Formation of N\textsuperscript{ε}-(Hexanonyl)lysine in Protein Exposed to Lipid Hydroperoxide

A PLAUSIBLE MARKER FOR LIPID HYDROPEROXIDE-DERIVED PROTEIN MODIFICATION\textsuperscript{*}

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The objectives of this study were to estimate the structure of the lipid hydroperoxide-modified lysine residue and to prove the presence of the adducts in vivo. The reaction of lipid hydroperoxide toward the lysine moiety was investigated employing N-benzyol-glycyl-L-lysine (Bz-Gly-Lys) as a model compound of Lys residues in protein and 13-hydroperoxyoctadecadienoic acid (13-HPODE) as a model of the lipid hydroperoxides. One of the products, compound X, was isolated from the reaction mixture of 13-HPODE and Bz-Gly-Lys and was then identified as N-benzyol-glycyl-N\textsuperscript{ε}-(hexanonyl)lysine. To prove the formation of N\textsuperscript{ε}-(hexanonyl)lysine, named HEL, in protein exposed to the lipid hydroperoxide, the antibody to the synthetic hexanonyl protein was prepared and then characterized in detail. Using the anti-HEL antibody, the presence of HEL in the lipid hydroperoxide-modified proteins and oxidized LDL was confirmed. Furthermore, the positive staining by anti-HEL antibody was observed in human atherosclerotic lesions using an immunohistochemical technique. The amide-type adduct may be a useful marker for the lipid hydroperoxide-derived modification of biomolecules.

During lipid peroxidation, biomolecules such as proteins or aminolipids can be covalently modified by lipid decomposition products. For the case of aliphatic aldehydes (alkanals) such as 1-hexanal or 1-nonanal, the N\textsuperscript{ε}-amino groups of the lysine residues in protein can be modified through the formation of a Schiff base. a,\textgreek{b}-Unsaturated aldehydes (alkenals) such as acrolein or 4-hydroxy-2-nonalenal react with lysine, cysteine, and histidine through a Michael-type addition (1,2). On the other hand, lipid hydroperoxide might covalently react with protein without serious decomposition of its structure (3). Keto fatty acid (4), which is one of the products by lipoxygenase reaction, can also react with protein and amino acids as previously suggested (5-7). In addition, the pyrrole compounds from long chain epoxides and lysine were identified (8). However, the mechanism of lipid hydroperoxide-derived protein modification is not so clear.

To estimate the structure after lipid hydroperoxide-derived lysine modification, the reaction of 13-hydroperoxyoctadecadienoic acid (13-HPODE)\textsuperscript{3} with N-benzyol-glycyl-L-lysine (Bz-Gly-Lys) was investigated. In this study, a novel compound, N-benzyol-glycyl-N\textsuperscript{ε}-(hexanonyl)lysine (named HEL), was identified as one of the lipid hydroperoxide-modified lysine residues. The formation of HEL in lipid hydroperoxide-modified proteins including oxidatively modified LDL was confirmed using the specific antibody to the HEL residue. In addition, the HEL moiety was detected in human atherosclerotic plaques by immunohistochemical approach. As far as we know, the formation of an amide-type adduct has not been previously reported. This novel adduct derived from lipid hydroperoxide may become an initial marker for the oxidative damage of biological molecules in vivo.

**EXPERIMENTAL PROCEDURES**

\textbf{Materials—}The chemicals used were from the following sources. Bz-Gly-Lys and N-acetyl-glycyl-L-lysine methyl ester (AGLME) were purchased from Peptide, Inc. Soybean lipoxygenase, lipid-free BSA (product number A7511, initial fractionation by cold alcohol precipitation, \textgreek{e} 97\% albumin, essentially fatty acid free), arachidonic acid, methylgligoxal, 2-hexenal, cardiolipin, and N\textsuperscript{ε}-carboxybenzoyl-L-lysine methyl ester were obtained from Sigma. Linoleic acid, glyoxal, 1-nonanal, 2-nonenal, hexanoic acid, acetic acid, N\textsuperscript{ε}-hydroxysuccinimide (NHS), and benzoyl-glycine were purchased from Wako Pure Chemicals Industries. Methyl linolate and 1-hexanal were obtained from Nacarai Tesque, Inc. 1-Ethyl-3(3-dimethyaminopropyl)carbodimide (EDC), N\textsuperscript{ε}-hydroxysulfosuccinimide (sulf-NHS), and keyhole limpet hemocyanin (KLH) were obtained from Pierce. 4-Hydroxy-2-nonalenal was synthesized and provided by Dr. Koji Uchida (Nagoya University). Propionic acid, butyric acid, valeric acid, heptanoic acid, octanoic acid, nonanoic acid, decanoic acid, and undecanoic acid were purchased from GL Science, Inc. Monomethyl azelate was supplied by Larodan Fine Chemicals. Glutaric anhydride and malonaldehyde bis(dimethylacetal) were purchased from Aldrich.

