Michael Addition-Type 4-Hydroxy-2-nonenal Adducts in Modified Low-Density Lipoproteins: Markers for Atherosclerosis

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ABSTRACT: It has been proposed that plasma low-density lipoprotein (LDL) undergoes oxidative modification before it can give rise to foam cells in atherosclerosis. Oxidation of LDL generates a variety of reactive aldehyde products including 4-hydroxy-2-nonenal (HNE), which may covalently attach to the LDL apolipoproteins. We here present direct evidence that HNE derivatization of LDL forms Michael addition-type adducts of HNE with histidine and lysine residues of apolipoprotein B-100 (apo B) and also demonstrate the utility of an antibody specific to the HNE adducts generated in the LDL treated with HNE or oxidatively modified by Cu^{2+} or cultured endothelial cells. HNE adducts present in the LDL that had been treated with HNE were attested to be Michael addition-type adducts on the basis of the fact that incubation of LDL with 1 mM HNE (2 h, 37 °C) resulted primarily in the formation of Michael addition-type HNE–histidine (39.9 mol/mol of LDL) and HNE–lysine (19.3 mol/mol of LDL) adducts. An enzyme-linked immunosorbent assay (ELISA) and an SDS–polyacrylamide gel electrophoresis (SDS–PAGE)/immunoblot analysis of HNE-modified LDL demonstrated that these HNE adducts were detectable with the HNE-specific antibody affinity-purified with the Michael adduct (HNE–histidine) as a ligand. The following lines of evidence indicated the presence of Michael addition-type HNE adducts in the oxidatively modified LDL *in vitro*: (i) Amino acid analysis of LDL that had been treated with Cu^{2+} (24 h, 37 °C) demonstrated the presence of a Michael addition-type HNE–histidine adduct (7–9 mol/mol of LDL). (ii) Competitive ELISA demonstrated the inhibition of antibody binding to HNE-modified LDL by the addition of Cu^{2+}-oxidized LDL. (iii) SDS–PAGE/immunoblot analysis of the Cu^{2+}-oxidized LDL demonstrated the appearance of HNE adducts, which were abolished by the addition of a HNE-modified histidyl peptide. Furthermore, HNE adducts were detected in the LDL that had been treated with cultured bovine aortic endothelial cells (24 h, 37 °C). Taken together, this evidence suggests that the Michael addition-type HNE adducts may be reliable markers for atherosclerosis.

Various lines of evidence indicate that an important part of the pathogenesis of atherosclerosis is the oxidative modification of plasma low-density lipoproteins (LDLs) (Steinberg et al., 1989; Ylä-Herttuala et al., 1989, 1990; Palinski et al., 1989; Rosenfeld et al., 1990). It has been proposed that LDL must undergo oxidative modification before it can give rise to foam cells, the key component of progression of atherosclerosis. It has been believed that oxidation of LDL *in vivo* can be reproduced by *in vitro* incubation of LDL with cultured cells such as endothelial cells (Henriksen et al., 1981; Morel et al., 1984; Steinbrecher et al., 1984, 1987), smooth muscle cells (Morel at al., 1984; Heinecke et al., 1986), and macrophages (Cathcart et al., 1985) or by autoxidation catalyzed by cupric ion in the absence of cells (Lenz et al., 1990). These cell-mediated or metal-catalyzed modifications convert LDL to a form that is recognized by macrophages far more readily than normal LDL (Henriksen et al., 1981, 1983). Uptake of oxidized LDL leads to the formation of foam cells, the dominant cells in atherosclerotic lesions (Rosenfeld et al., 1990).

During incubation of LDL with cells, the LDL molecule undergoes a large number of structural changes that alter its metabolism (Steinberg et al., 1989). Although the detailed mechanism for oxidative modification of LDL has not been established, it is generally accepted that the primary generation of lipid hydroperoxide derivatives initiates a reaction cascade leading to rapid propagation and to amplification in the number of reactive oxygen species formed; this leads ultimately to extensive fragmentation of the fatty acid chains (Esterbauer et al., 1992) and conversion of the LDL to a more atherogenic form (Quinn et al., 1987). Some of the lipid peroxidation products, such as malondialdehyde (MDA) and 4-hydroxy-2-alkenals, become covalently attached to the LDL apolipoprotein B-100 (apo B) (Haberland et al., 1982; Jürgens et al., 1986; Hoff et al., 1988) and thereby may contribute to the development of fatty streaks and atheromatous plaques and to progression of atherosclerosis (Ylä-Herttuala et al., 1989;

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[1] Abbreviations: HNE, 4-hydroxy-2-nonenal; apo B, apolipoprotein B-100; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; KLH, keyhole limpet hemocyanin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; MDA, malondialdehyde; TBARS, 2-thiobarbituric acid-reactive substances; EDTA, ethylenediaminetetraacetic acid; ECL, enhanced chemiluminescence; OPA, *o*-phthaldehyde; $NaBH_4$, sodium borotetrahydride; TBS, Tris-buffered saline.

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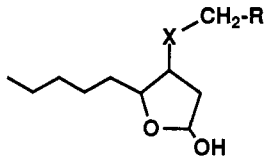

FIGURE 1: Structure of the Michael addition-type HNE adduct. X represents histidyl imidazolyl (HNE–histidine adduct), lysyl ε-amino (HNE–lysine adduct), and cysteinyl sulfhydryl (HNE–cysteine adduct) groups of proteins (Uchida et al., 1993).

