Protein-bound 4-hydroxy-2-hexenal as a marker of oxidized n-3 polyunsaturated fatty acids[S]

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Abstract **In the present study, to investigate the contribution of n-3 PUFAs in the oxidative modification of protein in vivo, we characterize the covalent binding of 4-hydroxy-2-hexenal (HHE), a potent cytotoxic aldehyde originating from the peroxidation of n-3 PUFAs, to protein and describe the production of this aldehyde in oxidatively modified LDL and in human atherosclerotic lesions. Upon incubation with BSA, HHE was rapidly incorporated into the protein and generated the protein-linked carbonyl derivative, a potential marker of oxidatively modified proteins under oxidative stress. To detect the protein-bound HHE in vivo, we raised monoclonal antibody HHE53 (MAb HHE53) directed to the HHE-modified protein and identified the Michael addition-type HHE-histidine adduct as the major epitope. This antibody reacted with copper-oxidized LDL, suggesting that HHE was produced during the oxidative modification of LDL. In addition, we demonstrated that the materials immunoreactive to MAb HHE53 indeed constituted the atherosclerotic lesions, in which intense positivity was associated primarily with macrophage-derived foam cells. The results of this study suggest that the reaction between oxidized n-3 PUFAs and protein might represent a process common to the formation of degenerative proteins during aging and its related diseases.**—Yamada, S., T. Funada, N. Shibata, M. Kobayashi, Y. Kawai, E. Tatsuda, A. Furuhata, and K. Uchida. **Protein-bound 4-hydroxy-2-hexenal as a marker of oxidized n-3 polyunsaturated fatty acids.** ***J. Lipid Res.*** **2004.** 45: **626–634.**

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Various lines of evidence indicate that the oxidative modification of protein and the subsequent accumulation of the degenerated proteins are found in cells and tissues during aging, oxidative stress, and in a variety of pathological states, including premature diseases, muscular dystrophy, rheumatoid arthritis, and atherosclerosis (1–4). The critical agents that give rise to the degeneration of protein may be represented by reactive aldehyde intermediates, such as 2-alkenals and 4-hydroxy-2-alkenals (1, 5, 6). These highly reactive compounds are considered important mediators of cell damage because of their ability to covalently modify biomolecules, which can disrupt important cellular functions and cause mutations (5). Furthermore, the adduction of aldehydes to apolipoprotein B in LDLs has been implicated as the mechanism by which LDL is converted to an atherogenic form that is recognized by macrophage scavenger receptors far more readily than normal LDL, leading to the formation of cholesterol-engorged foam cells (7, 8).

4-Hydroxy-2-alkenals represent the most prominent aldehyde substances generated during the peroxidation of PUFAs. Among them, 4-hydroxy-2-nonenal (HNE) is known to be the major aldehyde formed during the lipid peroxidation of n-6 PUFAs, such as linoleic acid and arachidonic acid. It has been suggested that HNE accumulates in membranes at concentrations of 10 μM to 5 mM in response to oxidative insults (5). The development of specific antibodies against protein-bound HNE has made it possible for us to obtain highly probable evidence for the occurrence of oxidative stress in vivo. On the other hand, the peroxidation of n-3 PUFAs, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), generates a closely related compound, 4-hydroxy-2-hexenal (HHE). However, to our knowledge, the endogenous production of HHE in vivo has not been determined. In the present study, to investigate the mechanisms contributing to the modification of LDL and to examine the pos-

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HHE, 4-hydroxy-2-hexenal; HNE, 4-hydroxy-2-nonenal; KLH, keyhole limpet hemocyanin; LC-MS, liquid chromatography-mass spectrometry; MAb, monoclonal antibody; TBARS, 2-thiobarbituric acid-reactive substance.

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[S] The online version of this article (available at http://www.jlr.org) contains an additional two figures.

sible involvement of oxidized n-3 PUFAs in the oxidative modification of proteins in vivo, we raised a monoclonal antibody (MAb) directed to the protein-bound HHE as an index of the peroxidation of n-3 PUFAs and determined its production in oxidatively modified LDL and in human atherosclerotic lesions.

EXPERIMENTAL PROCEDURES

Materials

The stock solutions of *trans*-4-hydroxy-2-alkenals were prepared by acid treatment (1 mM HCl) of 4-hydroxy-2-alkenal dimethylacetal, which was synthesized according to the procedure of De Montarby, Mosset, and Gree (9). The concentration of 4-hydroxy-2-alkenal stock solutions was determined by the measurement of ultraviolet (UV) absorbance at 224 nm. BSA, ovalbumin, human hemoglobin, human serum albumin, N^{α}-acetyl-L-lysine, N^{α}-acetyl-L-histidine, and N^{α}-acetyl-L-cysteine were obtained from Sigma Chemical Co. Keyhole limpet hemocyanin (KLH) was obtained from ICI. Horseradish peroxidase-linked anti-mouse IgG was obtained either from Bio-Rad or Dako (Glostrup, Denmark). A ninety-six-well microtiter plate was obtained from Nunc. Enhanced chemiluminescence (ECL) Western blotting detection reagents were obtained from Amersham Biosciences (Buckinghamshire, UK). Other chemicals were of the best grade available from commercial sources.

General procedures

NMR spectra were recorded with a Varian Mercury 400V (400 MHz) instrument. UV absorption spectra were measured with a JASCO (Tokyo, Japan) V-550 spectrophotometer, and microtiter plate UV adsorption was measured with a Molecular Devices Spectra Max 250 microtiter plate reader. Liquid chromatography-mass spectrometry (LC-MS) was carried out with a Micromass (Manchester, UK) Platform LCZ (ZMD4000) instrument.

Reaction of proteins with aldehydes

Modification of the protein by aldehydes was performed by incubating proteins (1 mg/ml) with 1–2 mM aldehydes in 1 ml of 50 mM sodium phosphate buffer, pH 7.2, at 37°C. After incubation, unbound HHE was removed by extensive dialysis against 50 mM sodium phosphate buffer, pH 7.2, or by gel filtration on a PD-10 column (Pharmacia LKB), which had been equilibrated with 50 mM sodium phosphate buffer, pH 7.2.

