroxyl radicals. Incubation of LDL with DHF and Fe^{3+}-ADP resulted in the formation of

TABLE 5. The effect of antioxidants or media not supporting oxidation on the modification of LDL by endothelial cells

Incubation Condition	Thiobarbituric Acid-Reactive Materials (nmol MDA/ml)	Macrophage Degradation (μg ^{125}I-labeled LDL per 5 hr per mg cell protein)
Unincubated LDL	0.30	1.33
LDL incubated with endothelial cells in:		
F-10	3.25	11.31
RPMI	0.26	1.06
F-10 plus 20 μM BHT	0.36	0.94
F-10 plus 1 $\times$ 10^{-4} M EDTA	1.02	0.99

^{125}I-Labeled LDL (100 μg/ml) was incubated with endothelial cells in RPMI 1640 medium or in F-10 medium with or without the addition of butylated hydroxytoluene (40 nmol added in 20 μl of ethanol) or EDTA (1 $\times$ 10^{-4} M) in a 2-ml volume at 37°C for 24 hr. The amount of TBA-reactive materials generated and macrophage degradation were measured and expressed as described in Materials and Methods. The presence of ethanol during the incubation with cells or the addition of BHT or EDTA at the end of the incubation had no effect on the generation of TBA-reactive materials or macrophage degradation. The mean values of duplicate samples of a representative experiment are shown.

TBA-reactive materials **(Table 6)**, accompanied by an increased macrophage degradation of the oxidized LDL. ApoB was completely broken down under these conditions. In the absence of any component of the complete oxidation system, there was little or no TBA-reactive material generated nor apoB breakdown and the LDL incubated under such conditions was not degraded more rapidly by macrophages than control incubated LDL.

DISCUSSION

We have presented several lines of evidence that the breakdown of apoB-100 during oxidative modification of LDL is not catalyzed by proteolytic enzymes associated with the LDL or released by cultured endothelial cells: *a*) none of the inhibitors of proteolytic enzymes had any effect; *b*) heat treatment of LDL had no effect (65–70°C for 11 min); *c*) ^{125}I-labeled BSA incubated with native or EC-modified LDL underwent no breakdown; *d*) attempts to mimic the pattern of apoB degradation occurring during oxidative modification by incubation with purified proteolytic enzymes were negative; *e*) apoB degradation occurred only in media supporting oxidation and was inhibited by antioxidants such as BHT or EDTA. The breakdown of apoB during the isolation and storage of lipoproteins has been frequently noted (15–18) but most investigators have presumed that it reflected the presence of proteolytic contaminants and have shown that the addition of inhibitors of bacterial growth and inhibitors of proteolysis protects the apoB (15, 18). Indeed, loss of intact apoB during LDL preparation undoubtedly occurs at times on this basis. The present studies show that under appropriate conditions apoB breakdown can occur in vitro simply on the basis of oxidative damage, without necessarily invoking proteolytic enxymes as the cause. In the present studies, isolation of LDL in the presence of antibiotics and of inhibitors of both kallikrein and thrombin failed to prevent the subsequent breakdown of apoB during incubations under the oxidative conditions as described above.

The general pattern of breakdown for different preparations of LDL was similar and the pattern was similar whether the LDL was oxidized by endothelial cells or in