**Preparation of Lipid Hydroperoxides**—13-HPODE was prepared by the enzymatic reaction of lipoxygenase with linoleic acid (9,10). 15-Hydroperoxyoctadecatrienoic acid (15-HPOTE) was prepared as described previously (9,10). Methyl linolate hydroperoxide (MLOOH) was prepared by the reaction of soybean lipoxgenase with methyl linolate (ML). A 200-mg sample of ML and sodium deoxycholate (1.62 g) was dissolved in 240 ml of 200 mM borate buffer (pH 9.0). Lipoygenase (100 mg, Sigma type II-B) was added to the solution and reacted for 3 h at room temperature. The formed peroxide was extracted twice with an

\textbf{enolic acid; Bz-Gly-Lys, N-benzyol-glycyl-L-lysine methyl ester; NHS, N-hydroxysuccinimide; HEL, N\textsuperscript{ε}-(hexanonyl)lysine; LDL, low density lipoprotein; sulfo-NHS, N-hydroxysulfosuccinimide; EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide; KLH, keyhole limpet hemocyanin; 15-HPETE, 15-hydroperoxyoctadecatrienoic acid; BSA, bovine serum albumin; LC-MS, liquid chromatography-mass spectrometry; PBS, phosphate-buffered saline; AAPH, 2,2\'-azobis(2-amidinopropane)dihydrochloride; ELISA, enzyme-linked immunosorbent assay; 13-HODE, 13-hydroxyoctadecadienoic acid; FAB-MS, fast-atom bombardment MS; ESP, electrospray ionization; ML, methyl linolate; MLOOH, ML hydroperoxide; HPLC, high performance liquid chromatography.
equal amount of chloroform/methanol (1:1). The collected chloroform layer was evaporated. The obtained peroxide was purified by thin layer chromatography (TLC) and developed with n-hexane/ether (6:4). The peroxide was extracted with CHCl₃, and then evaporated. The amount of MLOOH was calculated from the molar coefficient, ε₂₄₅nm = 25000 m⁻¹ cm⁻¹. The reaction was stopped as already described, and the residual product was purified by reversed-phase HPLC on a Develosil ODS-HG-5 (8 × 250 mm) equilibrated with 0.1% trifluoroacetic acid, CH₃CN (5/3) at a flow rate of 2.0 ml/min. The elution was estimated by UV absorbance at 234 nm, and identification of the synthetic deuterified hexanonyl compound was performed by liquid chromatography-mass spectrometry (LC-MS).

**Synthesis of N-acetyl-glycyl-N'-(hexanonyl)-l-lysine Methyl Ester**—

The N-acetyl-glycyl-N'-(hexanonyl)-l-lysine methyl ester (N'-hexanonyl AGLME) was prepared by conjugation between AGLME and hexanoic acid using EDC as the coupling reagent and NHS as the enhancer, as described previously. The synthetic compound could not be purified by silica gel chromatography because of its high water solubility. Therefore, the reaction mixture was diluted with 0.1% trifluoroacetic acid and passed through a Sep-Pak cartridge. The cartridge was washed with 0.1% trifluoroacetic acid, and the products were then eluted with 0.1% trifluoroacetic acid, CH₃CN (1/1). The eluent was concentrated and applied to preparative reversed-phase HPLC (Develosil ODS-5 (20 × 250 mm)) using 0.1% trifluoroacetic acid, CH₃CN (7:3) as the eluent. The elution was monitored by absorbance at 215 nm. The peak was collected and concentrated. The obtained N-acetyl-glycyl-N'-(hexanonyl)-l-lysine methyl ester (N'-hexanonyl AGLME) was identified by ¹H NMR and mass spectroscopy (LC-MS). The spectral data of N'-hexanonyl AGLME are as follows: ¹H NMR (CDCl₃) (ppm) 0.71 (t, J = 7.1 Hz, 3H), 1.09 (m, 2H), 1.20 (m, 2H), 1.21 (m, 2H), 1.31 (m, 2H), 1.49 (m, 1H), 1.57 (m, 1H), 1.77 (t, J = 7.2 Hz, 2H), 2.06 (t, J = 7.0 Hz, 2H), 2.07 (t, J = 7.0 Hz, 2H), 2.09 (t, J = 7.0 Hz, 2H), 4.22 (m, 1H), 4.34 (m, 1H), 7.37 (t, J = 1.2 Hz, 1H), 7.45 (t, J = 1.2 Hz, 1H), 7.65 (s, 2H), 7.70 (d, J = 7.0 Hz, 2H); FAB-MS m/z 406 (M + H)⁺, 428 (M + Na)⁺.