Palinski et al., 1989; Haberland et al., 1988). One of the major products of membrane peroxidation, 4-hydroxy-2-nonenal (HNE), has been shown to have a number of adverse biological effects (Esterbauer et al., 1991). Oxidation of LDL leads to significant changes in the structural and functional properties, including an increased electronegative charge (Jürgens et al., 1986), a loss of lysine residues (Jürgens et al., 1986), an increased fluorescence (Esterbauer et al., 1986; Koller et al., 1986), and an enhanced recognition by macrophages (Jessup et al., 1986). The possibility that HNE may play a role in the pathogenesis of atherosclerosis is suggested by the facts that (i) high concentrations of HNE can be generated during the oxidation of LDL phospholipids (Esterbauer et al., 1990, 1991, 1992) and (ii) the structural and functional changes associated with the oxidation of LDL can be produced also by direct interaction of LDL with HNE.

We have recently developed procedures for the detection of HNE adducts generated in HNE-modified proteins and shown that HNE forms stable Michael addition-type adducts (Figure 1) with histidine (Uchida & Stadtman, 1992b, 1993) and lysine residues (Szweda et al., 1993) of proteins, in addition to cysteine (Uchida & Stadtman, 1992a). An immunochemical procedure that uses an antibody specific to the HNE adducts was also developed. The selectivity and sensitivity of this procedure were established by results of studies in which the HNE-specific antibody was used to detect HNE-modified proteins in rat liver hepatocytes exposed to HNE or oxidative stress (Uchida et al., 1993).

In the present study, we present direct evidence that HNE reacts primarily with histidine and lysine residues of LDL apoB to form Michael addition-type HNE adducts. Furthermore, the antibody affinity-purified with the Michael-type HNE adduct as a ligand is used for the immunochemical detection of HNE adducts generated in LDL oxidatively modified by Cu^{2+} or cultured endothelial cells.

EXPERIMENTAL PROCEDURES

Materials. LDL (1.019–1.063 g/mL) was prepared from plasma of healthy humans by sequential ultracentrifugation and then extensive dialysis against phosphate-buffered saline (10 mM sodium phosphate buffer, pH 7.2, containing 150 mM NaCl) containing 0.01% EDTA at 4 °C. LDL used for oxidative modification by Cu^{2+} or cultured endothelial cells was dialyzed against a 1000-fold volume of phosphate-buffered saline at 4 °C. The stock solution of *trans*-4-hydroxy-2-nonenal was prepared by acid treatment (1 mM HCl) of 4-hydroxynonenal diethyl acetal, which was synthesized according to the procedure of Gree et al. (1986). The concentration of the HNE stock solution was determined by the measurement of UV absorbance at 224 nm (Schauenstein et al., 1971). Keyhole limpet hemocyanin (KLH) was obtained from Pierce. Horseradish peroxidase-linked anti-rabbit IgG immunoglobulin and ECL (enhanced chemiluminescence) Western blotting detection reagents were obtained from Amersham. Affi-gel 10 was obtained from Bio-Rad. Nα-Acetyl-L-histidine, Nα-acetyl-L-lysine, glutathione (reduced form), and bovine serum albumin (BSA) were obtained from Sigma. Gly_3-His-Gly_3 methyl ester and Ala_3-His-Ala_3 methyl ester were obtained from Research Genetics (Huntsville, AL). 2-Thiobarbituric acid was obtained from J. T. Baker, and malondialdehyde bis(dimethyl acetal) was from Aldrich. The protein concentration was measured using the BCA protein assay reagent obtained from Pierce.

Reaction of LDL with HNE or Cupric Ion. HNE-modified LDL was prepared by incubating 50 μg of LDL with HNE (0–1 mM) in 0.1 mL of 50 mM sodium phosphate buffer, pH 7.2, for 2 h at 37 °C. Copper-oxidized LDL was prepared by incubating 50 μg of LDL with 10 or 100 μM $CuSO_4$ in 0.1 mL of 50 mM sodium phosphate buffer, pH 7.2, for 24 h at 37 °C.

Oxidative Modification of LDL by Cultured Bovine Aortic Endothelial Cells. The endothelial cells used were bovine aortic endothelial cells obtained from the NIA Aging Cell Culture Repository. These cells were grown to confluence in 60-mm plastic tissue culture plates in Medium 199 containing 20% fetal bovine serum and 50 μg/mL gentamicin sulfate. The cells were washed three times with serum-free Ham's F-10 medium and then incubated for 24 h at 37 °C with 2 mL of serum-free Ham's F-10 medium with or without 100 μg of LDL protein/mL. At the end of incubation, the medium was harvested and any detached cells were removed by low-speed centrifugation. Incubation of LDL alone was carried out in parallel using 60-mm plates without cells.

Lipid Peroxidation Assay. Lipid peroxidation of LDL was determined by measurement of 2-thiobarbituric acid-reactive substances (TBARS), according to the methods of Masaki et al. (1989). LDL incubated with and without oxidants (Cu^{2+} or endothelial cells) in 0.1 mL of reaction mixture was treated with 0.5 mL of 2.8% (w/v) trichloroacetic acid and 0.5 mL of 1% 2-thiobarbituric acid in 0.05 N NaOH and then boiled for 20 min. After cooling, the sample was centrifuged (11000*g*, 3 min), and the absorbance of the supernatant solution was measured at 534 nm. Malondialdehyde bis(dimethyl acetal), which yields malondialdehyde (MDA) by acid treatment, was used as a standard.