Protein carbonyl

An aliquot (0.5 ml) of the protein samples was treated with an equal volume of 0.1% (w/v) 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCl and incubated for 1 h at room temperature. This mixture was treated with 0.5 ml of 20% trichloroacetic acid (w/v, final concentration), and after centrifugation, the precipitate was extracted three times with ethanol-ethyl acetate (1:1, v/v). The protein sample was then dissolved with 2 ml of 8 M guanidine hydrochloride, 13 mM EDTA, and 133 mM Tris solution (pH 7.4), and the UV absorbance was measured at 365 nm. The results were expressed as moles of DNPH incorporated per protein (mol/mol) based on an average absorptivity of 21.0 mM^{-1} cm^{-1} (10).

Amino acid analysis

An aliquot (0.1 ml) of the protein samples incubated with or without HHE was treated with 10 mM EDTA (10 μl), 1 N NaOH (10 μl), and 100 mM $NaBH_4$ (10 μl). After incubation for 1 h at 37°C, 10% trichloroacetic acid was added to the reaction mixture. After centrifugation at 10,000 *g* for 3 min, the precipitates were hydrolyzed in vacuo with 6 N HCl for 24 h at 105°C. The hydrolysates were then concentrated and dissolved in 50 mM sodium phosphate buffer, pH 7.4. The amino acid analysis was performed using a JEOL JLC-500 amino acid analyzer equipped with a JEOL LC30-DK20 data-analyzing system.

Measurement of lipid peroxidation

2-Thiobarbituric acid-reacting substance (TBARS) production was measured with a microtiter plate method as follows. An aliquot of the lipid sample (0.5 ml) was mixed with 1% (w/v) 2-thiobarbituric acid (0.5 ml) and 2.8% (w/v) trichloroacetic acid (0.5 ml) in 0.05 N NaOH. The mixture was boiled for 15 min. The mixture was immediately cooled and then centrifuged to remove undissolved materials. The supernatant was transferred to a microtiter plate well and measured at 532 nm by a microplate reader. The amount of TBARS was calculated from comparison with authentic malondialdehyde bis (dimethylacetal).

Preparation of MAb

To raise the anti-HHE MAb, female BALB/c mice were subcutaneously immunized four times with HHE-conjugated KLH in 50% Freund's complete adjuvant 1 or 2 weeks apart. Titers to HHE-modified BSA in immunized mice sera were measured by ELISA as described below. Two months after the initial immunization, the immunized mice were given an intraperitoneal boost of HHE-conjugated KLH. A few days later, the splenic lymphocytes from an immunized mouse that had the highest titer to HHE-modified BSA were fused to P3U1 murine myeloma cells in the presence of polyethylene glycol (11). Hybrid cells were cultured in hypoxanthine-aminopterin-thymidine selection medium. Culture supernatants of the hybridoma were screened by ELISA. Hybridoma cells corresponding to supernatants that were positive on HHE-BSA and negative on BSA were then cloned by limiting dilution. After repeated screening, 13 clones were obtained. Finally, seven cell lines were selected for further use because they showed a positive reaction to HHE-BSA but a negative reaction to BSA through two successive subclonings by the limiting dilution. Finally, the higher and good growth antibody from these cell lines was designated HHE53. This cell line was injected into BALB/c mice for the production of ascites fluid. The antibody was precipitated from the ascites fluid by ammonium sulfate (40% saturation). The precipitate was dissolved in PBS, dialyzed against the same buffer, and further purified by a Protein A-affinity chromatography.

ELISA

Direct ELISA. A coating antigen was prepared by incubating 1 mg of BSA with 1 mM aldehyde compounds in 1 ml of 50 mM sodium phosphate buffer, pH 7.2, for 24 h at 37°C. A 100 μl aliquot of the antigen solution (10 μg protein/ml) was added to each well of a 96-well microtiter plate and incubated overnight at 4°C. The antigen solution was then removed, and the plate was washed two times with PBS. Each well was filled with 200 μl of 0.5% gelatin containing PBS, 20% Blocking Reagent N101, or 20% Blocking Reagent N102 (NOF Corp., Tokyo, Japan). A total of 1 μg/ml of the primary antibody (MAb HHE53) in 0.5% BSA containing PBS was then added to the wells at 100 μl/well and incubated for 3 h at room temperature or overnight at 4°C. After discarding the primary antibody solution, the plate was washed four times with 0.05% Tween 20 containing PBS (PBST). This was followed by the addition of 100 μl of a 1:10,000 dilution of goat anti-mouse IgG conjugated to peroxidase in 0.1% BSA containing PBS and incubation for 4 h at room temperature. After washing four times with PBST, enzyme-linked antibody bound to the well was revealed by adding 3,3′,5,5′-tetramethylbentidine

(TMBZ) or *o*-phenilenediamine (OPD)/H_2O_2 containing a color reagent. Finally, the absorbance at 450 nm (TMBZ) or 492 nm (OPD) was read on a microplate reader.

Competitive ELISA. A 100-μl aliquot of the antigen solution containing HHE-treated BSA (10 μg of protein) was added to each well of a 96-well microtiter plate and incubated overnight at 4°C. The antigen solution was then removed, and the plate was washed two times with PBS. Each well was filled with 200 μl of 0.5% gelatin containing PBS, 20% Blocking Reagent N101, or 20% Blocking Reagent N102 (NOF Corp.). A variable concentration of a competitor in PBS was added to the wells at 50 μl/well, and 1 μg/ml of the primary antibody solution (MAb HHE53) in PBS was then added to the wells at 50 μl/well and incubated for 3 h at room temperature or overnight at 4°C. After discarding the antibody-competitor mixture, the plate was washed four times with PBST. A peroxidase-conjugated antibody solution was added and incubated for 4 h at room temperature. After washing, peroxidase-conjugated antibody bound to the well was revealed as previously described. The results were expressed as the ratio B/B_0, where B = absorbance in the presence of the competitor minus background absorbance (no antibody) and B_0 = absorbance in the absence of the competitor minus background absorbance (no antibody).

Reaction of amino acid derivatives with HHE

HHE conjugates of N^{α}-acetyllysine, N^{α}-acetylhistidine, and N^{α}-acetylcysteine were prepared as previously reported (12).