**Amino Acid Analysis**—Samples were hydrolyzed with 6 N HCl in vacuo at 105 °C. The hydrolysates were dissolved in citrate buffer (pH 2.2), and then applied to an amino acid analyzer, JLC-500 (JEOL).

**Preparation of Antibody against Hexanonyl Keyhole Limpet Hemocyanin**—The conjugation of hexanoic acid with proteins was performed as follows. Hexanoic acid (2.3 mg), EDC (4.5 mg), and sulfo-NHS (5 mg) were dissolved in 400 µl of dimethylformamide, and the reaction mixture was incubated for 24 h at room temperature. To the solution, 0.95 ml of KLH or BSA (10 mg in 0.1 M phosphate buffer (pH 7.4)) was added and further incubated for 4 h at room temperature. The obtained hexanonyl proteins were dialyzed against phosphate-buffered saline (PBS) for 3 days at 4 °C. The hexanonyl KLH emulsified with an equal volume of complete Freund’s adjuvant to a final concentration of 0.5 mg/ml, and 1 ml of the solution was then intramuscularly injected into a New Zealand White rabbit. After 4 weeks, 1 ml of the hexanonyl KLH emulsified with an equal volume of incomplete adjuvant (0.5 mg/ml) was injected as a booster every 2 weeks until an adequate amount of hexanonyl BSA conjugate was obtained for evaluation of the antibody generation specific to hexanonyl protein.

**Preparation of Chemically Modified Proteins**—Conjugates of acetic acid (C2), propionic acid (C3), butyric acid (C4), valeric acid (C5), heptanoic acid (C7), octanoic acid (C8), nonanoic acid (C9), decanoic acid (C10), and undecanoic acid (C11) with BSA were prepared using EDC and NHS as coupling agents as described previously. Glutaric acid-BSA conjugate was prepared from glutaric anhydride, lipid-free BSA conjugate. The obtained BSA was mixed with an equal volume of saturated sodium acetate. Under ice-cold conditions, glutaric anhydride (3 ml) was added and reacted for 1 h. The modified BSA was dialyzed against water at 4 °C for 24 h. The dialyzed BSA conjugate was prepared as follows. First, the monomethylazelaic acid (50 mg), EDC (52.2 mg), and NHS (31.3 mg) in dimethylformamide (1 ml) were incubated at room temperature for 24 h. Five milliliters of BSA solution (30 mg/ml in 0.1 M phosphate buffer (pH 7.4)) was then added to the solution and then incubated at room temperature for 16 h. The reaction mixture was dialyzed against PBS at 4 °C for 3 days. Azelaic acid-BSA conjugate was prepared from obtained monomethylazelaic acid-BSA by saponification. Alkaline solution (0.25 M NaOH) was added to the monomethylazelaic acid-BSA and further incubated for 1 h. After neutralization with HCl, the reaction mixture was dialyzed against PBS at 4 °C for 24 h. These conjugates were evaluated by the trinitrobenzenesulfonic acid method (14). The losses (%) of lysine residue were as follows: C2, 72%: C3, 40%; C4, 17%; C5, 90%: C6 (hexanonyl BSA), 91%; C7, 91%; C8, 89%; C9, 89%; C10, 94%; C11, 90%; glutaric acid-BSA, 38%; azelaic acid-BSA, 62%.