Amino Acid Composition. Changes in the amino acid composition and formation of HNE adducts of histidine and lysine in LDL that had been treated with HNE or Cu^{2+} were assessed by amino acid analysis (Uchida & Stadtman, 1993). An aliquot (0.1 mL) of LDL incubated in the absence or presence of HNE or Cu^{2+} was treated with 10 mM EDTA (10 μL)/1 N NaOH (10 μL)/100 mM $NaBH_4$ (10 μL). After incubation for 1 h at 37 °C, the mixture was treated with 1 N HCl (30 μL), evaporated to dryness, and then hydrolyzed with 6 N HCl (0.2 mL) for 20 h at 110 °C under a nitrogen atmosphere. The hydrolyzed sample was evaporated to dryness and redisolved in 200 μL of 50 mM sodium phosphate buffer (pH 8.0) containing 1 mM EDTA. After filtration with a Millipore filter (0.45 μm), an aliquot (10 μL) was labeled with *o*-phthaldehyde (OPA) for determination of amino acid composition by HPLC. Reversed-phase HPLC was performed on a Hewlett-Packard Model 1090 chromatograph equipped with a Hewlett-Packard Model 1046A programmable fluorescence detector.

HNE-Specific Antibody. Polyclonal antiserum that recognizes the HNE–protein epitopes has been raised by immunizing New Zealand white rabbits with HNE-treated KLH as reported previously (Uchida et al., 1993). The polyclonal antibody was partially purified by affinity chromatography on a HNE–histidyl peptide column. Affi-gel 10

was derivatized by incubation of the gel slurry with the HNE-derivatized Ala_3-His-Ala_3 methyl ester in 0.1 M Hepes, pH 8.0, for 20 h at 4 °C. The antiserum (2 mL) was diluted to 20 mL with 0.1 M Hepes buffer, pH 8.0, and passed three times over a column containing 1 mL of affinity resin. Unbound proteins were removed by washing with 5 mL of 0.1 M Hepes buffer, pH 8.0, followed by 5 mL of 100 mM NaCl, and bound antibodies were eluted with 5 mL of 0.1 M glycine, pH 2.5. The eluate was neutralized immediately with 1 M Tris·HCl, pH 8.0, and stored at −70 °C.

Later, competitive ELISA study demonstrated that the binding of these antibodies to HNE-modified LDL not only was blocked by the HNE–*N*-acetylhistidine adduct but also was blocked by HNE–*N*-acetyllysine and HNE–glutathione adducts. It is thus evident that these antibodies recognize the HNE moieties only of the Michael addition products.

Enzyme-Linked Immunosorbent Assay (ELISA). A coating antigen was prepared by incubating 1 mg of LDL with 2 mM HNE in 1 mL of 50 mM sodium phosphate buffer, pH 7.2, for 2 h at 37 °C. A 100-μL aliquot of the antigen solution containing 0.4 μg of LDL was added to each well of a 96-well microtiter plate and incubated for 20 h at 4 °C. The antigen solution was then removed, and the plate was washed with Tris-buffered saline (TBS) containing 10% Tween 20 (TBS/Tween). Each well was incubated with 200 μL of 1% BSA in TBS/Tween for 30 min at 37 °C in a moist chamber to block the unsaturated plastic surface. The plate was then washed once with TBS/Tween. After the supernatants were discarded and the wells were washed three times with TBS/Tween, 100 μL of a 5×10^4 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase in TBS/Tween was added. After incubation for 1 h at 37 °C, the supernatant was discarded, and the plates were washed three times with TBS/Tween. Enzyme-linked antibody bound to the well was revealed by adding 100 μL/well of 1,2-phenylenediamine in 0.1 M citrate/phosphate buffer (pH 5.0) containing 0.003% H_2O_2. The reaction was terminated by the addition of 50 μL of 2 M sulfuric acid, and absorbance at 490 nm was read on a micro-ELISA plate reader.

In the competitive ELISA study, a competitor was incubated with HNE-specific antibody for 20 h at 4 °C to yield competitor/antibody mixtures containing antibody at 25 ng/mL and variable concentrations of competitor. A 100-μL aliquot of competitor/antibody mixtures was added to each well and incubated for 1 h at 37 °C. After the supernatants were discarded and the wells were washed three times with TBS/Tween, the second antibody was added and enzyme-linked antibody bound to the well was revealed as described above. Results were expressed as the ratio B/B_0, where B = [(absorbance in presence of competitor) − (background absorbance (no antibody))] and B_0 = [(absorbance in absence of competitor) − (background absorbance)].

SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE)/Immunoblot Analysis. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli (1970). The apo B of LDL that had been treated with HNE or oxidatively modified by Cu^{2+} or endothelial cells was delipidated twice with 0.5 mL of chroloform/methanol (1:1) and then treated with Laemmli sample buffer for 3–5 min at 100 °C. The samples were run on two 6% SDS–PAGE slab gels. One gel was used for staining with Coomassie Brilliant Blue; the other was transblotted to nitrocellulose membranes, incubated with 2% BSA in TBS/Tween for blocking, washed, and treated with the HNE-specific antibody (2 μg/mL). This procedure was followed by addition of horseradish peroxidase conjugated to goat anti-rabbit IgG immunoglobulin and ECL reagents. The bands were visualized by exposure of membranes to autoradiography film.