HPLC analysis of HHE-conjugated N^{α}-acetylhistidine

The reaction mixture (10 ml) contained 10 mM HHE and 10 mM N^{α}-acetylhistidine in 50 mM sodium phosphate buffer, pH 7.2. After incubation for 48–72 h at 37°C, the reaction mixtures were analyzed by reverse-phase HPLC using an Inertsil ODS-3V column (4.6 × 250 mm; GL Science, Inc., Tokyo, Japan) equilibrated in a solution of 5% acetonitrile in 10 mM ammonium acetate at a flow rate of 1.0 ml/min. The elution profiles were monitored by absorbance at 210 nm. The reaction gave four products, which were isolated by reverse-phase HPLC using an Inertsil ODS-3 column (20 × 250 mm; GL Science, Inc.) equilibrated in a solution of 2.5% acetonitrile in 10 mM ammonium acetate at a flow rate of 15 ml/min. The chemical structure of the product was characterized by LC-MS and ^{1}H NMR spectrometry.

In vitro modification of BSA with PUFAs

The Cu^{2+}-catalyzed oxidation of PUFA ethyl ester in the presence of BSA was performed by incubating BSA (200 μg/ml) with 0.4 mM PUFA in the presence of 10 μM Cu^{2+} and 2 mM ascorbic acid in 1 ml of 50 mM sodium phosphate buffer, pH 7.2, in atmospheric oxygen at 37°C.

In vitro peroxidation of LDL

LDL was prepared from healthy human plasma by sequential ultracentrifugation (13) and then extensively dialyzed against PBS (10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl) containing 0.25 M EDTA at 4°C. LDL used for the oxidative modification by Cu^{2+} was dialyzed against a 1,000-fold volume of PBS at 4°C. Cu^{2+} oxidation of LDL was prepared by incubating 0.5 mg of LDL with $CuSO_4$ (5 μM) in 1 ml of PBS (10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl) at 37°C. After incubation, the reaction mixture was added and dialyzed against a 1,000-fold volume of PBS.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed with the Titan Gel lipoprotein system (Helena Laboratories, Saitama, Japan) for lipoprotein samples and the Titan Gel high-resolution protein system for protein samples. The samples were run on two separate gels. One gel was used for staining with Coomassie Brilliant Blue or Fat Red 7B, and the other was used for immunoblot analysis.

Immunoblot analysis

Proteins were transblotted to nitrocellulose membranes, incubated with 20% Blocking Reagent N101 or Blocking Reagent N102 (NOF Corp.) for blocking, washed with PBST, and treated with MAb HHE53 (1 μg/ml). This procedure was followed by the addition of peroxidase conjugated to goat anti-mouse IgG and ECL reagents. The bands were visualized by exposure of the membranes to autoradiography film.

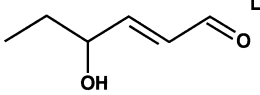
Immunohistochemical detection of the HHE-histidine adduct in human atherosclerotic aorta

Aortic wall samples were obtained at autopsy from five patients with arterial atherosclerosis and used for histopathological and immunohistochemical examinations. Each autopsy was performed at Tokyo Women's Medical University, after family members granted informed consent in accordance with the Ethical Guidelines of Human Materials in Tokyo Women's Medical University and the Helsinki Declaration of 1983. Tissue samples from each case were processed to make frozen or paraffin-embedded materials. For frozen materials, samples were fixed in 10% formalin, immersed in 30% sucrose in PBS, embedded at OCT (Sakura, Tokyo, Japan), and stored at −80°C. For paraffin-embedded materials, samples were fixed in 10% formalin, dehydrated, embedded in paraffin, and stored at room temperature. Multiple 6 μm thick sections were cut from frozen and paraffin materials and used for hematoxylin and eosin (H and E) staining or immunohistochemical staining. Before immunostaining, the frozen sections were rehydrated, and the paraffin sections were deparaffinized and rehydrated. These sections were quenched for 15 min with 3% hydrogen peroxide, rinsed in PBS, pretreated for 30 min with 3% nonimmune animal serum in PBS, and then incubated overnight at 4°C with a mouse anti-CD68 MAb (KP-1; Dako) at a dilution of 1:500, a mouse anti-HNE histidine MAb (HNEJ2) (14) at a dilution of 0.1 mg/ml, or a mouse anti-HHE histidine MAb (HHE53) at a dilution of 0.1 mg/ml. Antibody binding was visualized by the avidin-biotin-immunoperoxidase complex method using the Vectastain ABC kit (Vector, Burlingame, CA) according to the manufacturer's instructions. 3,3′-Diaminobenzidine tetrahydrochloride was used as the chromogen, and hematoxylin was used as the counterstain. Sections from which the primary antibodies were omitted served as negative reaction controls. Some sections were incubated with MAb HHE53 preabsorbed with an excess of the appropriate antigen, HHE-*N*-acetyl-histidine. The localization of MAb HHE53 immunoreactivity was verified by consecutive sections stained with H and E and immunostained for CD68.

RESULTS

Covalent binding of HHE to protein

HHE (**Fig. 1**) is a strong electrophile and readily reacts with nucleophiles. Hence, we examined the potential reactivity of the HHE toward proteins using BSA as a convenient model protein. The binding of HHE to BSA was suggested by a mobility shift of the protein bands during agarose gel electrophoresis analysis (data not shown). To further evaluate the covalent binding of HHE to the protein, we examined the generation of the protein-linked carbonyl groups and changes in the amino acid composi-



4-Hydroxy-2-hexenal (HHE)

Fig. 1. Chemical structure of 4-hydroxy-2-hexenal (HHE).

tion. Spectrophotometric measurement of the protein carbonyls, after their reaction with DNPH, is a simple and accurate technique that has been widely used to reveal increased levels of covalently modified proteins during aging and disease (1–4). As shown in **Fig. 2A**, the exposure of BSA to HHE resulted in a dose-dependent increase in protein carbonyl formation. The incorporation of HHE into the protein was accompanied by the selective loss of histidine and lysine residues (Fig. 2B).

