Immunochemical detection of a novel lysine adduct using an antibody to linoleic acid hydroperoxide-modified protein

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Abstract **We have previously prepared the polyclonal antibody to the 13-hydroperoxyoctadecadienoic acid-modified protein (13Ab) (Kato et al. 1997. *J. Lipid Res.* 38: 1334–1346), however, the epitopes have not yet been structurally identified. In this study, we identified a novel amide-type adduct as one of the major epitopes of 13Ab and characterized the endogenous formation. Upon incubation of the lysine derivative with peroxidized linoleic acid, the formation of N^{ε}-(azelayl)lysine (AZL) was confirmed using liquid chromatography-mass spectrometry. The chemically synthesized azelayl protein was significantly recognized by 13Ab. The peroxidation products of different polyunsaturated fatty acids also generated several analogous carboxyalkylamide-type adducts to AZL by the reaction with the lysine derivative, whereas 13Ab specifically recognized AZL, suggesting that the AZL moiety may be one of the major epitopes of 13Ab. The immunoreactive materials of 13Ab were immunohistochemically detected in atherosclerotic lesions from hypercholesterolemic rabbits. More strikingly, the immunoreactivity was significantly enhanced when the sections were treated with alkali or phospholipase A_2 for hydrolyzing the ester bonds prior to the staining. These results suggest that the lipid hydroperoxide-derived carboxylic adducts, such as AZL, and their esters linked with phospholipids may be generated in vivo and involved in the pathogenesis of atherosclerosis associated with oxidative stress.**—Kawai, Y., Y. Kato, H. Fujii, Y. Makino, Y. Mori, M. Naito, and T. Osawa. **Immunochemical detection of a novel lysine adduct using an antibody to linoleic acid hydroperoxide-modified protein. *J. Lipid Res.* 2003.** 44: **1124–1131.**

Supplementary key words protein modification • lipid peroxidation • 13-hydroperoxyoctadecadienoic acid

Oxidative modification of an LDL is thought to be involved in the etiology of atherosclerosis (1, 2). The oxidation of LDL leads to the loss of ε-amino groups from the lysine residues in apolipoprotein B (apoB) due to the covalent adduction by the oxidative decomposed products of polyunsaturated fatty acid esters (3, 4). Aldehydes such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) are highly reactive to ε-amino groups in proteins, and therefore, aldehyde-derived protein adducts have been extensively investigated. The adduction of aldehydes to apoB of LDL has been implicated in the uncontrolled uptake of these lipoproteins by macrophage scavenger receptors, and the following formation of foam cells leading to fatty streaks. Indeed, MDA-lysine (5), HNE-lysine (6, 7), and acrolein-lysine adducts (8, 9) have been detected in oxidized LDL (oxLDL) and atherosclerotic lesions. On the other hand, the esterified aldehydes bound to phospholipids or cholesteryl esters (called "core aldehydes") are generated during the lipid peroxidation as well as "free" aldehydes. For example, 9-oxononanoyl esters and 5-oxovaleroyl esters have been detected during the oxidation of linoleic acid and arachidonic acid (AA) esters, respectively (10, 11). These core aldehydes, i.e., phospholipids/cholesteryl esters in which there are aldehydic fatty acid fragments still esterified to the glycerol/cholesterol backbone, can also react with the amino groups of proteins and form the esterified lipid-protein adducts (12). Protein adducts from levuglandin E_2 esters (13) and hydroxyalkenal esters (14) of the phospholipids have also been discovered in oxLDL.

In contrast to the reactivity of aldehydes, the lipid hy-

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Abbreviations: AA, arachidonic acid; α-LNA, α-linolenic acid; AZL, N^{ε}-(azelayl)lysine; Bz-Gly-Lys, N^{α}-benzoyl-glycyl-L-lysine; CML, N^{ε}-(carboxymethyl)lysine; DHA, docosahexaenoic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EPA, eicosapentaenoic acid; GLL, N^{ε}-(glutaroyl)lysine; HEL, N^{ε}-(hexanoyl)lysine; HNE, 4-hydroxy-2-nonenal; 15-HPETE, 15-hydroperoxyeicosatetraenoic acid; 13-HPODE, 13-hydroperoxyoctadecadienoic acid; LC-MS, liquid chromatography-mass spectrometry; MDA, malondialdehyde; sulfo-NHS, *N*-hydroxysulfosuccinimide; NMR, nuclear magnetic resonance; SUL, N^{ε}-(succinyl)lysine.

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droperoxides, the primary products of lipid peroxidation, can also modify the amino groups of proteins. Kim et al. have shown the first demonstration that the immuno-positive materials of a polyclonal antibody to lipid hydroperoxide-modified proteins were generated in atherosclerotic lesions (15). We have also prepared the polyclonal antibodies to the 13-hydroperoxyoctadecadienoic acid (13-HPODE)-modified protein (16) and 15-hydroperoxyeicosatetraenoic acid (15-HPETE)-modified protein (17). Although the precise epitopes of these antibodies have not been identified, several aldehyde-modified proteins could not be recognized by these antibodies, suggesting the existence of specific epitopes derived from the lipid hydroperoxides. These polyclonal antibodies (named 13Ab and 15Ab) weakly recognized the intact oxLDL, whereas the immunoreactivity was enhanced by the alkali-treatment of oxLDL for hydrolyzing the carboxyl esters (16, 17). These characterizations suggest that the lipid hydroperoxide-derived esterified adducts are generated in oxLDL.