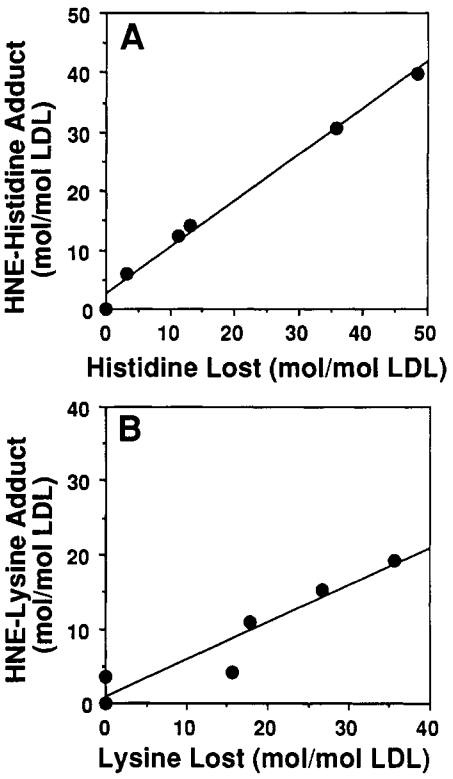

FIGURE 2: Stoichiometry of the loss of amino acids (histidine and lysine) and the formation of their HNE adducts in HNE-modified LDL. LDL (50 μg) in 0.1 mL of 50 mM sodium phosphate buffer, pH 7.2, was incubated with 0–1 mM HNE for 2 h at 37 °C. Loss of amino acids and formation of HNE adducts were determined by amino acid analysis as described. (A) Loss of histidine and formation of HNE–histidine adduct; (B) loss of lysine and formation of HNE–lysine adduct.

The hapten for HNE-specific antibody was prepared by incubating Gly_3-His-Gly_3 methyl ester (5 mg) with 6 mM HNE in 1 mL of 50 mM sodium phosphate buffer, pH 7.2. After 20 h of incubation at 37 °C, the adduct was purified by reversed-phase HPLC on a TSK-GEL ODS-80TM column (0.46 × 25 cm) (TOSO HAAS). The adduct was eluted with a linear gradient of 0.05% trifluoroacetic acid in water (solvent A)/acetonitrile (solvent B) (t = 0, 100% A; t = 20 min, 0% A) at a flow rate of 1 mL/min. The presence of HNE–histidine adduct in the product was determined by amino acid analysis. The hapten (Gly_3-HNE-His-Gly_3 methyl ester) was incubated with HNE-specific antibody at 4 °C for 20 h to yield hapten/antibody mixtures containing antibody at 2 μg/mL and variable concentrations of hapten.

RESULTS

Michael Addition-Type HNE Adducts Generated in the HNE-Modified LDL. The HNE adducts present in HNE-modified LDL have been identified by direct amino acid analysis. As shown in Figure 2, incubation of LDL with various concentrations of HNE (0–1.0 mM) for 2 h at 37 °C led to a loss of up to 50 histidine residues (Figure 2A) and 40 lysine residues (Figure 2B) per molecule of LDL (the total number of histidine and lysine residues per molecule of LDL apoB obtained by amino acid analysis was 106 and 303 molecules, respectively). These losses were accompanied by the formation of Michael addition-type adducts, accounting for 80% and 49%, respectively, of the histidine and lysine residues that had disappeared.

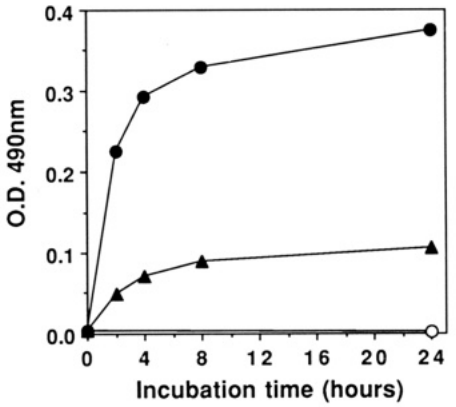

FIGURE 3: ELISA for binding of the HNE-specific antibody to HNE-modified LDL. LDL (50 μg) in 0.1 mL of 50 mM sodium phosphate buffer, pH 7.2, was incubated at 37 °C in the absence (○) and the presence of 0.1 (▲) or 1 mM (●) HNE. Aliquots were sampled at the indicated times and analyzed for HNE–protein epitopes by ELISA.

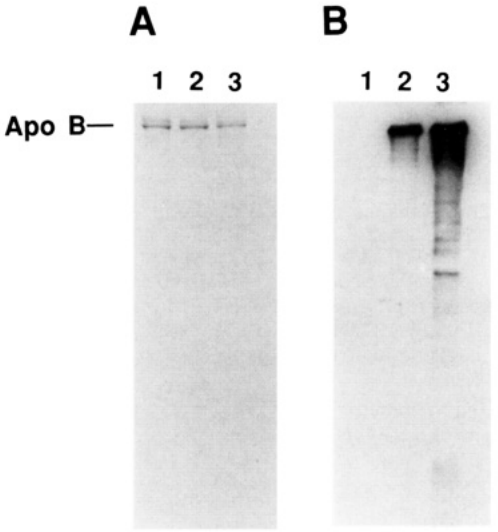

FIGURE 4: SDS–PAGE and subsequent immunoblot analysis of HNE-treated LDL. (A) SDS–PAGE. (B) Immunoblot. LDL (50 μg) in 0.1 mL of 50 mM sodium phosphate buffer, pH 7.2, was incubated for 2 h at 37 °C in the absence (lanes 1) and the presence of 0.1 (lanes 2) or 1 mM (lanes 3) HNE. Apolipoproteins from LDL samples were loaded on an SDS–PAGE gel and transblotted to a nitrocellulose membrane. Subsequently, the membrane was incubated with the antibody (2 μg/mL).