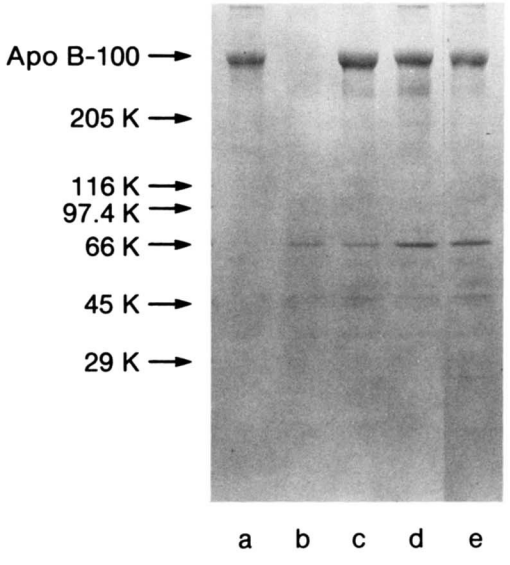

Fig. 8. Effect of antioxidants or media not supporting oxidation on the breakdown of apoB-100 by endothelial cells. LDL was incubated with EC in F-10 RPMI 1640 or F-10 with 20 μM BHT or 10^{-4} M EDTA. Aliquots of the media (30 μg of LDL protein) were then analyzed by SDS-PAGE. Lane a, unincubated LDL; lane b, LDL incubated with EC in F-10; lane c, LDL incubated with EC in RPMI 1640; lane d, LDL incubated with EC in F-10 and BHT; lane e, LDL incubated with EC in F-10 medium with EDTA.

TABLE 6. Modification of LDL by dihydroxyfumaric acid and Fe^{3+}-ADP

Incubation Condition	Thiobarbituric Acid-Reactive Materials (nmol MDA/ml)	Macrophage Degradation (μg ^{125}I-labeled LDL per 5 hr per mg cell protein)	Intact ApoB Remaining
Unincubated LDL (F-10)	0.44	1.86	yes
LDL incubated with Cu^{2+} (F-10)	8.18	14.74	none
LDL incubated with:			
Buffer	0.44	2.09	yes
Fe^{3+}-ADP	0.80	2.32	yes
DHF	1.21	1.41	yes
Fe^{3+}-ADP + DHF	8.90	11.48	none

^{125}I-Labeled LDL (100 μg/ml) was incubated with dihydroxyfumaric acid (0.83 mM) and Fe^{3+}-ADP (0.025 mM $FeCl_3$/0.25 mM ADP) in 0.15 M NaCl buffered with 10 mM phosphate (pH 7.2) at 37°C for 20 hr. Unincubated LDL or LDL incubated with 5 μM Cu^{2+} in F-10 medium at 37°C for 20 hr were included as controls. The generation of TBA-reactive materials in the medium (0.25 ml) and the macrophage degradation of the incubated LDL (10 μg) were measured as described in Materials and Methods. The results are expressed as the amount of malondialdehyde formed in nmol/ml and the amount of trichloroacetic acid-soluble non-iodide radioactivity generated after 5 hr per mg of cell protein, respectively. The mean values of duplicate samples of a representative experiment are shown. The breakdown of apoB-100 was determined by SDS-PAGE.

the presence of copper ions. These results suggest that certain areas in the apoB protein are peculiarly sensitive to cleavage. However, the peptide bands tend to be broad and incompletely resolved. The reasons for this are not clear but may relate to the varying degrees of oxidation of amino acid side chains involved.

The reproducibility of the changes in amino acid composition during oxidative modification of LDL (Table 1) indicates that certain residues are uniquely sensitive to oxidative damage. Only histidine, lysine, and proline showed decreases of more than 10% and there may have been a significant but very small decrease in methionine (5.9%). There was a small but significant increase in aspartic acid content, compatible with the previously reported oxidative opening of the histidine-imidazole ring to convert it to aspartic acid (52). The specificity of the changes in amino acid composition may reside in part in the intrinsic susceptibility of different side chains to oxidative damage but may also reflect the relationship between the polypeptide chain of apoB and its lipid environment. The close association of the hydrophobic regions of the protein with fatty acyl chains could provide opportunities for nascent lipid peroxide radicals to interact *selectively* with adjacent portions of the polypeptide chain. Intermediates of lipid peroxidation have been reported to react with both protein and DNA, resulting in damage to amino acids (45), cross-linking of proteins (53), and cleavage of DNA strands (54).