During the reaction of 13-HPODE with lysine derivatives, we have recently identified the formation of N^{ε}-(hexanoyl) lysine (HEL), a unique amide-type lysine adduct (18). The amide-type adduct is a new class of protein adducts derived from lipid peroxidation. The presence of the HEL moiety in oxLDL and human atherosclerotic lesions has been revealed by using a specific polyclonal antibody to the HEL moiety (18). In view of the analogy between the action of free aldehydes and core aldehydes, it is possible that the esterified amide adducts are generated during the reaction of lipid hydroperoxide-esters with proteins as well as HEL formation. In the present study, we characterized the epitopes of 13Ab for assessing the implication of the lipid hydroperoxide-derived esterified adduct formation in atherosclerosis, and discovered the formation of carboxyalkylamide-type novel lysine adducts. The results in this study suggest that the lipid hydroperoxide-derived esterified adducts are endogenously generated and may be implicated in the pathogenesis of atherosclerosis.

EXPERIMENTAL PROCEDURES

Materials

N^{α}-benzoyl-glycyl-L-lysine (Bz-Gly-Lys) was obtained from Peptide, Inc. (Osaka, Japan). Monomethylazelaic acid, monomethylglutaric acid, and monomethylsuccinic acid were obtained from Fluka (Buchs, Switzerland). Bovine serum albumin (BSA) and phospholipase A_2 (EC 3.1.1.4) from bee venom were obtained from Sigma Chemical Co. (St. Louis, MO). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysulfosuccinimide (sulfo-NHS) were obtained from Pierce Chemical Co. (Rockford, IL). 13-HPODE was prepared by the lipoxygenase-catalyzed enzymatic oxidation of linoleic acid as previously described (16). The polyclonal antibodies to 13-HPODE-modified protein (13Ab) and 15-HPETE-modified protein (15Ab) were prepared as previously described (16, 17).

Enzyme-linked immunosorbent assay

The competitive indirect enzyme-linked immunosorbent assay (ELISA) was done as described (16) with some modification. Briefly, 100 μl of 13-HPODE-modified BSA [0.5 μg/well in phosphate-buffered saline (PBS)] was coated into the wells and kept at 4°C overnight. The 13-HPODE-modified BSA was prepared by incubating 13-HPODE (5 mM) with BSA (1 mg/ml) in phosphate buffer (pH 7.4) at 37°C for 3 days. At the same time, 50 μl of 13Ab [1:500 in PBS containing 0.05% Tween 20 (TPBS)] and 50 μl of competitor (in PBS) were mixed in a tube and reacted overnight at 4°C. The plate was washed and blocked with 4% Block Ace (Dainihon Seiyaku Co., Osaka), and 90 μl of the competitor solution was then added to a well and further incubated. The binding of the residual antibody on the coated modified BSA was indirectly estimated by the binding of the peroxidase-labeled anti-rabbit IgG secondary antibody. The color development reaction was performed by adding *o*-phenylenediamine solution (0.5 mg/ml containing 0.03% hydrogen peroxide), and the optical density was measured at 490 nm. The result of the competitive ELISA was expressed as B/B0, where B is the amount of antibody bound in the presence and B0 is the amount in the absence of the competitor. Each point represents the mean of duplicate determinations.

The noncompetitive indirect ELISA was performed as described (16) with some modifications. Briefly, 50 μl of protein (0.06–5.55 μg/ml) was dispensed into wells and kept at 4°C overnight. The plate was then incubated with 13Ab (1:1,000, v/v in TPBS), and the binding of the antibody to the protein was evaluated by incubation with the peroxidase-labeled anti-rabbit IgG antibody. The data represent the mean of duplicate determinations.

Synthesis of amide-type lysine adducts

The N^{ε}-(azelayl)lysine (AZL) derivative [N^{α}-benzoyl-glycyl-N^{ε}-(azelayl)lysine] was prepared by the carbodiimide method. Briefly, monomethylazelaic acid (50 mg), EDC (52.2 mg), and sulfo-NHS (31.3 mg) in dimethylformamide (800 μl) were magnetically stirred for 24 h at room temperature. After the incubation, Bz-Gly-Lys (83.3 mg) in 2.2 ml phosphate buffer (pH 7.4) was added and further stirred for 4 h at room temperature. The formed monomethylazelayl derivative was extracted by ethyl acetate and separated by reverse-phase high performance liquid chromatography (HPLC) equipped with a Develosil ODS-HG-5 column (8 × 250 mm, Nomura Chemical Co., Aichi, Japan) and isocratic conditions, with 37.5% aqueous CH_3CN containing 0.1% trifluoroacetic acid as the eluent at a flow rate of 2.0 ml/min (detected at UV 234 nm). The obtained monomethylazelayl derivative was incubated in 1 N NaOH at 37°C for 1 h, and the formed AZL derivative was separated by reverse-phase HPLC as described above. The purified AZL was identified by ^{1}H-nuclear magnetic resonance (NMR) (Bruker ARX-400) and liquid chromatography-mass spectrometry (LC-MS, VG Biotech PLATFORM II) in the electrospray ionization positive (ESP+) mode. The spectral data were as follows: ^{1}H-NMR (CD_3OD) (ppm) 1.33 (m, 6H), 1.43 (m, 2H), 1.51 (m, 2H), 1.59 (m, 4H), 1.75 (m, 1H), 1.92 (m, 1H), 2.16 (t, J = 7.6, 2H), 2.27 (t, J = 7.4, 2H), 3.16 (t, J = 6.8, 2H), 4.11 (m, 2H), 4.44 (m, 1H), 7.47 (t, J = 7.4, 2H), 7.55 (t, J = 7.4, 1H), 7.87 (d, J = 7.2, 2H); LC-MS (ESP+) m/z 478 $(M+H)^+$.