To determine whether the Michael addition-type HNE adducts of histidine and lysine residues formed in HNE-modified LDL could cross react with the affinity-purified HNE-specific antibody, LDL treated with HNE was subjected to an enzyme-linked immunosorbent assay (ELISA) and an SDS–PAGE/immunoblot analysis with the HNE antibody. As shown in Figure 3, incubation of LDL with HNE (0.1 and 1 mM) generated epitopes recognized by the HNE-specific antibody. Immunoblot analysis of HNE-modified LDL also demonstrated that the immunoreactivity of LDL apo B clearly depends upon the HNE concentration used (Figure 4B), although treatment of LDL with HNE did not affect the pattern of electrophoretic mobilities of apolipoproteins detected by Coomassie Brilliant Blue staining (Figure 4A). As is evident from the results in Figures 3 and 4, little or no immunoreactivity was detected in native apo B with either an ELISA or an immunoblot analysis. HNE-derivatized irrelevant proteins acted as competitive inhibitors of antibody binding to HNE–LDL (data not shown). Furthermore, the antibody binding to the HNE adducts generated in the HNE-modified LDL was completely inhibited by incubating the antibody (25 ng/mL) with 50 μM simple Michael addition-type HNE adducts (HNE–*N*-acetylhistidine, HNE–*N*-acetyllysine, and HNE–glutathione).

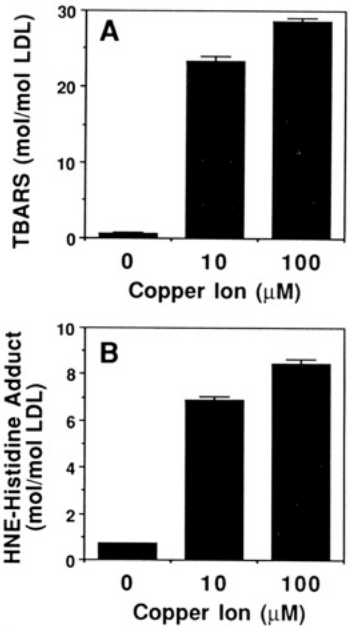

FIGURE 5: Cu^{2+}-catalyzed LDL peroxidation (A) and concomitant formation of HNE–histidine adduct (B). LDL (50 μg) in 0.1 mL of 50 mM sodium phosphate buffer, pH 7.2, was incubated for 24 h at 37 °C in the absence and the presence of Cu^{2+}. LDL peroxidation was determined in terms of TBARS, and the formation of HNE–histidine adduct was determined by amino acid analysis as described.

In addition to HNE, several other highly reactive aldehydes produced during lipid peroxidation can form covalent bonds with proteins (Esterbauer et al., 1991). Among these, malondialdehyde (MDA), a major product of arachidonic and linoleic acids peroxidation, has received much attention because it reacts readily with lysine residues of proteins (Chio & Tappel, 1969). It is noteworthy that the HNE-specific antibody exhibited no cross reactivity with MDA-modified LDL as judged by an ELISA technique (data not shown). This attests to the specificity of this antibody for the detection of HNE–protein epitopes in oxidatively modified LDL and therefore its utility in studies on the role of HNE in the pathogenesis of atherosclerosis.

HNE Adducts Generated in the Oxidatively Modified LDL by Cu^{2+}. In view of the fact that HNE is produced during the oxidation of LDL and the observation that exogenously added HNE reacts with histidine and lysine residues of LDL apo B (Figure 2), it was of interest to demonstrate directly that the oxidation of LDL leads to HNE adducts that can be recognized by the HNE-specific antibodies. To this end, LDL was incubated with 10 or 100 μM Cu^{2+} (24 h, 37 °C). Oxidation of endogenous fatty acid was verified by the formation of MDA as determined by the production of 2-thiobarbituric acid-reactive substances (TBARS) (Figure 5A), and the generation of HNE was confirmed by the results of amino acid analysis showing that a Michael addition-type HNE–histidine adduct (7–9 mol/mol LDL) was produced (Figure 5B) as well as trace amounts of HNE–lysine adducts (data not shown).

The fact that the Cu^{2+}-catalyzed oxidation of LDL leads to the generation of HNE–apo B adducts was confirmed by the results of three different experimental approaches in which the presence of HNE–apo B complexes was analyzed by ELISA competition and/or SDS–PAGE/immunoblot techniques.

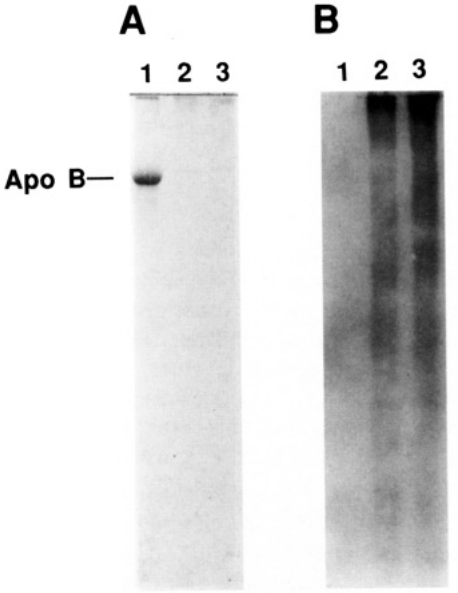

FIGURE 6: SDS–PAGE and subsequent immunoblot analysis of Cu^{2+}-oxidized LDL. (A) SDS–PAGE. (B) Immunoblot. LDL (50 μg) in 0.1 mL of 50 mM sodium phosphate buffer, pH 7.2, was incubated for 24 h at 37 °C in the absence (lanes 1) and the presence of 10 (lanes 2) or 100 μM (lanes 3) Cu^{2+}. Apolipoproteins from LDL samples were loaded on an SDS–PAGE gel and transblotted to a nitrocellulose membrane. Subsequently, the membrane was incubated with the antibody (2 μg/mL).