MAbs directed to protein-bound HHE

There is increasing evidence that aldehydes endogenously generated during the degradation process of biological molecules are involved in many pathologies (5). HHE has also been reported to be produced during lipid peroxidation (15); however, to our knowledge, very few papers have addressed the endogenous production of the HHE protein adduct in vivo. Therefore, to evaluate the contribution of protein modification by HHE in the pathogenesis of various diseases associated with oxidative stress, we attempted to raise a MAb specific to the protein-bound HHE.

Hybridomas were prepared by the fusion of myeloma cells with the spleen cells of mice immunized with the HHE-modified KLH. Hybridomas secreting antibodies against HHE-modified protein were detected by ELISA on plates coated with HHE-modified BSA. Among the seven clones obtained, four clones named HHE52, HHE53, HHE55, and HHE57 showed relatively high reactivity (data not shown). Finally, one clone, HHE53, that showed

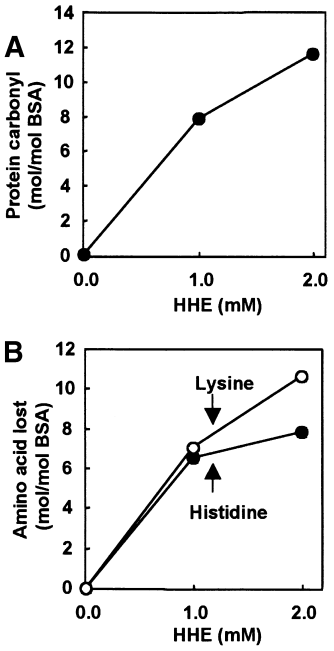


Fig. 2. Covalent binding of HHE to protein. BSA (1 mg/ml) was incubated with 0–2 mM HHE in 50 mM sodium phosphate buffer, pH 7.2, for 24 h at 37°C. A: Introduction of carbonyl groups into protein upon reaction with HHE. The protein carbonyl content was determined by a procedure using 2,4-dinitrophenylhydrazine. B: Loss of histidine and lysine residues. An aliquot (0.1 ml) was taken from the reaction mixture, and the amount of amino acids was determined by amino acid analysis as described in Experimental Procedures.

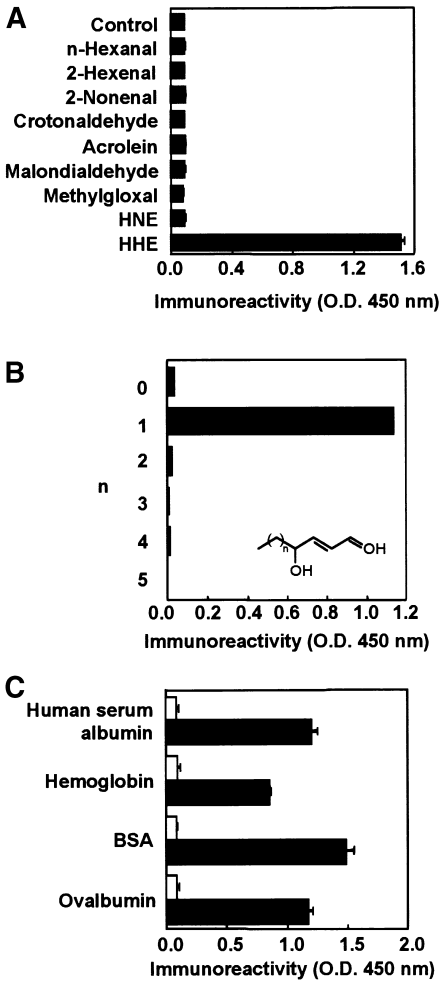


Fig. 3. Specificity of monoclonal antibody (MAb) HHE53. A: Immunoreactivity of MAb HHE53 to aldehyde-treated BSA. The affinity of MAb HHE53 was determined by a direct ELISA using aldehyde-treated BSA as the absorbed antigen. A coating antigen was prepared by incubating 1 mg of BSA with 1 mM aldehyde in 1 ml of 50 mM sodium phosphate buffer, pH 7.2, for 2 h at 37°C. HNE, 4-hydroxy-2-nonenal; O.D., optical density. B: Immunoreactivity of MAb HHE53 to the 4-hydroxy-2-alkenal-treated BSA. The affinity of MAb HHE53 was determined by a direct ELISA using 4-hydroxy-2-alkenal-treated BSA as the absorbed antigen. A coating antigen was prepared as previously described. C: Immunoreactivity of MAb HHE53 to the HHE-treated proteins. The affinity of MAb HHE53 was determined by a direct ELISA using HHE-treated proteins as the absorbed antigen. A coating antigen was prepared by incubating 1 mg of protein with 1 mM HHE in 1 ml of 50 mM sodium phosphate buffer, pH 7.2, for 2 h at 37°C. Bars indicate standard deviations (n = 3).

good growth and distinctive recognition of HHE-modified BSA was selected and used in the present study.

Specificity of MAb HHE53

In spite of an extensive screening of hybridomas that produced monoclonal antibodies specific to the HHE-modified BSA, it is still conceivable that the antibody recognizes epitope originating from other lipid peroxidation products. Therefore, we examined the immunoreactivity of MAb HHE53 toward aldehyde-treated BSA by a direct ELISA. As shown in **Fig. 3A**, 2-alkenals and ketoaldehydes did not generate immunoreactive structures in the protein. In addition, the antibody specifically reacted with the protein treated with HHE but scarcely reacted with the protein treated with other 4-hydroxy-2-alkenals, such as 4-hydroxy-2-pentenal, 4-hydroxy-2-heptenal, 4-hydroxy-2-octenal, HNE, and 4-hydroxy-2-decenal (Fig. 3B), strongly suggesting that HHE was the only source of immunoreactive materials generated in the protein. The antibody-recognized epitope was generated on any of a variety of different proteins treated with HHE (Fig. 3C).