Oxidized lipid-modified proteins were prepared as follows. Lipid (linoleic acid and i,6-docosahexaenoic acid), cholesterol, and cardiolipin oxidized by 5% ascobic acid and 0.05 mM FeCl₃ for 24 h at 37 °C in PBS containing 20% methanol. To the reaction mixture, lipid-free BSA (final concentration, 5 mg/ml) was added and further incubated at 37 °C for 3 days. To isolate the modified proteins, an equal amount of CHCl₃/CH₂OH (2:1) was added, vigorously mixed, and then centrifuged for 10 min at 4 °C. The lower layer was discarded, and an equal amount of
Modification of LDL—Human LDL was isolated from healthy volunteers using density centrifugation (15). The modification of LDL was performed by copper ion and 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) as described previously (9). The modification by copper ion was performed against water for 2 days at 4 °C. Aldehyde-modified proteins were prepared as already described (9, 10). The formation of lipid peroxide was measured by a BCA assay kit (Pierce).

LC-MS Measurement—The sample was applied to a liquid chromatograph on a Develosil ODS-HG-5 (4.6 × 250 mm, Nomura Chemical Co.) using a linear gradient of the two-solvent system at a flow rate of 0.8 ml/min. The elution was monitored by absorbance at 234 nm. Solvent A (0.01% trifluoroacetic acid) and solvent B (CH3CN) were used for the gradient. The gradient employed was as follows: B 0% to B 60% in 5 min, B 60% to B 60% in 5 min, B 60% to B 0% in 10 min.

ChCl3 was added and mixed. After centrifugation, the lower layer was discarded again. To the residual upper layer, 9 volumes of ice-cool ethanol was added and kept for 45 min at 4 °C. After centrifugation, the pellet was dissolved in water with sonication. The protein solution was dialyzed against water for 2 days. Aldehyde-modified proteins were prepared as already described (9, 10). The preparation of lipid hydroperoxide-modified proteins was as follows. The hydroperoxides (13-HPDOE or 15-HPETE) were incubated with lipid-free BSA at 37 °C in 0.1 M phosphate buffer (pH 7.4) for 3 days. After incubation, the sample was stored at −70 °C until HPLC analysis. The amount of adduct was estimated by comparison with the synthetic compound.

In this experiment, we focused on the reaction of lipid peroxides with a methylene blue derivative in the presence of hemoglobin (16).

Enzyme-linked Immunosorbent Assay (ELISA)—Indirect noncompetitive ELISA was performed as already described (9, 10). Briefly, 50 μl of antigen in PBS was dispensed into a well and kept at 4 °C overnight. After blocking with Block Ace (Dainihon Seiyaku, Osaka, Japan), 100 μl of antiserum (1/5000 in PBS containing 0.5% BSA) was added to the well. The binding of the antibody on the coated antigen was evaluated as already described (9, 10).

The cross-reactivity of the low molecular weight compound with antibody was investigated by indirect competitive ELISA (9, 10). As a coating agent, 50 μl of hexanoyl BSA (0.5 μg/ml) was pipetted onto wells and kept at 4 °C overnight. At the same time, 50 μl of antiserum (1/2500 in PBS containing 1% BSA) and 50 μl of sample were mixed in an Eppendorf tube and reacted at 4 °C overnight. The plate was washed, and 90 μl of the reacted solution was pipetted onto a well. The binding of the residual antibody on coated hexanoyl BSA was estimated as described previously (9, 10).

Reaction between Preincubated 13-HPDOE and Lysine Residue—The

FIG. 1. Separation of lipid-Lys adducts derived from the reaction of 13-HPDOE and Bz-Gly-Lys. A, the reaction mixture was concentrated and extracted with methanol. The extract was applied to gel filtration chromatography (HW-40). The elution was monitored by absorbance at 234 nm and fluorescence (excitation, 350 nm; emission, 420 nm). Fractions 29–34 were used for further isolation. B, the concentrated fractions were further fractionated using Sep-Pak cartridge. An aliquot of the 20% methanol fraction was applied to reversed-phase HPLC on a Develosil ODS-HG-5 (4.6 × 250 mm, Nomura Chemical Co.) using a linear gradient of the two-solvent system at a flow rate of 0.8 ml/min. The elution was monitored by absorbance at 234 nm. Solvent A (0.01% trifluoroacetic acid) and solvent B (CH3CN) were used for the gradient. The gradient employed was as follows: B 0% to B 60% in 60 min, B 60% to B 60% in 5 min, B 60% to B 0% in 10 min.

Dialyzed against 0.1 M phosphate buffer (pH 7.4) for 3 days. Oxidized BSA was used as a coating agent, 50 μl of the reacted solution was pipetted onto a well. The binding of the antibody on coated hexanoyl BSA was estimated as described previously (9, 10).