(i) SDS–PAGE/Immunoblot Analysis. As shown in Figure 6, when subjected to SDS–polyacrylamide gel electrophoresis, the native (unoxidized) form of apo B migrates as a single protein band which is stained by Coomassie Brilliant Blue (Figure 6A, lane 1) but does not bind HNE-specific antibody (Figure 6B, lane 1). However, upon Cu^{2+}-catalyzed oxidation, LDL–apo B is converted to both higher and lower molecular weight protein species, none of which react with Coomassie Brilliant Blue (Figure 6A, lanes 2 and 3). The reduced detection of oxidized LDL–apo B protein by Coomassie Brilliant Blue has previously received attention (Parthasarathy et al., 1985). In other studies, it was demonstrated that treatment of proteins with HNE leads to a loss in Coomassie Brilliant Blue staining characteristics (B. S. Berlett and E. R. Stadtman, unpublished data). Therefore, the inability to detect oxidized apo B derivatives by Coomassie Brilliant Blue staining may be due in part to the formation of aldehyde-modified proteins including HNE–apo B adducts.

(ii) Competiton between Oxidized apo B and HNE-Treated LDL for Binding of HNE Antibody. The ability of native and oxidized forms of LDL to inhibit the binding of HNE-specific antibody to HNE-modified LDL was examined by the use of the ELISA technique. As shown in Figure 7, Cu^{2+}-oxidized LDL at concentrations of 100–350 μg/mL inhibited the binding of HNE antibody to HNE-modified LDL by 35–50%, whereas native LDL at comparable concentrations did not inhibit antibody binding. Under the same conditions, HNE-modified LDL at the concentration of 10 μg/mL inhibited the antibody binding to the coating antigen (HNE-modified LDL) by 50%.

(iii) Ability of HNE-Modified Peptides To Inhibit Binding of HNE Antibodies to Oxidized LDL. A symmetrical heptapeptide containing six glycine residues and a single HNE-modified histidine residue (Gly_3-HNE-His-Gly_3 methyl ester) was tested for its ability to compete with oxidized LDL for the binding of HNE antibody. As shown in Figure 8, at concentrations of 0.1 (lane B) and 1.0 μg/mL (lane C) the heptapeptide partially inhibited binding of HNE antibody to

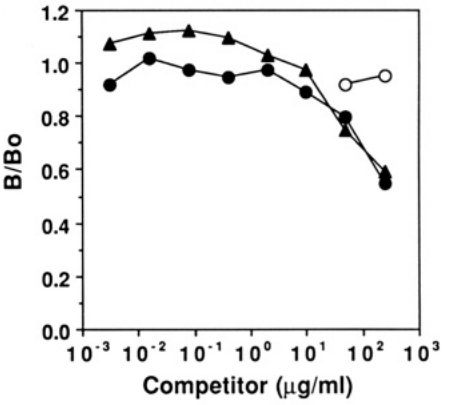

FIGURE 7: Competitve ELISA for binding of the HNE-specific antibody to HNE-modified LDL by native or Cu^{2+}-oxidized LDL. Assays were done as described and used HNE-modified LDL as the absorbed antigen. Numbers on the abscissa indicate the concentration of competitors when the antibody was preincubated with competitors at 4 °C for 20 h. Competitors were as follows: ○, native LDL; ▲, Cu^{2+} (10 μM)-oxidized LDL; ●, Cu^{2+} (100 μM)-oxidized LDL.

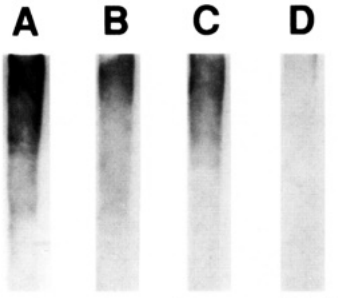

FIGURE 8: Effect of the addition of HNE-modified heptapeptide on antibody binding to the Cu^{2+}-oxidized LDL. LDL (50 μg) in 0.1 mL of 50 mM sodium phosphate buffer, pH 7.2, was incubated for 24 h at 37 °C in the presence of 10 μM Cu^{2+}. Apolipoproteins from oxidized LDL were loaded on an SDS–PAGE gel and transblotted to a nitrocellulose membrane. Subsequently, the membrane was incubated with the HNE-specific antibody (2 μg/mL) in the absence and the presence of the HNE-modified heptapeptide. The HNE-modified peptide Gly_3-HNE-His-Gly_3 methyl ester was used as the competitor at the following concentrations (μg/mL): A, 0; B, 0.1; C, 1; D, 10.

oxidized LDL, and at a concentration of 10 μg/mL the binding to oxidized LDL was completely prevented (lane D).

HNE Adducts Generated in the Oxidatively Modified LDL by Cultured Bovine Aortic Endothelial Cells. On the basis of the observation that cultured endothelial cells, as well as cupric ions in the absence of cells, are capable of inducing lipid peroxidation of LDL and oxidative modification of apo B (Henriksen et al., 1981; Morel et al., 1984; Steinbrecher et al., 1984, 1987), we examined the possibility that adducts that react with the HNE antibody are formed when LDL is incubated with cultured bovine aortic endothelial cells.