We then characterized the antibodies' ability to recognize specific molecular targets in their native three-dimensional structure. Based on the facts that 4-hydroxy-2-alkenals primarily react with lysine, histidine, and cysteine residues of protein (12), we prepared reaction mixtures of HHE/N^{α}-acetyllysine, HHE/N^{α}-acetylhistidine, and HHE/N^{α}-acetylcysteine and examined their immunoreactivities with MAb HHE53. As shown **Fig. 4A**, the binding of the HHE-modified BSA to MAb HHE53 was only slightly inhibited by the reaction mixtures of HHE/N^{α}-acetyllysine and HHE/N^{α}-acetylcysteine but significantly inhibited by the reaction mixture of HHE/N^{α}-acetylhistidine. To identify the HHE-histidine adduct recognized by MAb HHE53, the immunoreactivity of the reaction products of HHE with N^{α}-acetylhistidine was characterized. The ELISA analysis of the HPLC fractions for immunoreactivity with MAb HHE53 showed that the antibody immunoreacted with these products (Fig. 4B). The LC-MS analysis of the major products eluted at 5–7 min showed a pseudomolecular ion peak at m/z 311.9 $(M+H)^+$, corresponding to the formation of HHE-N^{α}-acetylhistidine monoadduct (supplementary data Fig. S1). Based on the structural characterization by NMR (supplementary data Fig. S2), it was determined that these products represent the HHE-N^{α}-acetylhistidine Michael addition adducts. Because of the presence of three chiral centers at C-2, C-4, and C-5 in the tetrahydrofuran moiety, the 4-hydroxy-2-alkenal-histidine Michael adducts, including HHE-histidine adducts, are theoretically composed of at least eight configurational isomers (16). Indeed, the reaction of N^{α}-acetylhistidine with HHE gave multiple products. The isolated HHE-N^{α}-acetylhistidine Michael adducts inhibited antibody binding to the coated antigen in a dose-dependent manner (Fig. 4C). These results suggest that MAb HHE53 exclusively recognizes the HHE-histidine Michael adducts (Fig. 4D).

Formation of the HHE-histidine adduct during metal-catalyzed peroxidation of n-3 PUFAs in the presence of protein

Consistent with the observation (Fig. 2) that HHE was rapidly incorporated into BSA, the epitope structure recognized by MAb HHE53 was formed upon the incubation

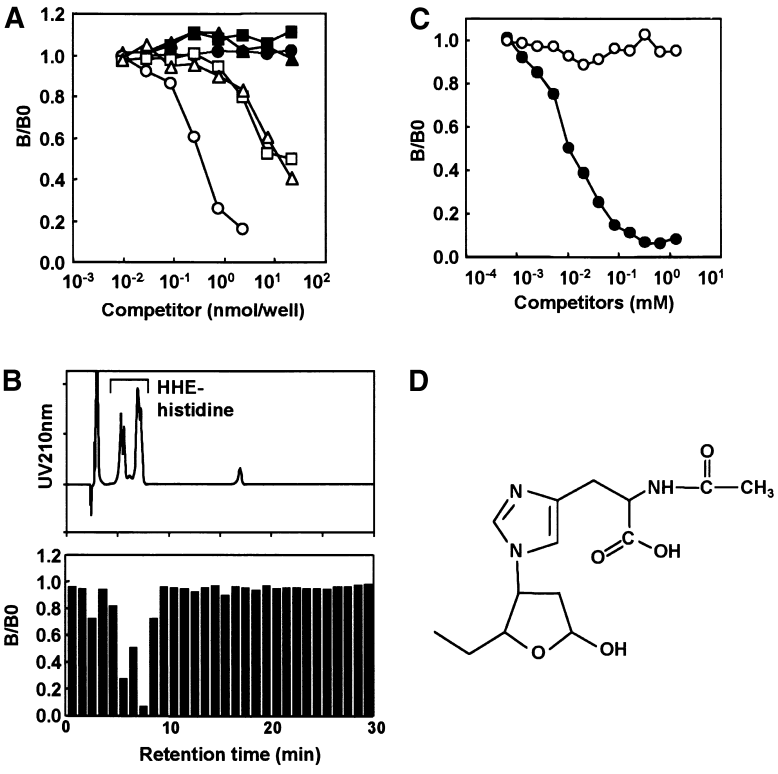


Fig. 4. Identification of antigenic structures recognized by MAb HHE53. A: Competitive ELISA analysis with the reaction mixtures of amino acid derivatives and HHE. Competitors were prepared by incubating 10 mM amino acid derivatives, N^{α}-acetylcysteine, N^{α}-acetylhistidine, or N^{α}-acetyllysine, in the presence or absence of 10 mM HHE in 50 mM sodium phosphate buffer, pH 7.2, for 24 h at 37°C. Competitors: open circles, HHE/N^{α}-acetylhistidine; open triangles, HHE/N^{α}-acetyllysine; open squares, HHE/N^{α}-acetylcysteine; closed circles, N^{α}-acetylhistidine; closed triangles, N^{α}-acetyllysine; closed squares, N^{α}-acetylcysteine. The amounts of material in the ELISA were normalized to the concentration of the starting materials (amino acid derivatives). The ratio B/B_0 is explained in Experimental Procedures. B: Competitive ELISA analysis of HPLC fractions for immunoreactivity with MAb HHE53. The reaction was performed by incubating 10 mM N^{α}-acetylhistidine with 10 mM HHE in 50 mM sodium phosphate buffer, pH 7.2, for 24 h at 37°C. The upper panel shows a profile of ultraviolet (UV) absorbance at 210 nm. The lower panel shows competitive ELISA analysis of HPLC fractions for immunoreactivity with MAb HHE53. C: Competitive ELISA analysis of Michael addition-type HHE-N^{α}-acetylhistidine adducts. Competitors: closed circles, Michael addition-type HHE-N^{α}-acetylhistidine adducts; open circles, N^{α}-acetylhistidine. D: An epitope structure recognized by MAb HHE53.

of BSA with HHE (**Fig. 5A**). Similar results were obtained from experiments using human plasma (Fig. 5B). To further examine whether immunoreactive materials with MAb HHE53 are produced during lipid peroxidation, we analyzed the protein incubated with PUFAs in the presence of a metal/ascorbate-mediated free radical-generating system. As shown in **Fig. 6A**, lipid peroxidation monitored by TBARS formation was significantly induced by incubating PUFAs with the metal/ascorbate system. Agarose gel electrophoresis/immunoblot analysis of BSA treated with iron/ascorbate/DHA clearly revealed the formation of immunoreactive materials (Fig. 6B). In addition, ELISA analysis demonstrated that peroxidation of n-3 PUFAs, such as DHA and EPA, with metal/ascorbate in the presence of BSA resulted in a time-dependent increase in the antigenicity of the protein, whereas the peroxidation of arachidonic acid in the presence of the protein scarcely generated antigenic materials (Fig. 6C). These results suggest that MAb HHE53 is capable of distinguishing the peroxidation of n-3 and n-6 PUFAs.