FIG. 2. Proposed structure of the N-(Hexanonyl)lysine adduct with the parent molecules (A) and time-dependent formation of N-hexanonyl adduct during incubation of 13-HPDOE with Bz-Gly-Lys (B). A, the proposed structure of a novel adduct with parent molecules. B, Bz-Gly-Lys (5 mM) was reacted with 13-HPDOE (5 mM) in 0.1 M phosphate buffer (pH 7.4) at 37 °C for 3 days. After incubation, the sample was stored at −70 °C until HPLC analysis. The amount of adduct was estimated by comparison with the synthetic compound.
Isolation of Lipid Hydroperoxide-modified Lysine Derivative—To search for the specific hydroperoxide-derived lysine modification, we used Bz-Gly-Lys as the substrate, and the isolation of the 13-HPODE-modified lysine derivative was performed. To remove large amounts of unreacted Bz-Gly-Lys, the reaction mixture was concentrated and then applied to gel filtration chromatography using TOYOPEARL HW-40 (TOSOH) as a gel. The fractions were monitored by absorbance at 234 nm and lipofuscin-like fluorescence (Fig. 1A). As a result of the HPLC analysis of each fraction, fractions 29–34 contained the fluorescent fractions were used for further isolation. The HPLC analysis of each fraction, fractions 35–40 contained the lipid decomposition product-modified molecules. Therefore, the fluorescent fractions were used for further isolation. The mixed fractions 29–34 were applied to a Sep-Pak cartridge. The fluorescent fractions were collected and further purified by reversed-phase HPLC analysis (see above). The structure of the isolated compound X nor the synthetic (Hexanonyl)lysine by Lipid Hydroperoxide

FIG. 3. Comparison of HPLC-MS between synthetic N-hexanonyl AGLME and 13-HPODE-modified AGLME. The structure of N-hexanonyl AGLME is shown in the chart (A). The aliquots of the reaction mixture and synthetic compound were analyzed by reversed-phase HPLC on a Develosil ODS-HG-5 column as described under “Experimental Procedures” (B).

FIG. 4. Formation of N-(Hexanonyl)lysine by lipid hydroperoxides. An aliquot of the reaction mixture was applied to HPLC connected with MS (PLATFORM II, VG Biotech.). The chromatogram was scanned by m/z 358 as the (M + H)+ ion of N-(hexanonyl) AGLME. The total current ion is shown in the figure. A, 13-HPODE-modified AGLME; B, 15-HPETE-modified AGLME; C, MLOOH-modified AGLME.

lecular weight of X, 405, was confirmed by FAB-MS. Compound X was hydrolyzed with 6 N HCl at 105 °C in vacuo and submitted for amino acid analysis. Interestingly, both Gly and Lys were completely recovered from the acid hydrolysates of compound X (Gly/Lys = 0.96). This suggested that the bond between the lipid-derived structure and Bz-Gly-Lys was acid liable such as an amide bond or a Schiff base. The structure of X was elucidated using 1H NMR. The proposed structure of X with the parent molecules is shown in Fig. 2A. To confirm the structure of compound X, the synthesis of the N-(hexanonyl)-lysine adduct was performed by carbodiimide conjugation of the lysine derivative with hexanoic acid. The instrumental analysis of the synthetic N-benzoyl-glycyl-N-(hexanonyl)-lysine almost agreed with that of isolated compound X. Neither the isolated compound X nor the synthetic N-(hexanonyl)lysine adduct had any fluorescence. The time-dependent changes in Bz-Gly-Lys during incubation with 13-HPODE were examined. As shown in Fig. 2B, a loss of Bz-Gly-Lys was observed in a time-dependent fashion, and the formation of N-benzoyl-glycyl-N-(hexanonyl)lysine, compound X, was confirmed. The conversion yield of compound X from the loss of Bz-Gly-Lys after a 3-day incubation with 13-HPODE was 5.6%.
To further confirm the formation of the N\textsuperscript{e}-(hexanonyl)lysine named "HEL" in the 13-HPODE-modified Lys, the AGLME was incubated with 13-HPODE, and the formation of the HEL derivative was investigated. After a 3-day incubation, an aliquot of the reaction mixture was applied to LC-MS. The product, which shows \( m/z \) 358 as an (M+H\textsuperscript{+}) ion, corresponding to \( \text{N}^\text{e}-\text{hexanonyl AGLME} (M, 357) \), was eluted at a retention time of 40.79 min, and this completely agreed with the elution time of the synthetic \( \text{N}^\text{e}-\text{hexanonyl AGLME} \) (Fig. 3). The mass charts of both the product and the synthetic \( \text{N}^\text{e}-\text{hexanonyl AGLME} \) showed the same fragmentation pattern.