Incubation of LDL with endothelial cells in Ham's F-10 medium (24 h, 37 °C) led to oxidation of LDL as assessed by the formation of TBARS (data not shown). Neither endothelial cells without LDL nor control LDL incubated in the medium produced any detectable amount of TBARS. After delipidation and separation by the SDS–PAGE technique, the native form of apo B in LDL appears as a discrete protein band that is readily visualized by Coomassie Brilliant Blue staining (Figure 9A, lane 1). In contrast, the unmodified form of apo B is absent in LDL samples that had been incubated with endothelial cells; instead, the cell-treated sample contained a number of higher molecular weight (polymerized forms) as well as some lower molecular weight protein components (Figure 9A, lane 2). Immunoblot analysis demonstrated that

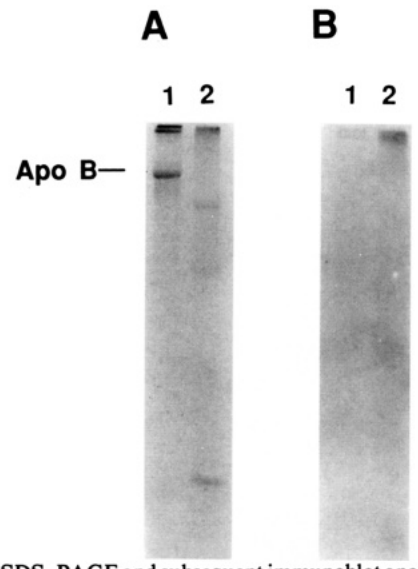

FIGURE 9: SDS–PAGE and subsequent immunoblot analysis of LDL incubated with cultured bovine aortic endothelial cells. (A) SDS-PAGE. (B) Immunoblot after incubation for 24 h at 37 °C with 2 mL of serum-free Ham's F-10 medium in the absence (lanes 1) and presence (lanes 2) of endothelial cells. Apolipoproteins from LDL samples were loaded on an SDS–PAGE gel and transblotted to a nitrocellulose membrane. Subsequently, the membrane was incubated with the antibody (2 μg/mL).

only the high molecular weight fraction of the cell-treated LDL possessed epitopes that were recognized by the HNE-specific antibody (Figure 9B, lane 2). Efforts to identify the HNE–amino acid adduct by direct amino acid analysis were unsuccessful, probably because the level of such adducts was below the limit of detectability by this technique.

DISCUSSION

Chemistry of HNE Modification of LDL. The proposition that HNE produced in the oxidation of LDL plays a role in atherogenesis is supported by the results of earlier studies showing that antibodies raised against HNE-modified LDL bind to oxidized forms of LDL (Palinski et al., 1989; Chen et al., 1992) and to epitopes in atherosclerotic lesions (Palinski et al., 1989). The demonstration here that treatment of LDL with HNE leads to the loss of lysine and histidine residues is in agreement with the results of other workers (Jürgens et al., 1986; Esterbauer et al., 1986; Chen et al., 1992) and supports the view that HNE adducts of these two amino acids are among the epitopes recognized by the anti-HNE antibodies. Nevertheless, the structures of the HNE adducts formed and the specificity of the antibodies for various epitopes remain to be established. These questions are partly resolved by the results of an earlier study (Uchida et al., 1993) and the results reported here. We showed previously (Uchida & Stadtman, 1992a,b, 1993; Szweda et al., 1993) that the imidazole moiety of histidine residues (Uchida & Stadtman, 1992a), the ε-amino group of lysine residues (Szweda et al., 1993), and the sulfhydryl group of cysteine residues (Uchida & Stadtman, 1992b) all undergo Michael addition-type reactions to the α,β double bond of HNE. This and the further observation that reduction of the aldehyde group of these primary Michael addition products with $NaBH_4$ converts them to hydroxy derivatives that are stable to strong acid hydrolysis (Uchida & Stadtman, 1992a) formed the basis of methods for the identification and quantification of the HNE adducts of proteins by conventional amino acid analytical techniques.

By means of these techniques, it was established in the present study that at least 80% of the histidine residues that are lost when LDL is treated with HNE can be accounted for as the Michael addition products (Figure 2A). In contrast, the Michael addtion products accounted for only 49% of the lysine residues that disappeared upon HNE treatment (Figure 2B). Low recovery of the HNE–lysine adduct probably reflects the occurrence of secondary reactions in which the aldehyde moiety of the primary Michael addition products reacts further with another lysine residue, leading to intra- and intermolecular Schiff base cross-linked derivatives. These secondary reactions are likely responsible for the HNE-provoked polymerization of glyceraldehyde-3-phosphate dehydrogenase (Uchida & Stadtman,1993) and glucose-6-phosphate dehydrogenase (B. Friguet, E. R. Stadtman, and L. I. Szweda, unpublished observations) and may explain also the presence of higher molecular weight forms of apo B in oxidized preparations of LDL (Figures 6 and 9).