Formation of HHE-histidine in oxidized LDL

In view of the observations that the peroxidation of n-3 PUFAs with metal/ascorbate in the presence of protein resulted in the formation of antigenic materials with MAb HHE53, it was of interest to directly demonstrate that the oxidation of LDL leads to HHE-histidine adducts that can be recognized by the antibody. To this end, LDL was incubated with 5 μM Cu^{2+} (24 h, 37°C). Oxidation of endogenous fatty acid was verified by the formation of malondialdehyde as determined by the production of TBARS (**Fig. 7A**). After separation by agarose gel electrophoresis, the native form of the LDL appeared as a single protein band that was readily visualized by Fat Red 7B staining (Fig. 7B, upper panel); however, the LDL incubated with 5 μM Cu^{2+} exhibited enhanced anodic mobility compared with the native LDL, indicating the increased negative charge of the molecule, probably attributable to the modification of the ε-amino group in the lysine residues. Agarose gel electrophoresis/immunoblot analysis of the Cu^{2+}-oxidized LDL using MAb HHE53 revealed the generation of immunoreactive materials that were not detected in the native LDL (Fig. 7B, lower panel). These data confirmed the generation of HHE-derived epitopes in the Cu^{2+}-oxidized LDL.

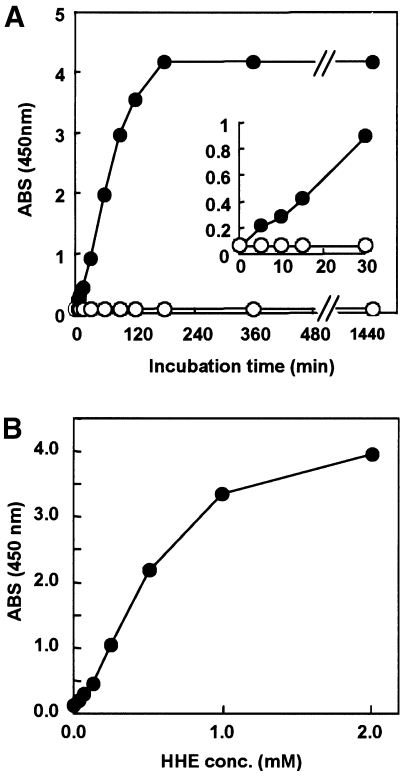


Fig. 5. Formation of the HHE-histidine adduct in the HHE-treated protein and human plasma. BSA (1 mg/ml) was incubated with 1 mM HHE in 1 ml of 50 mM sodium phosphate buffer, pH 7.2, at 37°C. A: Immunoreactivity of MAb HHE53 to the BSA exposed to the HHE. The coating antigen was prepared as described for Fig. 3. Closed circles, HHE-BSA; open circles, BSA. ABS, absorbance. B: Human plasma samples (1 mg protein/ml) were incubated with 0–1 mM HHE in 1 ml of 50 mM sodium phosphate buffer, pH 7.2, for 24 h at 37°C. conc., concentration.

Immunohistochemical localization of the HHE-histidine adduct in human atherosclerotic aorta

The findings that the level of aldehyde-modified LDL increases in the plasma of patients with atherosclerosis (17) and that the monoclonal antibodies raised against aldehyde-modified LDL bind to epitopes in atherosclerotic lesions (18, 19) suggest that the protein-bound aldehydes, including HHE, are most likely accumulated in an atherosclerotic lesion. Hence, consecutive frozen sections from an unstable lesion of a human atherosclerotic plaque were immunohistochemically examined for the HHE-histidine Michael addition-type adducts using MAb HHE53. Immunoreactivity with MAb HHE53 was localized in the cytoplasm of spindle or foamy macrophages that were seen in atherosclerotic plaques (**Fig. 8D**). These macrophages were identified by histopathological and immunohistochemical features on H and E-stained (Fig. 8A) or CD68-immunostained (Fig. 8B) sections. These cells are also immunoreactive with MAb HNEJ2 (Fig. 8C), which recognizes protein-bound HNE (14). However, MAb HHE53 and MAb HNEJ2 at this concentration (0.1 mg IgG/ml) have similar immunoreactivity toward HHE-modified and HNE-modified proteins, respectively, suggesting that HNE may be more abundant than HHE in human atherosclerotic lesions. On the other hand, no immunoreaction product deposits were detectable in sections immunostained with MAb HHE53 preincubated with an excess of HHE-N^{α}-acetylhistidine (Fig. 8E) or in sections processed with the omission of the primary antibodies (Fig. 8F). We examined the variability in staining among the five independent samples and confirmed that the immunoreactivity with MAb HHE53 was commonly detected in the macrophage-derived foam cells in all samples. In addition, there was no significant difference in the immunohistochemical localization of HHE-histidine adduct between frozen and paraffin-embedded sections (data not shown). These data suggest that the generation of