The Mechanism for Formation of HEL—HEL was formed by the reaction of AGLME with 15-HPETE as well as 13-HPODE (Fig. 4, A and B). The reaction of methyl linoleate hydroperoxide with AGLME could also generate the HEL derivative (Fig. 4C). The relative ratio of the formation of HEL from 13-HPODE, 15-HPETE, and MLOOH was 1:0.49:0.47. These results suggested that the adduct should be formed from not only free fatty acid hydroperoxides but also esterified fatty acid hydroperoxides, such as cholesteryl ester hydroperoxide and phosphatidylcholine hydroperoxide.

Hexanal, one of the lipid decomposition products, can react with lysine and form a Schiff base. However, HEL does not have a Schiff base in its structure. To deny the participation of 1-hexanal in the amide bond formation, the formation of \( \text{N}^\text{e}-\text{hexanonyl AGLME} \) during incubation of AGLME with aldehyde was examined by LC-MS. HEL was not formed from the reaction of 1-hexanal with AGLME.

Furthermore, the catalytic activity of peroxide on the formation of the amide bond was also investigated. The \( \text{N}^\text{e}-\text{hexanonyl adduct} \) was not generated by the reaction of 5 mM AGLME with 5 mM hexanoic acid (1-hexanal) in the absence or presence of 5 mM tert-butyl hydroperoxide. This result revealed that the formation of HEL was not derived from the decomposition prod-
ucts, hexanal or hexanoic acid. The existence of other unknown precursors is suggested.

Preparation of Antibody to HEL—

It is difficult to detect HEL moiety in protein molecules or tissue samples by chemical methods because HEL is unstable for acid hydrolysis from its amide linkage and does not have any specific absorbance. Therefore, the preparation of an antibody specific to HEL was planned. The antibody was prepared by injection of hexanonyl KLH as an immunogen, and the production of the antibody, which reacts with hexanonyl BSA, was observed. A detailed characterization of the obtained antiserum was then performed. At first, we examined the cross-reactivity of the antibody with amide-type synthetic adducts (CH3-(CH2)n-CO-NH-Lys; n=5-9) by ELISA. As shown in Fig. 5, hexanonyl (n=4) protein has been strongly recognized. Heptanonyl (n=5) and pentanonyl (n=3) proteins could be reacted with the antibody to a lesser extent. It is important that propanonyl (n=1) BSA could not be bound by the antibody, suggesting that the antibody can be used for the oxidative modification of \( \omega-6 \) fatty acids but not \( \omega-3 \) ones. Similar results were obtained by indirect competitive ELISA (data not shown). In addition, a carboxyalkylated protein (HOOC-(CH2)n-CO-NH-Lys) such as glutaric acid-BSA (n=3) or azelaic acid-BSA (n=7) could not be recognized by the anti-HEL antibody.

Lipid oxidation leads to the formation of reactive aldehydes such as 1-hexanal, malondialdehyde, and 4-hydroxy-2-nonenal. These reactive aldehydes can react with biological molecules such as proteins (1, 2). The cross-reactivity of aldehyde-modified proteins with the anti-HEL antibody was examined by ELISA. As shown in Fig. 6, aldehyde-modified proteins used were not recognized by the antibody, whereas hexanonyl BSA was.

To prove that the epitope of the antibody is \( \text{N}^e-(\text{hexanonyl})\text{lysine} \) (HEL), the cross-reactivity of the synthetic peptide-containing HEL moiety with the antibody was investigated by competitive ELISA. Fig. 7 shows that N-acetyl-glycyl-\( \text{N}^e-(\text{hexanonyl})\text{lysine} \) methyl ester and N-benzoyl-glycyl-\( \text{N}^e-(\text{hexanonyl})\text{lysine} \) could be recognized by the antibody. This suggested that the antibody is specific to the \( \text{N}^e-(\text{hexanonyl})\text{lysine} \) moiety.