Oxidative modification of specific amino acid residues in enzymes with accompanying loss of enzyme activity has been reported (20, 55) and increased proteolytic susceptibility subsequent to oxidative modification has also been reported (56). More recently, direct peptide bond scission by oxygen radicals has been observed. For example, hydroxyl radical (23) and superoxide anion (24) have been reported to cleave collagen into smaller fragments. Recent studies of Kim, Rhee, and Stadtman (31) have suggested the iron-catalyzed oxidative degradation of several proteins in the presence of reduced thiols. Samuni, Chevion, and Czapski (57), Gutteridge and Wilkins (19), and Shinar, Navok, and Chevion (21) have reported the site-specific oxidative damage of proteins by hydroxyl radicals generated locally at sites of protein-bound copper ions. If apoB is able to bind copper ions at specific sites, it is possible that this could be the basis for the cleavage of the apoB chain.

Exactly how the configuration recognized by the acetyl LDL receptor is generated during oxidative modification of LDL remains uncertain. However, studies in this laboratory have recently established that the recognition site is on the peptide chain (58). It was shown that completely delipidated apoB isolated from oxidative modified LDL was recognized and taken up by the acetyl LDL receptor and competed with radioactive acetyl LDL for uptake. However, not every pattern of apoB breakdown yields a molecule recognized by this receptor. As shown in the present study, for example, there can be a complete loss of intact apoB resulting from the action of certain proteolytic enzymes, and yet no enhanced macrophage degradation of the degraded LDL. Moreover, oxidative modification, even when it is sufficient to result in loss of much of the intact apoB, does not necessarily lead to enhanced macrophage degradation. This lack of correlation suggests that rather specific changes in apoB structure are required. These could include addition of lipid peroxide intermediates to the peptide chain (59–61) and/or specific conformational changes leading to exposure of previously buried hydrophobic regions (62).

We thank Joellen Barnett, Jennifer Pattison, and Lorna Joy for their expert technical assistance, and Mary Ellen Seifert for preparation of the manuscript. We also thank Dr. Russell F. Doolittle for the amino acid compositional analyses. This work was supported in part by Grant HL14197-15 and Training Grant HL07276-09 from the National Heart, Lung, and Blood Institute.

Manuscript received 16 April 1987 and in revised form 3 June 1987.