Other alkyl lengths of the carboxyalkylamide-type lysine adducts were also synthesized using the corresponding alkyl length of the monomethylcarboxylic acid. These synthetic derivatives were identified by ^{1}H-NMR and LC-MS measurements (unpublished observations). The HEL derivative was synthesized as previously described (18).

Reaction of peroxidized fatty acids with lysine derivative

The fatty acid (final concentration, 5 mM) was oxidized in the presence of 0.5 mM ascorbate and 0.05 mM $FeSO_4$ for 24 h at 37°C in phosphate buffer (pH 7.4) containing 20% methanol. To the solution, Bz-Gly-Lys (final concentration, 5 mM) was added

and further incubated at 37°C for 3 days. After the incubation, the reaction mixtures were analyzed by LC-MS. The gradient elution of HPLC was done using two solvents: solvent A, 10% CH_3CN containing 0.1% acetate; and solvent B, 100% CH_3CN containing 0.1% acetic acid. The sample was injected into a Develosil ODS HG-5 column (4.6 × 250 mm) and eluted with a linear gradient from 100% A to 65% B for 40 min at 0.8 ml/min. For the quantification of AZL and HEL, *tert*-butoxycarbonyl isoleucine was added to the samples at a final concentration of 0.1 mM as the internal standard. Analyses were performed by monitoring ions of *m/z* 478 (AZL), 406 (HEL), and 232 (internal standard). A standard curve was produced by plotting the synthetic adduct level against the quotient of the peak area of the adduct divided by the peak areas of the internal standard.

Preparation of chemically modified proteins containing carboxylic adducts

The azelayl BSA (AZL-BSA), N^{ε}-(glutaroyl)lysine BSA (GLL-BSA), and N^{ε}-(succinyl)lysine BSA (SUL-BSA) were prepared as follows. Monomethylcarboxylic acid (monomethylazelaic acid, monomethylglutaroic acid, or monomethylsuccinic acid) was conjugated with BSA using the EDC/sulfo-NHS system as previously described (18). The methyl esters of the adducts in the proteins were hydrolyzed by incubation in 0.5 N NaOH for 1 h at room temperature. After the incubation, the mixture was neutralized with an equal volume of 0.5 N HCl. These modified proteins were then dialyzed against PBS for 3 days at 4°C. The preparation of the carboxymethyl BSA was performed as previously reported (19).

Animals and immunohistochemistry

Male New Zealand White rabbits (2.5 kg; Kitayama, Japan) were allowed free access to water and a commercial rabbit diet containing 1% cholesterol (20). After 12 weeks, the rabbits were sacrificed and their aortas were removed. Formalin-fixed, paraffin-embedded aorta sections (5 μm thick) were prepared and used for the immunohistochemical analyses. Briefly, deparaffinized sections were incubated with normal serum for 30 min to block the nonspecific binding of the secondary antibody and then with a primary antibody (1:50, v/v) at 4°C overnight. For the ester bond hydrolysis with alkali, prior to blocking, the sections were incubated with 0.25 N NaOH for 1 h at room temperature. For the enzymatic hydrolysis, the sections were incubated with phospholipase A_2 (1.0 U/μl) for 6 h at room temperature. Immunostaining was performed using the avidin-biotin complex method with the Vectastain ABC-AP (alkaline phosphatase) kit and Vector Alkaline Phosphatase Substrate Kit II (Vector Laboratories, Inc., Burlingame, CA). A competitive experiment was performed by staining with 13Ab preincubated with an excess of AZL derivative (25 mM) at 37°C for 1 h.

RESULTS

The importance of carboxyl groups for the recognition of 13Ab

To examine the lipid hydroperoxide-derived protein modification, we previously prepared the polyclonal antibody 13Ab against the 13-HPODE-modified protein (16); however, the structural identification of the epitope(s) has not yet been performed. Amino acid analyses of the lipid hydroperoxide-modifed proteins have shown that lysine residues are the potential targets by the reaction of lipid hydroperoxides (16, 17). We have previously identified the formation of HEL (**Fig. 1A**) as one of the major products formed by the reaction of 13-HPODE with a lysine derivative (18). HEL is a unique amide-type adduct, which has the C6 saturated aliphatic chain containing the CH_3-terminal of 13-HPODE; however, the HEL moiety was scarcely recognized by the antibody (Fig. 1B), showing that HEL could not be the epitope of 13Ab. This result supports our previous characterization of 13Ab that the carboxyl groups derived from 13-HPODE are important for the recognition of 13Ab (16).