Formation of HEL by Peroxidized \( \omega-6 \) Fatty Acids—

As shown in Fig. 8, the formation of HEL was observed by incubation of BSA with ascorbate/Fe\(^2+\)-oxidized linoleic acid, arachidonic acid, and cardiolipin. The treatments of BSA with 15-HPETE as well as 13-HPODE also generated antigenic compounds. The result may suggest that the \( \text{N}^e-(\text{hexanonyl})\text{lysine} \), HEL, becomes a marker for the oxidative modification of lysine by oxidized \( \omega-6 \) fatty acids including phospholipids containing esterified \( \omega-6 \) fatty acids. In addition, oxidized BSA, which was prepared by the oxidation of protein by hydrogen peroxide/iron, could not generate the antigenic materials.

The Effect of the Preincubation of 13-HPODE on the Formation of HEL—

To clarify whether the formation of HEL becomes a marker for the oxidative damage of protein by lipid hydroperoxide, the effect of the preincubation of 13-HPODE on the formation of \( \text{N}^e-(\text{hexanonyl})\text{lysine} \) was examined by chemical and immunochemical methods (Scheme I). The loss of 13-HPODE during preincubation was then performed. At first, we examined the cross-reactivity of the antibody with amide-type synthetic adducts (CH3-(CH2)n-CO-NH-Lys; n=0-9) by ELISA. As shown in Fig. 5, hexanonyl (n=4) protein has been strongly recognized. Heptanonyl (n=5) and pentanonyl (n=3) proteins could be reacted with the antibody to a lesser extent. It is important that propanonyl (n=1) BSA could not be bound by the antibody, suggesting that the antibody can be used for the oxidative modification of \( \omega-6 \) fatty acids but not \( \omega-3 \) ones. Similar results were obtained by indirect competitive ELISA (data not shown). In addition, a carboxyalkylated protein (HOOC-(CH2)n-CO-NH-Lys) such as glutaric acid-BSA (n=3) or azelaic acid-BSA (n=7) could not be recognized by the anti-HEL antibody.

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FORMATION OF HEL BY LIPID HYDROPEROXIDE

FIG. 9. Effect of preincubation of 13-HPODE on the formation of HEL moiety. The 13-HPODE was incubated in 0.1 M phosphate-buffered saline (pH 7.4) at 37 °C at various intervals. An aliquot of the solution was harvested and analyzed. A, the residual amounts of 13-HPODE were estimated after conversion to the 13-ODE, followed by HPLC analysis. B, squares, the preincubated 13-HPODE was incubated with Bz-Gly-Lys for 3 days at 37 °C, and the formation of \( \text{N}^e \)-hexanonyl compounds was estimated by LC-MS. B, triangle, the preincubated 13-HPODE was incubated with BSA for 3 days at 37 °C, and the formation of HEL residues in the protein was evaluated by ELISA.

SCHEME I. Flow diagram of the preincubation experiment. The 13-HPODE was prepared from linoleic acid by lipoygenase. The obtained 13-HPODE was incubated at neutral pH. An aliquot of the preincubated solution was used for the estimation of the residual 13-HPODE. The preincubated solution was divided, one portion was further incubated with Bz-Gly-Lys, and the other was further incubated with BSA. The detailed protocol is shown under "Experimental Procedures," and the result is summarized in Fig. 9.
reduction with NaBH₄ followed by HPLC analysis. The preincubated 13-HPODE was reacted with Bz-Gly-Lys and further incubated for 3 days at 37 °C. The formation of HEL moiety was evaluated by LC-MS. During a 3-day preincubation, about 90% of 13-HPODE was decomposed (Fig. 9A). The formation of HEL from Bz-Gly-Lys and 13-HPODE was also decreased with increasing preincubation time (Fig. 9B, squares). The preincubated 13-HPODE was also mixed with lipid-free BSA, and the formed HEL moiety in BSA was then estimated by the anti-HEL antibody. A similar result was observed using BSA as a
Formation of N\(^\text{e}\)-(Hexanonyl)lysine by Lipid Hydroperoxide

**DISCUSSION**

Lipid decomposition products can modify biological materials. Among the products, the reactivities of malondialdehyde and 4-hydroxy-2-nonenal have been investigated in detail. The chemical structures of modified amino acid residues were identified \textit{in vitro} (1). The antibodies raised to malondialdehyde or 4-hydroxy-2-nonenal-modified protein were prepared, and the immunopositive materials were detected in various tissues such as atherosclerotic plaques (17, 18). These findings suggested that the modification of lipid decomposition products (advanced lipid peroxidation end products) occurred \textit{in vivo}. On the other hand, considerable amounts of lipid hydroperoxides exist \textit{in vivo} (19).