REFERENCES

1. Fowler, S., H. Shio, and N. J. Haley. 1979. Characterization of lipid-laden aortic cells from cholesterol-fed rabbits. IV. Investigation of macrophage-like properties of aortic cell populations. *Lab. Invest.* **41:** 372–378.
2. Schaffner, T., K. Taylor, E. J. Bartucci, K. Fisher-Dzoga, J. H. Beeson, S. Glagov, and R. W. Wissler. 1980. Arterial foam cells with distinctive immunomorphologic and histochemical features of macrophages. *Am. J. Pathol.* **100:** 57–73.
3. Gerrity, R. G. 1981. The role of the monocyte in atherogenesis. I. Transition of blood-borne monocytes into foam cells in fatty lesions. *Am. J. Pathol.* **103:** 181–190.
4. Goldstein, J. L., Y. K. Ho, S. K. Basu, and M. S. Brown. 1979. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc. Natl. Acad. Sci. USA.* **76:** 333–337.
5. Fogelman, A. M., I. Shechter, J. Seager, M. Hokom, J. S. Child, and P. E. Edwards. 1980. Malondialdehyde alteration of low density lipoproteins leads to cholesteryl ester accumulation in human monocyte-macrophages. *Proc. Natl. Acad. Sci. USA.* **77:** 2214–2218.
6. Henriksen, T., E. M. Mahoney, and D. Steinberg. 1981. Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: recognition by receptors for acetylated low density lipoproteins. *Proc. Natl. Acad. Sci. USA.* **78:** 6499–6503.
7. Morel, D. W., P. E. DiCorleto, and G. M. Chisolm. 1984. Endothelial and smooth muscle cells alter low density lipoprotein in vitro by free radical oxidation. *Arteriosclerosis.* **4:** 357–364.
8. Henriksen, T., E. M. Mahoney, and D. Steinberg. 1983. Enhanced macrophage degradation of biologically modified low density lipoprotein. *Arteriosclerosis.* **3:** 149–159.
9. Heinecke, J. W., L. Baker, H. Rosen, and A. Chait. 1986. Superoxide-mediated modification of low density lipoprotein by arterial smooth muscle cells. *J. Clin. Invest.* **77:** 757–761.
10. Cathcart, M. K., D. W. Morel, and G. M. Chisolm. 1985. Monocytes and neutrophils oxidize low density lipoprotein making it cytotoxic. *J. Leukocyte Biol.* **38:** 341–350.
11. Parthasarathy, S., D. J. Printz, D. Boyd, L. Joy, and D. Steinberg. 1986. Macrophage oxidation of low density lipoprotein generates a modified form recognized by the scavenger receptor. *Arteriosclerosis.* **6:** 505–510.
12. Hiramatsu, K., H. Rosen, J. W. Heinecke, G. Wolfbauer, and A. Chait. 1987. Superoxide initiates oxidation of low density lipoprotein by human monocytes. *Arteriosclerosis.* **7:** 55–60.
13. Steinbrecher, U. P., S. Parthasarathy, D. S. Leake, J. L. Witztum, and D. Steinberg. 1984. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc. Natl. Acad. Sci. USA.* **81:** 3883–3887.
14. Parthasarathy, S., U. P. Steinbrecher, J. Barnett, J. L. Witztum, and D. Steinberg. 1985. Essential role of phospholipase A_2 activity in endothelial cell-induced modification of low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **82:** 3000–3004.