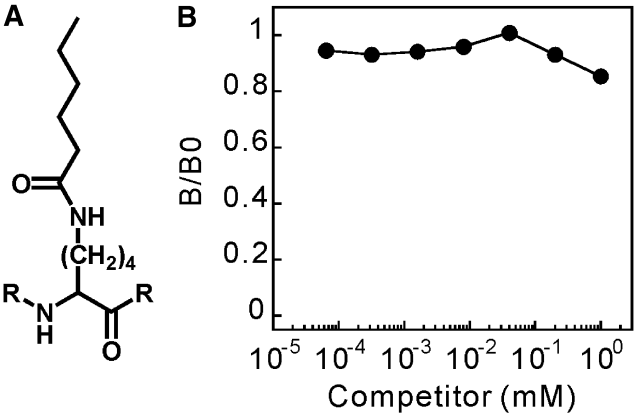


Fig. 1. Cross-reactivity of 13Ab to N^{ε}-(hexanoyl)lysine (HEL). A: Chemical structure of HEL. B: Cross-reactivity of 13Ab to HEL, estimated by competitive ELISA as described in Experimental Procedures. The HEL derivative was synthesized by conjugating N^{α}-benzoyl-glycyl-L-lysine (Bz-Gly-Lys) with hexanoic acid using the 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/*N*-hydroxysulfosuccinimide system, and used as a competitor. The 13-hydroperoxyoctadecadienoic acid-modified BSA was prepared, diluted with PBS, and used as a coating agent (0.5 μg/well).

Identification of a novel amide-type lysine adduct from the reaction of oxidized linoleic acid and a lysine derivative

Based on the information of HEL generation during the reaction of 13-HPODE with lysine derivatives (18) and the characterization of 13Ab (16) (Fig. 1), we presumed the formation of AZL, a novel amide-type adduct containing a carboxyl group derived from linoleic acid, as a plausible epitope of the antibody. The scheme for the proposed formation of AZL and the synthetic scheme for this adduct are illustrated in **Fig. 2**. To examine the formation of AZL, the reaction of the peroxidized linoleic acid with a lysine derivative (Bz-Gly-Lys) was carried out as the model system, and the reaction mixture was then analyzed by LC-MS. As expected, the molecular ion peak at *m/z* 478 was detected from the reaction mixture (**Fig. 3A**). Compared with the retention time and the molecular ion peak of the synthetic AZL derivative, the obtained peak was identified as AZL. The quantitative analyses using LC-MS showed that the amount of AZL generated in the reaction mixture was comparable to that of HEL (Fig. 3B). We have previously shown that the conversion yield of HEL from the loss of Bz-Gly-Lys after a 3 day incubation with 13-HPODE was 5.6% (18). These observations suggest that AZL may be one of the major lysine adducts generated from the reaction with peroxidized linoleic acid as well as HEL.

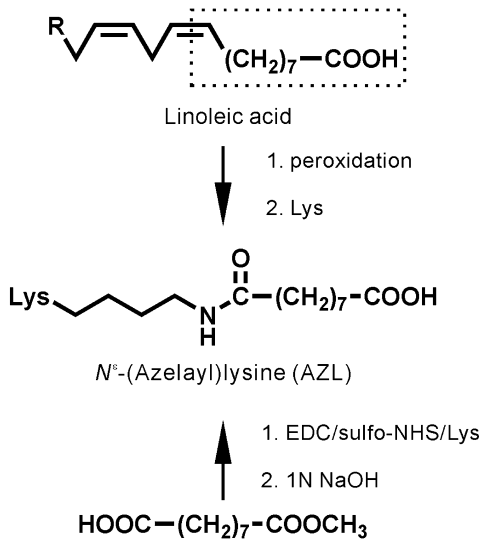


Fig. 2. Scheme for the proposed formation of N^{ε}-(azelayl)lysine (AZL) during lipid peroxidation and the procedure for the chemical synthesis.

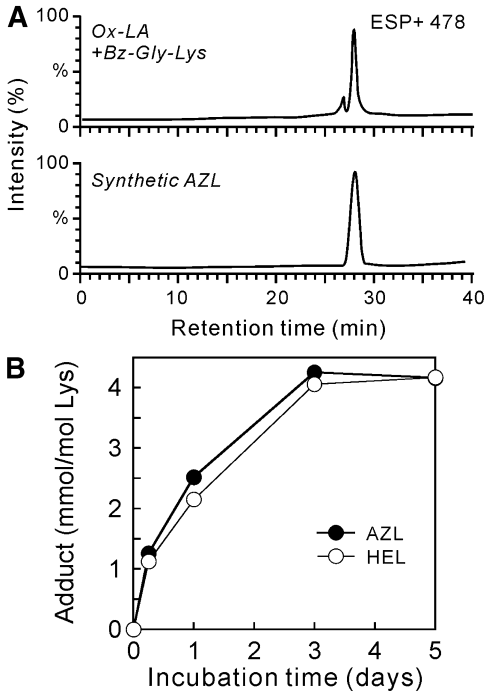


Fig. 3. Formation of AZL as one of the major products from the reaction of oxidized linoleic acid with Bz-Gly-Lys. A: Analysis of AZL adduct by liquid chromatography-mass spectrometry (LC-MS). Linoleic acid (5 mM) was oxidized by Fe^{2+}-ascorbate for 24 h at 37°C, and then incubated with Bz-Gly-Lys (5 mM) for 3 days at 37°C. LC-MS measurements were performed by monitoring ions at *m/z* 478. The authentic standard was synthesized as described in Experimental Procedures. B: Time-dependent formation of AZL and HEL during the reaction of oxidized linoleic acid with Bz-Gly-Lys, estimated by LC-MS. The amount of adducts was calculated from the standard curves developed by using synthetic standards with *tert*-butoxycarbonyl isoleucine as the internal standard.