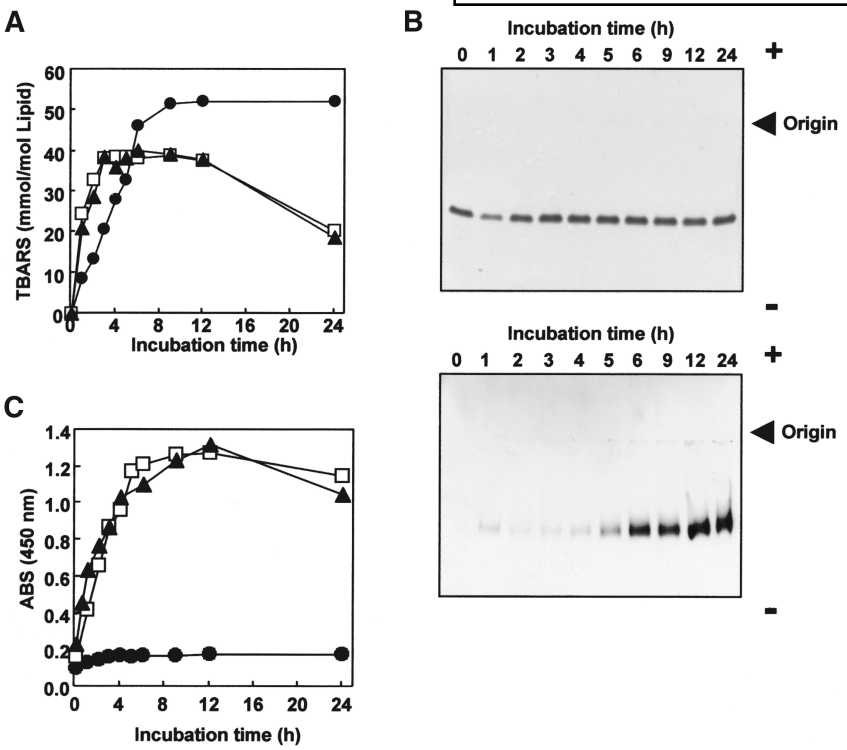


Fig. 6. Formation of the HHE-histidine adduct in the protein exposed to Cu^{2+}/ascorbate oxidation of PUFAs. BSA (0.2 mg/ml) was incubated with 0.4 mM PUFAs in the presence of 10 μM Cu^{2+} and 2 mM ascorbate in 1 ml of 50 mM sodium phosphate buffer, pH 7.2, at 37°C. A: 2-Thiobarbituric acid-reactive substance (TBARS) formation via PUFA peroxidation. PUFAs: closed circles, arachidonic acid (ARA); open squares, eicosapentaenoic acid (EPA); closed triangles, docosahexaenoic acid (DHA). B: Agarose gel electrophoresis/immunoblot analysis of the BSA exposed to Cu^{2+}/ascorbate oxidation of DHA. The upper panel shows the results of agarose gel electrophoresis. The lower panel shows the results of agarose gel electrophoresis/immunoblot analysis with MAb HHE53. C: Immunoreactivity of MAb HHE53 to BSA exposed to Cu^{2+}/ascorbate oxidation of PUFAs. The immunoreactivity of MAb HHE53 was determined by a direct ELISA using BSA exposed to Cu^{2+}/ascorbate oxidation of PUFAs as the absorbed antigen. The coating antigen was prepared as described for Fig. 3. PUFAs: closed circles, ARA; open squares, EPA; closed triangles, DHA.

epitopes recognized by the antibody during the dehydration, embedding, rehydration, and staining processes is unlikely and that the observed immunoreactivities reflect endogenous products generated in vivo. Thus, the detection of the HHE-histidine Michael addition-type adducts in atherosclerotic plaques supports the theory that the n-3 PUFAs were relatively oxidizable compounds in vivo.

DISCUSSION

Immunologic detection is a powerful tool that can be used to evaluate the presence of a desired target and its subcellular localization. Major advantages of this technique over the chemical approaches are the evaluation of small numbers of cells or archival tissues that may otherwise not be subject to analysis. Because of the increasing interest in 4-hydroxy-2-alkenal's modification of proteins under oxidative stress, it seemed useful to prepare an antibody that interacts specifically with the 4-hydroxy-2-alkenal-modified proteins; such antibodies have been prepared by immunizing rabbits with HNE-treated KLH (12), in which HNE adducts of histidine, lysine, and cysteine serve as the antigenic sites. Later, Toyokuni et al. (14) raised the MAb (HNEJ2) against HNE-modified KLH and found that the antibody cross-reacted specifically with HNE-modified proteins and had a higher affinity for the HNE-histidine adduct than for the HNE-lysine and HNE-cysteine adducts. However, most of the antibodies, including MAb HNEJ2, directed to the HNE-modified protein have been raised against a protein treated with HNE. Therefore, it is not known whether other 4-hydroxy-2-alkenal species are generated in vitro and in vivo. Hence, in the present study, we raised a novel MAb, HHE53, against HHE-treated KLH. This antibody turned out to be extremely specific for the HHE-treated proteins and hardly reacted with the proteins treated with other aldehydes (Fig. 3A, C). The lack of cross-reactivity of the antibodies for the 2-hexenal-treated protein can be ascribed to the absence of the 4-hydroxy group, which leads the primary Michael adduct to the tetrahydrofuran derivative through intramolecular cyclization. This and the observation (Fig. 3B) that MAb HHE53 did not cross-react with the proteins that had been treated with the HHE analogs, such as 4-hydroxy-2-pentenal, 4-hydroxy-2-heptenal, 4-hydroxy-2-octenal, HNE, and 4-hydroxy-2-decenal, suggest that both the tetrahydrofuran and butyl moieties of the HHE-histidine adduct may be critical for the antibody binding. In addition, the binding of these antibodies to the HHE-treated proteins was specifically inhibited by the reaction mixture of HHE/N^{α}-acetylhistidine (Fig. 4A), suggesting that the imidazole ring is also involved in the antibody binding. Using MAb HHE53, we demonstrated that the epitope structure recognized by the antibody was formed upon the incubation of BSA or human serum with HHE (Fig. 5). In addition, it was found that MAb HHE53 was capable of distinguishing the peroxidation of n-3 and n-6 PUFAs. This result suggests the usefulness of this antibody for future investigations aimed at elucidating the relative contribution of oxidized n-3 PUFAs to the accumulation of oxidatively modified proteins in vivo.