Specificity of HNE–Protein Antibodies. Chen et al. (1992) demonstrated that antibodies raised against HNE-modified LDL interact with epitopes on Cu^{2+}-oxidized LDL and other modified proteins. In competition experiments, they found that the antibody–protein interactions could be partly blocked by the HNE-modified forms of several polyamino acids, including polylysine, polytyrosine, polyarginine, and polyhistidine. This suggests, among other possibilities, that the specificity of the antibody is for the HNE moiety of the HNE–protein adduct rather than for a specific HNE–amino acid derivative. This possibility is reinforced by the results of our studies with the antibodies used in the present investigation. These antibodies were generated by immunization of rabbits with HNE-treated keyhole limpet hemocyanin in which HNE adducts of histidine, lysine, and cysteine may serve as the antigenic sites. Subsequently, the antibodies were purified by affinity chromatography on a solid matrix to which the heptapeptide Gly_3-HNE-His-Gly_3 methyl ester was covalently bound (Uchida et al., 1993). As shown here, these antibodies bind to HNE-modified LDL and to oxidized forms of LDL produced by incubation of LDL with Cu^{2+} or endothelial cells. However, contrary to expectations in competition studies (Uchida et al., 1993), it was demonstrated that the binding of these antibodies to HNE-modified proteins not only is blocked by the HNE–*N*-acetylhistidine Michael addition derivative but also is blocked by HNE derivatives of *N*-acetyllysine and the HNE thioether derivative of glutathione. Thus, it is evident that these antibodies recognize the HNE moieties only of the Michael addition products (Uchida et al., 1993). On the basis of these results, it is probably premature to conclude that the reaction of anti-HNE–lysine antibodies with proteins is a unique measure of the HNE–lysine content of the protein (Palinski et al., 1989).

Metal-Catalyzed Oxidative Modification of LDL. apo B degradation during cellular oxidation of LDL is not catalyzed by proteolytic enzymes released by cells but is a consequence of oxidative damage (Fong et al., 1987). On the basis of the fact that LDL oxidation by most cultured cells requires medium containing transition metal ions (Kalant et al., 1991; Kuzuya et al., 1992), it has been proposed that LDL apo B undergoes metal-catalyzed free radical oxidations during oxidative modification of LDL *in vitro* (Steinberg et al., 1989). As a matter of fact, the pattern of modification of LDL apo B tends to be broad and incompletely resolved upon SDS–PAGE (Figures 6 and 9), due probably to the varying degrees of oxidative cleavage of the polypeptide chain and the modification of amino acid side chains. This is supported by the fact that Cu^{2+} oxidation of LDL induces extensive degradation of apo B (Fong et al., 1987) together with the

formation of a significant amount of 2-oxohistidine (Uchida & Kawakishi, 1993), a major oxidation product of histidine residues exposed to the copper redox free radical generating system, and aspartic acid (Fong et al., 1987) in the hydrolysate of oxidized LDL. The reaction of Cu^{2+} bound to histidine with hydroperoxides may induce the formation of oxygen free radicals which damage LDL phospholipids and adjacent amino acid residues of apo B. Recent studies have suggested that metal-catalyzed oxidative modification of proteins, including LDL apo B, is closely related to aging and its related diseases (Stadtman & Oliver, 1991; Stadtman, 1991). It is known that various enzymic and nonenzymic systems catalyze the oxidative modification of proteins (Stadtman, 1990; Levine et al., 1981; Fucci et al., 1983; Stadtman & Wittenberger, 1985). Results of the mechanistic studies are consistent with the view that metal ions and hydroperoxides undergo site-specific Fenton reactions at the metal-binding sites on the proteins followed by generation of oxygen free radicals. These reactions involve the conversion of histidine to 2-oxohistidine (Uchida & Kawakishi, 1993) and asparagine (Farber & Levine, 1986); of proline to glutamic semialdehyde (Amici et al., 1989), 2-pyrrolidone (Uchida et al., 1990; Kato et al., 1992), and pyroglutamic or glutamic acid (Amici et al., 1989; Uchida et al., 1990; Kato et al., 1992); and of arginine to glutamic semialdehyde (Amici et al., 1989).

Michael Addition-Type HNE Adducts as Biological Markers for Atherosclerosis. The immunohistochemical analysis of atherosclerotic lesions from human aorta demonstrated that intense positivity was associated with cells, primarily macrophages (K. Uchida, S. Kawakishi, H. Hiai, and S. Toyokuni, unpublished data). This is consistent with the previous observations that most of the lesion volume is occupied by macrophages in fatty streaks and transitional lesions in Watanabe heritable hyperlipidemic rabbits (Rosenfeld et al., 1990). It is known from the *in vitro* studies that all of the major cell types within the atherosclerotic lesions are capable of promoting the oxidation of LDL (Henriksen et al., 1981; Morel et al., 1984; Steinbrecher et al., 1984, 1987; Heinecke et al., 1986; Cathcart et al., 1985); therefore, the cell-associated staining patterns we observed are likely attributable to the cellular oxidation of LDL by endothelial cells, macrophages, and smooth muscle cells. The resulting oxidized LDL may be taken up by cells and may be the ultimate source of the lipids that accumulate in atherosclerotic lesions. In addition, intracellular granular staining observed in atherosclerotic lesions represents the presence of HNE adducts that had already been taken up by macrophages and are present within the cell in cytoplasmic organelles (Rosenfeld et al., 1990). This leads to a speculation that the HNE modification renders proteins relatively resistant to intracellular proteolytic degradation, resulting in the marked accumulation of epitopes in macrophages.

In summary, we have demonstrated in the present study that LDL treated with HNE or oxidatively modified by Cu^{2+} or cultured endothelial cells gives rise to Michael addition-type HNE adducts that are detectable by the HNE-specific antibody. The immunochemical application of the HNE-specific antibody during pathogenesis of other free radical-related diseases may provide a reliable method to detect localized oxidatively modified proteins *in vivo*. As a matter of fact, using the HNE-specific antibody, we have recently demonstrated the presence of HNE–protein epitopes in the renal proximal tubules of rats exposed to a renal carcinogen, ferric nitrilotriacetate (Toyokuni et al., 1994). Taken together, Michael addition-type HNE adducts are useful biological markers in studies of a number of biological systems since lipid peroxidation and subsequent modification of proteins are involved in widespread biological processes.

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