Formation of carboxyalkylamide-type adducts from the reaction of various oxidized polyunsaturated fatty acids

The alkyl length of the azelayl moiety of AZL (C9) corresponds to the alkyl length from the COOH terminal to the first double bond on the original fatty acid. To examine the structural correlation between the formation of the carboxyalkylamide-type adducts such as AZL and the fatty acid sources, various polyunsaturated fatty acids were used for the reaction with the lysine derivative instead of linoleic acid. As shown in **Fig. 4**, as expected, AZL (*m/z* 478) was generated from the reaction of Bz-Gly-Lys with oxidized α-linolenic acid (α-LNA), which has a C9 carboxyalkyl moiety. The result exhibited a positive correlation with our previous immunochemical observations that 13Ab significantly reacted with the oxidized α-LNA-modified protein (16). The GLL (*m/z* 422) was generated from the reaction of Bz-Gly-Lys with oxidized AA or eicosapentaenoic acid (EPA). In addition, the SUL (*m/z* 408) was also generated from the reaction of Bz-Gly-Lys with oxidized docosahexaenoic acid (DHA). By comparison with authentic standards, the formation of these amide-type adducts was confirmed. The formation of AZL could not be observed from the reaction of the oxidized oleic acid containing the C9 carboxyalkyl moiety (unpublished observations), showing that monounsaturated fatty acids could not be the sources of the carboxyalkylamide-type adducts. These results show that these characteristic carboxyalkylamide-type lysine adducts are universal for the modification of lysine residues by various oxidized polyunsaturated fatty acids.

Cross-reactivity of 13Ab to carboxyalkylamide-type adducts

To confirm whether 13Ab can recognize the AZL adducts in protein, the azelayl BSA containing a large amount of AZL in the ε-amino groups of lysine was chemically synthesized and the cross-reactivity of 13Ab was examined. We also synthesized carboxymethyl BSA to examine the cross-reactivity of 13Ab to N^{ε}-(carboxymethyl)lysine (CML), because CML is a representative carboxylic lysine adduct and is reported to be formed not only during the Maillard reaction but also during lipid peroxidation in the presence of protein (21). **Figure 5A** shows that azelayl BSA was strongly recognized by 13Ab, whereas carboxymethyl BSA was not recognized. The cross-reactivity of 13Ab to the purified AZL derivative was also confirmed by competitive ELISA (unpublished observations).

In addition, Fig. 4 showed that the peroxidation of other polyunsaturated fatty acids also resulted in the formation of carboxyalkylamide-type adducts such as GLL and SUL upon the reaction with lysine residues. To investigate the specificity of 13Ab to these analogous carboxyalkylamide adducts, the cross-reactivity with the AZL-, GLL-, or SUL-containing proteins was examined. As shown in Fig. 5B, 13Ab recognized the AZL-conjugated protein, but not the GLL- and SUL-conjugated proteins. These results suggest that the AZL moiety should be one of the major epitopes of the antibody.

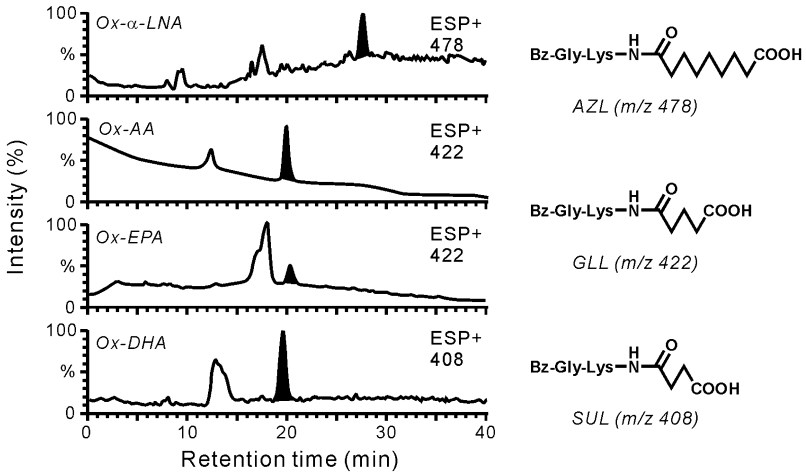


Fig. 4. Formation of carboxyalkylamide-type adducts by the reaction of Bz-Gly-Lys with various oxidized polyunsaturated fatty acids. Polyunsaturated fatty acids (final concentration, 5 mM) were oxidized by Fe^{2+}-ascorbate for 24 h at 37°C, and then incubated with Bz-Gly-Lys (final concentration, 5 mM) for 3 days at 37°C. Reaction mixtures were analyzed by LC-MS monitoring with *m/z* 478 (AZL), 422 [N^{ε}-(glutaroyl)lysine (GLL)], and 408 [N^{ε}-(succinyl)lysine (SUL)]. The closed peaks were identical with the synthetic amide-type adducts.