On the other hand, the modification of LDL involves oxidation of the unsaturated fatty acids included in the LDL particle with the appearance of lipid peroxidation products, including reactive aldehydes (20, 21). It has

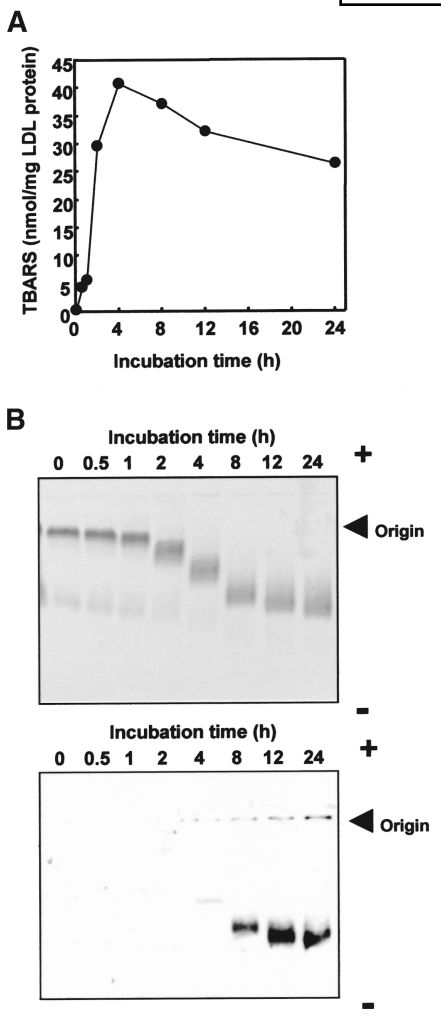


Fig. 7. In vitro formation of the HHE-histidine adduct in oxidized LDL. LDL (0.5 ml) was incubated with 5 μM Cu^{2+} in 1 ml of PBS at 37°C. A: TBARS formation of Cu^{2+}-oxidized LDL. B: Agarose gel electrophoresis/immunoblot analysis of Cu^{2+}-oxidized LDL. The upper panel shows results from agarose gel electrophoresis. The lower panels shows results from immunoblot analysis of Cu^{2+}-oxidized LDL with MAb HHE53.

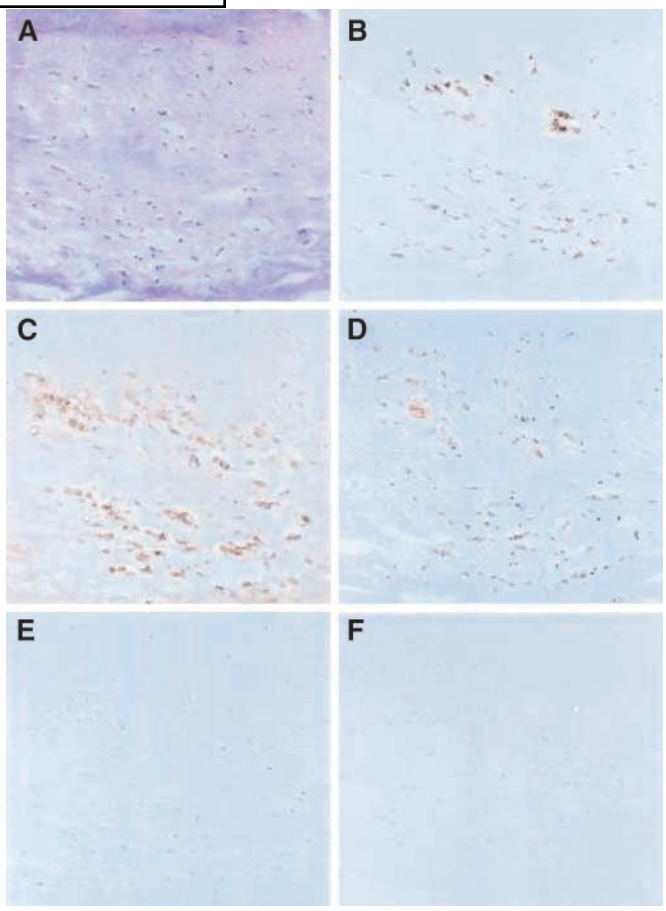


Fig. 8. Photomicrographs of consecutive frozen sections from an unstable lesion of a human atherosclerotic plaque. Many pleomorphic macrophages, infiltrating the shoulder of the plaque, are identified on a section stained with hematoxylin and eosin (A) and a section immunostained with MAb KP-1 for CD68 (B). These cells are also immunoreactive with MAb HNEJ2 (C) and MAb HHE53 (D). No immunoreaction is visible on a section immunostained with MAb HHE53 preincubated with an excess of HHE-N^{α}-acetylhistidine (E) or on a section from which the primary antibody (MAb HHE53) was omitted (F) as a negative reaction control.

been shown that these aldehydes form covalent bonds with the lysine amino groups of apolipoprotein B-100, leading to a decrease in the net negative charge of the LDL particle and to an increase in its electrophoretic mobility (22). To investigate the mechanisms contributing to the modification of LDL, we analyzed the Cu^{2+}-oxidized LDL by monitoring the production of n-3 PUFA-derived product HHE and observed that the peroxidation of LDL with Cu^{2+} resulted in the significant production of HHE-derived epitopes (Fig. 7). This is consistent with the previous findings that considerably higher concentrations of free HHE are generated in the Cu^{2+}-oxidized LDL (21).

The formation of lipid peroxidation products bound to proteins in vascular lesions, such as the atherosclerotic lesion, is a phenomenon common in most, if not all, types of vascular damage associated with oxidative stress. Hence, the in vivo detection of antigenic structures using MAb HHE53 was attempted in tissue samples from patients with atherosclerosis, which is considered a form of chronic inflammation resulting from the interaction between the modified lipoproteins, monocyte-derived macrophages, T cells, and the normal cellular elements of the arterial wall. We confirmed that atheromatous lesions indeed contained protein-bound HHE, colocalizing mainly with foamy macrophages (Fig. 8). It is known from in vitro studies that all of the major cell types within the atherosclerotic lesions are capable of promoting the oxidation of LDL (7, 8). Therefore, the observed cell-associated staining patterns 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, the intracellular granular staining observed in the atherosclerotic lesions represents the presence of protein-bound HHE that had already been taken up by the macrophages and that are present within the cell in cytoplasmic

organelles (23). This leads to the hypothesis that the modification by HHE renders proteins relatively resistant to intracellular proteolytic degradation, resulting in the marked accumulation of epitopes in the macrophages.

Long-chain n-3 PUFAs such as EPA and DHA are usually consumed in small quantities (24); therefore, they are reported in low proportions in plasma and most tissue lipids. However, increased consumption of these fatty acids raises their proportion in various blood and tissue lipid pools. A key observation from the previous study is that when long-chain n-3 PUFAs are consumed in a modest dose, they are readily incorporated into atherosclerotic-plaque lipids (25). Thus, it is not unlikely that the incorporation of n-3 PUFAs into plaque lipids may result in the enhanced production of HHE and its protein adducts in the lesions.

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