15. Chapman, M. J., and J. P. Kane. 1975. Stability of the apoprotein of human serum low density lipoprotein: absence of endogenous endopeptidase activity. *Biochem. Biophys. Res. Commun.* **66:** 1030–1036.
16. Schuh, J., G. F. Fairclough, Jr., and R. H. Haschemeyer. 1978. Oxygen-mediated heterogeneity of apo-low-density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **75:** 3173–3177.
17. Lee, D. M., A. J. Valente, W. H. Kuo, and H. Maeda. 1981. Properties of apolipoprotein B in urea and in aqueous buffers. The use of glutathione and nitrogen in its solubilization. *Biochim. Biophys. Acta.* **666:** 133–146.
18. Cardin, A. D., K. R. Witt, J. Chao, H. S. Margolius, V. H. Donaldson, and R. L. Jackson. 1984. Degradation of apolipoprotein B-100 of human plasma low density lipoproteins by tissue and plasma kallikreins. *J. Biol. Chem.* **259:** 8522–8528.
19. Gutteridge, J. M. C., and S. Wilkins. 1983. Copper salt-dependent hydroxyl radical formation damage to proteins acting as antioxidants. *Biochim. Biophys. Acta.* **759:** 38–41.
20. Levine, R. L. 1983. Oxidative modification of glutamine synthetase. II. Characterization of the ascorbate model system. *J. Biol. Chem.* **258:** 11828–11833.
21. Shinar, E., T. Navok, and M. Chevion. 1983. The analogous mechanisms of enzymatic inactivation induced by ascorbate and superoxide in the presence of copper. *J. Biol. Chem.* **258:** 14778–14783.
22. Marx, G., and M. Chevion. 1985. Site-specific modification of albumin by free radicals. *Biochem. J.* **236:** 397–400.
23. Curran, S. F., M. A. Amoruso, B. D. Goldstein, and R. A. Berg. 1984. Degradation of soluble collagen by ozone or hydroxyl radicals. *FEBS Lett.* **176:** 155–160.
24. Monboisse, J. C., P. Braquet, A. Randoux, and J. P. Borel. 1983. Nonenzymatic degradation of acid-soluble calf skin collagen by superoxide ion: protective effect of flavonoids. *Biochem. Pharmacol.* **32:** 53–58.
25. Lin, S. W., and K. J. A. Davies. 1985. General aspects of protein damage by oxygen radicals. *Fed. Proc.* **44:** 1093 (abstract).
26. Dean, R. T., C. R. Roberts, and W. Jessup. 1985. Fragmentation of extracellular and intracellular polypeptides by free radicals. *In* Intracellular Protein Catabolism. E. A. Khairallah, J. S. Bond, and J. W. C. Bird, editors. A. R. Liss, New York. 341–350.
27. Aft, R. L. and G. C. Mueller. 1984. Hemin-mediated oxidative degradation of proteins. *J. Biol. Chem.* **259:** 301–305.
28. Creeth, J. M., B. Cooper, A. S. R. Donald, and J. R. Clamp. 1983. Studies of the limited degradation of mucus glycoproteins. The effect of dilute hydrogen peroxide. *Biochem. J.* **211:** 323–332.
29. Schuessler, H., and A. Herget. 1980. Oxygen effect in the radiolysis of proteins. I. Lactate dehydrogenase. *Int. J. Radiat. Biol.* **37:** 71–80.
30. Schuessler, H., and K. Schilling. 1984. Oxygen effect in the radiolysis of proteins. Part 2. Bovine serum albumin. *Int. J. Radiat. Biol.* **45:** 267–281.