Immunohistochemical characterization of the epitopes of 13Ab in atherosclerotic lesions

To evaluate the endogenous generation of the epitopes of 13Ab, the antibody was applied to the immunohistochemical staining in aorta specimens from the hypercholesterolemic rabbits, an experimental animal model for atherosclerosis. The lipid peroxidation levels in the tissues and serum of the hypercholesterolemic rabbits were significantly increased compared with the control rabbits (unpublished observations). When the immunostaining using 13Ab was performed with the aorta sections of the hypercholesterolemic rabbits, immunopositive materials were observed, showing that the epitopes of the antibody, containing AZL, may be generated in vivo (**Fig. 6A**). More strikingly, alkali treatment of the sections prior to use of the primary antibody significantly enhanced the immunoreactivity of the antibody (Fig. 6B). Furthermore, when sections were enzymatically hydrolyzed with phospholipase A_2, which specifically cleaves the ester bonds of the *sn*-2 polyunsaturated fatty acids of glycerophospholipids, the enhanced immunoreactivity was also observed (Fig. 6C). The treatments of sections with hydrolyzing agents may saponify the ester bonds and expose the carboxylic epitopes of the antibody (**Scheme 1**). These results suggest that the esterified lipid-protein adducts, such as the AZL esters, were also generated in the rabbit atherosclerotic lesions, as well as the free carboxylic adducts. In addition, no positive staining was observed in the experiments using 13Ab preabsorbed by an excess of the AZL derivative (Fig. 6D) or using normal rabbit serum (Fig. 6E), suggesting the specificity of the immunostaining. Furthermore, no positive staining was observed in the nonatherosclerotic aortas obtained from normal rabbits (Fig. 6F), suggesting the possibility that the formation of the epitopes may be closely associated with the formation of atherosclerotic lesions.

DISCUSSION

We have previously prepared the polyclonal antibody against the 13-HPODE-modified protein (13Ab) for assessing lipid hydroperoxide-derived protein modifica-

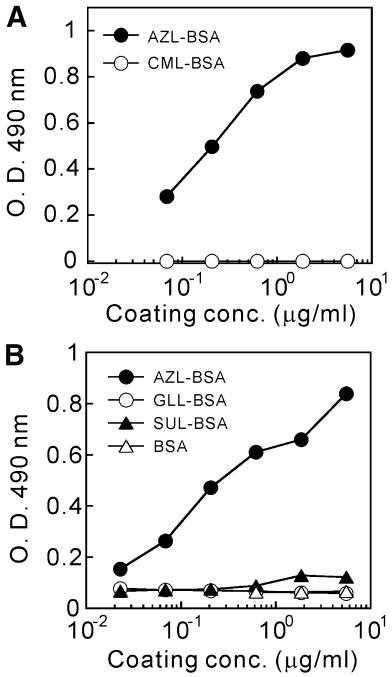


Fig. 5. Cross-reactivity of 13Ab to carboxylic adducts in protein. Carboxyalkylamide-conjugated BSA (AZL-BSA, GLL-BSA, and SUL-BSA) and N^{ε}-(carboxymethyl)lysine (CML)-BSA were prepared as described in Experimental Procedures, diluted with PBS, and coated on microtiter plates at 4°C overnight. The cross-reactivity of 13Ab to these carboxylic adduct-containing proteins was estimated by ELISA. A: Cross-reactivity of 13Ab to AZL-BSA and CML-BSA. B: Cross-reactivity of 13Ab to the carboxyalkylamide-conjugated BSA.

tions. Characterization of this antibody indicated that *i*) the fatty acid-derived carboxyl group is essential for the recognition, and *ii*) the epitopes are generated in oxLDL as the esterified form (16). In this study, we identified the formation of AZL, a carboxyalkylamide-type novel lysine adduct, as one of the major epitopes of 13Ab, and characterized the endogenous generation using the antibody. Covalent modifications of apoB in LDL by the oxidation products of the polyunsaturated fatty acids are believed to be involved in the pathogenesis of atherosclerosis (1–4). It has been reported that about 250 mol of amino groups per mole of apoB decreased by overnight copper-catalyzed oxidation (22). Among them, ~30% of the decreased lysine appeared to be due to the conjugation of the phospholipids. It has generally been assumed that these phospholipid-protein conjugations were due to the attachment of the phospholipid core aldehydes. As well as the aldehydes, several lines of evidence have suggested that the lipid hydroperoxides, the primary products of lipid peroxidation, can modify proteins (15, 23, 24). Alkali saponification of the oxidized phosphatidylcholine-modified BSA and oxLDL resulted in an increase in the immunoreactivity of 13Ab (16). The immunoreactivity of 13Ab was also observed when oxLDL was treated with phospholipase A_2 (unpublished observations). These results strongly suggest the occurrence of esterified lipid hydroperoxide-derived protein modifications in LDL. The importance of the esterified lipid-protein adducts was strongly suggested by the demonstration that a monoclonal autoantibody against oxLDL obtained from apoE-deficient mice could react with both the protein and lipid moieties of oxLDL and inhibit the macrophage binding of oxLDL (25, 26). In addition, the covalent conjugation of oxidized phospholipids with apoB is suggested to be one of the major ligands for macrophage scavenger receptors (22).