The fluorescence formation from lysine modification by linoleic acid hydroperoxide was previously observed (20). It has also been reported that the lipid hydroperoxide can react with protein, followed by the formation of the lipid-protein covalent adduct (3). However, the precise structures of the lipid hydroperoxide-lysine adduct are unknown. We report the identification of a novel adduct, N\(^\text{e}\)-(hexanonyl)lysine, which has an amide bond between the N\(^\text{e}\)-amino group and lipid-derived part, from the reaction mixture of 13-HPODE and Bz-Gly-Lys. As far as we know, the formation of an amide bond during lipid peroxidation has not been reported. The chemical approach for the detection of N\(^\text{e}\)-(hexanonyl)lysine, HEL, in a protein is probably difficult because the HEL moiety can be hydrolyzed with 6N HCl, which is often used for the detection of modified amino acid residues. Therefore, to detect HEL immunohistochemically, we prepared an antibody against the hexanonyl protein. The antibody reacted with N-benzyol-glycyl-N\(^\text{e}\)-(hexanonyl)-lysine (compound X) as well as the N\(^\text{e}\)-(hexanonyl)lysine residue in the protein. Using the anti-HEL antibody, the formation of N\(^\text{e}\)-(hexanonyl)lysine in both the 13-HPODE-modified protein and 15-HPETE-modified protein was proven (Fig. 8). This result suggests that the formation of the HEL may be a good marker for the oxidative modification by oxidized ω-6 fatty acids such as linoleic acid or arachidonic acid. The lipid hydroperoxide can become a precursor of the N\(^\text{e}\)-hexanonyl adduct, whereas it is unknown whether the reaction of the lipid hydroperoxide and lysine proceeds directly or indirectly.

We also observed the appearance of immunoreactivity against the anti-HEL antibody during the oxidation of LDL. The uptake of oxidized LDL by macrophage can be considered as one of the plausible contributors for foam cell formation, which may initiate atherosclerosis. The presence of HEL moiety in human atherosclerotic lesion was immunohistochemically proven by the anti-HEL antibody. However, the chemical identification of the HEL moiety in atherosclerotic lesion was not performed. More detailed studies are needed for the elucidation of the formation of the HEL moieties \textit{in vivo}.

Kim \textit{et al.} (21) prepared antibodies against lipid hydroperoxide-modified protein and reported the positive staining in early atherosclerotic lesion, whereas the precise epitopes were not so clear. Recently, we prepared antibodies to 13-HPODE- or 15-HPETE-modified proteins, which cannot recognize aldehyde-modified proteins (9, 10). The result assumed that the lipid hydroperoxide-specific modification can occur. However, N-benzyol-glycyl-N\(^\text{e}\)-(hexanonyl)-L-lysine (compound X) and the hexanoyl protein cannot cross-react with the antibodies raised against the 13-HPODE- or 15-HPETE-modified KLH (data not shown). This result agreed with the fact of the requirement of the carboxyl terminus of the lipid moiety in an adduct for the appearance of the antigenicity (9, 10). On the...
other hand, the synthetic carboxyalkylamides (HOOC-(CH₂)ₙ-CO-NH-Lys), glutaric acid-BSA and azelaic acid-BSA, were not reacted with the anti-HEL antibody (Fig. 6). The anti-HEL antibody may become a better tool for lipid hydroperoxide-derived oxidative modification than the anti-13-HPODE-KLH and 15-HPETE-KLH antibody because the anti-HEL antibody recognized the CH₃ terminus of the lipid-Lys adduct, which is formed from the reaction of lysine residues and peroxidation products, derived from not only free fatty acids but also esterified fatty acids (Fig. 4).

In summary, we isolated and identified a novel lipid-Lys adduct, N⁶-(hexanonyl)lysine, from the reaction between 13-HPODE and Lys, although the detailed mechanism of formation of the N⁶-hexanonyl linkage remains unknown. We also showed the preparation of the antibody to HEL and the formation of HEL in oxidatively modified LDL using the immunological method. The immunopositive materials were also observed in human atherosclerotic lesions. The adduct may become a marker for the initial stage of oxidative damage of biomolecules.

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