31. Kim, K., S. G. Rhee, and E. R. Stadtman. 1985. Nonenzymatic cleavage of proteins by reactive oxygen species generated by dithiothreitol and iron. *J. Biol. Chem.* **260:** 15394–15397.
32. Weinstein, D. B., T. E. Carew, and D. Steinberg. 1976. Uptake and degradation of low density lipoprotein by swine arterial smooth muscle cells with inhibition of cholesterol biosynthesis. *Biochim. Biophys. Acta.* **424:** 404–421.
33. Pittman, R. C., T. E. Carew, C. K. Glass, S. R. Green, C. A. Taylor, Jr., and A. D. Attie. 1983. A radioiodinated, intracellularly trapped ligand for determining the sites of plasma protein degradation in vivo. *Biochem. J.* **212:** 791–800.
34. Buonassisi, V., and J. C. Venter. 1976. Hormone and neurotransmitter receptors in an established vascular endothelial cell line. *Proc. Natl. Acad. Sci. USA.* **73:** 1612–1616.
35. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
36. Yagi, K. 1976. A simple fluorometric assay for lipoperoxide in blood plasma. *Biochem. Med.* **15:** 212–216.
37. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
38. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
39. Noble, R. P. 1968. Electrophoretic separation of plasma lipoproteins in agarose gel. *J. Lipid Res.* **9:** 693–700.
40. Yamamoto, M., S. Ranganathan, and B. A. Kottke. 1985. Structure and function of human low density lipoproteins. Studies using proteolytic cleavage by plasma kallikrein. *J. Biol. Chem.* **260:** 8509–8513.
41. Coetzee, G. A., W. Gevers, and D. R. van der Westhuyzen. 1980. Plasmin-treated low density lipoproteins: polypeptide analyses and metabolism by cultured smooth muscle cells. *Artery.* **7:** 1–15.
42. Mak, I. T., J. H. Kramer, and W. B. Weglicki. 1986. Potentiation of free radical-induced lipid peroxidative injury to sarcolemmal membranes by lipid amphiphiles. *J. Biol. Chem.* **261:** 1153–1157.
43. Tal, M., A. Silberstein, and E. Nusser. 1985. Why does Coomassie brilliant blue R interact differently with different proteins? *J. Biol. Chem.* **260:** 9976–9980.
44. Kane, J. P., D. A. Hardman, and H. P. Paulus. 1980. Heterogeneity of apolipoprotein B: isolation of a new species from human chylomicrons. *Proc. Natl. Acad. Sci. USA.* **77:** 2465–2469.
45. Roubal, W. T., and A. L. Tappel. 1966. Damage to proteins, enzymes, and amino acids by peroxidizing lipids. *Arch. Biochem. Biophys.* **113:** 5–8.
46. Gutteridge, J. M. C. 1982. Free-radical damage to lipids, amino acids, carbohydrates and nucleic acids determined by thiobarbituric acid reactivity. *Int. J. Biochem.* **14:** 649–653.
47. Krishnaiah, K. V., and H. Wiegandt. 1974. Demonstration of a protease-like activity in human serum low density lipoprotein. *FEBS Lett.* **40:** 265–268.
48. Makinen, K. K. 1972. Inhibition by bacitracin of some hydrolytic enzymes. *Int. J. Protein Res.* **4:** 21–28.
49. Carew, T. E., M. J. Chapman, S. Goldstein, and D. Steinberg. 1978. Enhanced degradation of trypsin-treated low density lipoprotein by fibroblasts from a patient with homozygous familial hypercholesterolemia. *Biochim. Biophys. Acta.* **529:** 171–175.
50. Chapman, M. J., T. L. Innerarity, K. S. Arnold, and R. W. Mahley. 1984. In vitro apo-B,E receptor binding and in vivo catabolism of trypsin-modified low density lipoproteins. *In* Latent Dyslipoproteinemias and Atherosclerosis. J. L. De Gennes, J. Polonovskiu, and R. Paoletti, editors. Raven Press, New York. 93–99.
51. Heinecke, J. W., H. Rosen, and A. Chait. 1984. Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. *J. Clin. Invest.* **74:** 1890–1894.
52. Tomita, M., M. Irie, and T. Ukita. 1969. Sensitized photooxidation of histidine and its derivatives. Products and mechanism of the reaction. *Biochemistry.* **8:** 5149–5160.
53. Roubal, W. T., and A. L. Tappel. 1966. Polymerization of proteins induced by free-radical lipid peroxidation. *Arch. Biochem. Biophys.* **113:** 150–155.
54. Morita, J., K. Ueda, K. Nakai, Y. Baba, and T. Komano. 1983. DNA strand breakage in vitro by autooxidized unsaturated fatty acids. *Agric. Biol. Chem.* **47:** 2977–2979.
55. Fucci, L., C. N. Oliver, M. J. Coon, and E. R. Stadtman. 1983. Inactivation of key metabolic enzymes by mixed-function oxidation reactions: possible implication in protein turnover and ageing. *Proc. Natl. Acad. Sci. USA.* **80:** 1521–1525.
56. Rivett, A. J. 1985. Preferential degradation of the oxidatively modified form of glutamine synthetase by intracellular mammalian proteases. *J. Biol. Chem.* **260:** 300–305.
57. Samuni, A., M. Chevion, and G. Czapski. 1981. Unusual copper-induced sensitization of the biological damage due to superoxide radicals. *J. Biol. Chem.* **256:** 12632–12635.
58. Parthasarathy, S., L. G. Fong, D. Otero, and D. Steinberg. 1987. Recognition of solubilized apoproteins from delipidated, oxidized low density lipoprotein (LDL) by the acetyl-LDL receptor. *Proc. Natl. Acad. Sci. USA.* **84:** 537–540.
59. Jurgens, G., J. Lang, and H. Esterbauer. 1986. Modification of human low-density lipoprotein by the lipid peroxidation product 4-hydroxynonenal. *Biochim. Biophys. Acta.* **875:** 103–114.
60. Haberland, M. E., C. L. Olch, and A. M. Fogelman. 1984. Role of lysines in mediating interaction of modified low density lipoproteins with the scavenger receptor of human monocyte macrophages. *J. Biol. Chem.* **259:** 11305–11311.
61. Steinbrecher, U. P. 1987. Oxidation of human low density lipoprotein results in derivatization of lysine residues of apolipoprotein B by liquid peroxide decomposition products. *J. Biol. Chem.* **262:** 3603–3608.
62. Haberland, M. E., and A. M. Fogelman. 1985. Scavenger receptor-mediated recognition of maleyl bovine plasma albumin and the demaleylated protein in human monocyte macrophages. *Proc. Natl. Acad. Sci. USA.* **82:** 2693–2697.