To characterize the formation of the esterified lipid hydroperoxide-derived protein adducts in vivo, 13Ab was applied to the immunohistochemical staining in the atherosclerotic aortas from hypercholesterolemic rabbits. Enhanced positive staining was obtained in the alkali-saponified sections from the atherosclerotic aortas, suggesting the contribution of the esterified lipid hydroperoxides as well as the free lipid hydroperoxides to the generation of the antigenic epitopes in vivo (Fig. 6). By using not only the alkali treatment but also enzymatic treatments for the hydrolysis of the sections, it was confirmed that the epitopes were linked with phospholipids in the lesions (Fig. 6C). As mentioned above, the esterified lipid-protein adducts may contribute to the pathogenesis of atherosclerosis. Therefore, the formation of the lipid hydroperoxide-derived esterified adducts may possess some consequence to atherosclerosis, and 13Ab may be a good tool for the characterization of these esterified adducts in vivo.

During the reaction of 13-HPODE with the lysine residues, HEL, a nonesterified amide-type lysine adduct, was identified as one of the major reaction products (18). HEL is also generated by the reaction of various oxidized ω-6 polyunsaturated fatty acids (18). Similarly, the oxidation of the ω-3 polyunsaturated fatty acids such as α-LNA, EPA, and DHA can also generate an amide-type adduct, N^{ε}-(propanoyl)lysine, by reacting with lysine residues (Kawai et al., unpublished observations). As well as the generation

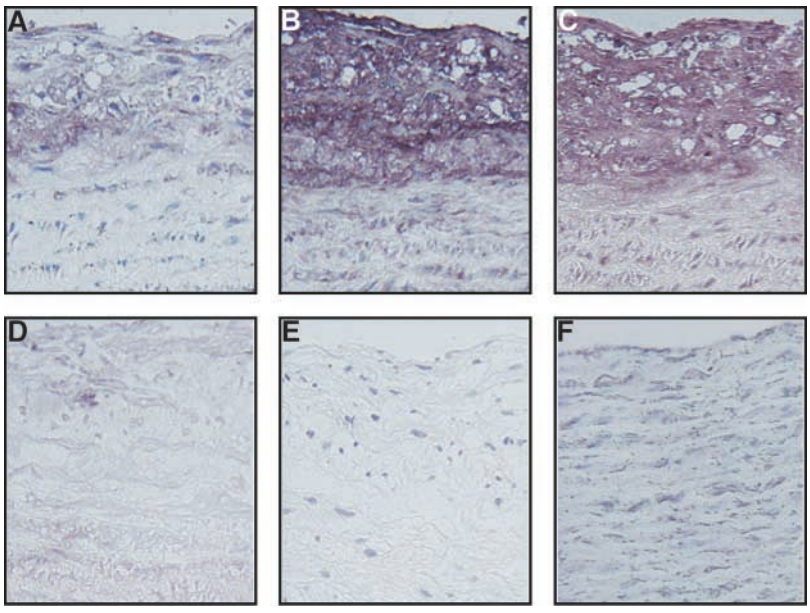
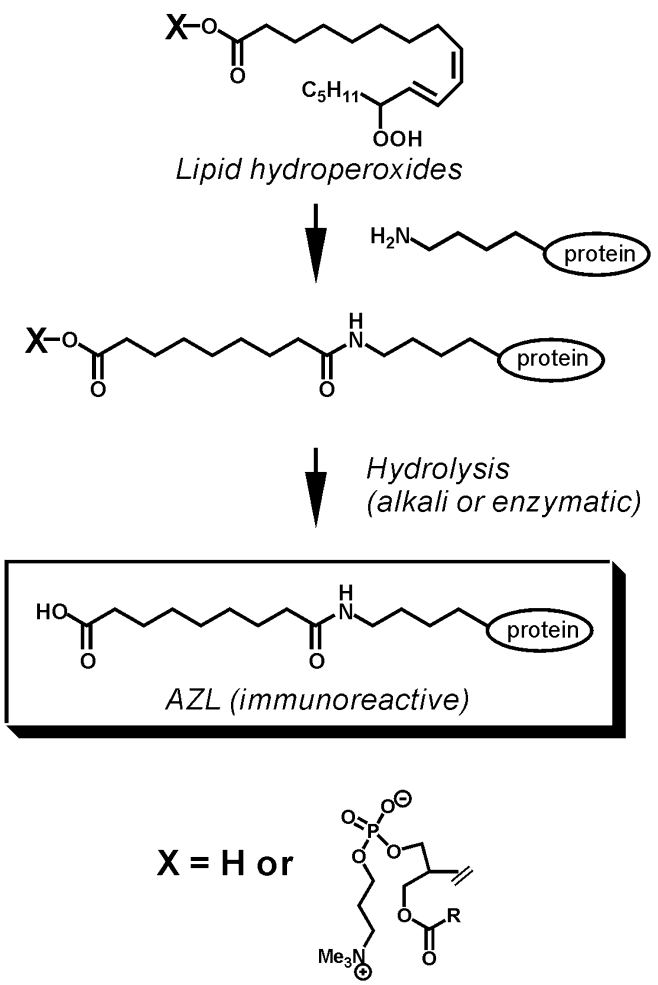


Fig. 6. Immunohistochemical detection of the epitopes of 13Ab in rabbit atherosclerotic aorta. Arterial tissue specimens with atherosclerosis from 1% cholesterol-fed rabbits were immunostained with 13Ab. Sections were treated with PBS (A), 0.25 N NaOH (B), and 1.0 U/μl phospholipase A_2 (C) prior to blocking, and then stained with 13Ab. Control experiments were performed by staining alkali-treated sections with 13Ab preabsorbed with an excess of AZL derivative (D) or normal rabbit serum (E) as the primary antibody. Non-atherosclerotic aorta obtained from normal rabbit was treated with alkali, and then stained with 13Ab (F). All sections were counterstained with hematoxylin. A–F, magnification ×40.



Scheme 1. Formation of free and/or phospholipid-esterifed N^{ε}-(azelayl)lysine adduct and the immunochemical detection by 13Ab.

of amide-type adducts containing the CH_3-terminal moiety of fatty acids, the amide-type adducts containing the fatty acid-derived COOH moiety are likely generated from the reaction of oxidized polyunsaturated fatty acids. Indeed, AZL was chemically identified from the reaction of the oxidized linoleic acid with a lysine derivative (Fig. 3). In addition, the oxidation of α-LNA, AA, EPA, and DHA in the presence of lysine residues could also generate carboxyalkylamide-type adducts (Fig. 4); however, 13Ab specifically recognized AZL but not other carboxyalkylamide-type adducts (Fig. 5B). We also confirmed that the GLL adduct formed from the reaction of the oxidized AA may be one of the major epitopes of the previously prepared 15Ab (ref. 17 and unpublished observations). Although linoleic acid and AA are the major polyunsaturated fatty acids in normal human serum phospholipids (27), the ω-3 polyunsaturated fatty acids such as EPA and DHA are highly susceptible to oxidation, and therefore may contribute to the oxidative modifications of protein in vivo. Studies on the DHA-derived SUL formation in DHA-administrated rodents are now in progress in our laboratories. Thus, these amide-type lysine adducts may be very useful markers for estimating the protein damage by lipid peroxidation.

Undoubtedly, other lipid-protein adducts and other oxidized lipids may play an important role in the LDL oxidation. For example, a high concentration of HNE (150 mM) has been detected in the lipid phase of LDL (28), and the presence of the HNE-modified protein adduct, the HNE-histidine Michael-type adduct (7–9 mol/mol LDL), has been estimated in oxLDL (6). Although amide-type adducts, such as HEL and AZL, have not yet been quantified in oxLDL, the predominance of the amide-type lysine adducts during lipid peroxidation was supported by the demonstration of Onorato et al. that the major products from the reaction of oxidized linoleic acid with pyridoxamine, an inhibitor of glycation and lipid peroxidation, are hexanoyl-pyridoxamine and azelayl-pyridoxamine (29). This raises the possibility that HEL and AZL may be one of the major lysine adducts in oxLDL, as well as the aldehyde-derived lysine adducts.

The precise mechanism for the formation of amide-type lysine adducts has not yet been elucidated. It is interesting that the generation of amide-type adducts such as HEL and AZL was attenuated by preincubating 13-HPODE prior to the reaction with a lysine derivative (ref. 18 and unpublished observations). Similarly, the immunoreactivity of 13Ab to 13-HPODE-modified protein was also attenuated by preincubating 13-HPODE (unpublished observations). These observations suggest that the initial decomposed intermediate(s), but not stable end-products such as aliphatic aldehydes, may contribute to the formation of these amide-type adducts/antigenic epitopes. Fruebis et al. (23) have reported the formation of fluorescent products upon the reaction of linoleic acid hydroperoxide with polylysine, although the structures of the presumed products remain to be proven. The report suggested a reaction mechanism initiated by the interaction of the amino group with the hydroperoxide group or the peroxy radical derived from a lipid hydroperoxide. This may in part support our prediction that initially formed unstable intermediate(s) may contribute to the formation of amide-type adducts, although amide-type adducts identified in this study were not fluorescent products. In addition to the modification of lysine residues, histidine and cysteine residues were also modified during the incubation with lipid hydroperoxides (16, 17), and the reaction mixtures of 13-HPODE with the histidine or cysteine derivatives were also recognized by 13Ab (unpublished observations), suggesting that 13Ab recognizes multiple epitopes other than the AZL adduct, although the epitope structures in these reaction mixtures have not yet been identified. The mechanism of the decomposition processes of lipid hydroperoxides under physiological conditions is complicated, and therefore not completely resolved. In order to assess the biological consequence of lipid hydroperoxides, the detailed mechanism for the formation of amide-type adducts (and other unidentified histidine/cysteine epitopes) must be examined in the future.

In summary, we found that one of the major epitopes of the antibody to the 13-HPODE-modified protein, 13Ab, was the novel AZL adduct. Using the antibody, we showed that the epitopes of 13Ab containing AZL and the esters

were generated in rabbit atherosclerotic lesions. Carboxyalkylamide-type adducts, such as AZL, were universally generated from the reaction of oxidized polyunsaturated fatty acids with lysine residues. These results suggest that carboxyalkylamide-type adducts may be potential markers for the lipid peroxidation-derived modification of proteins associated with atherosclerosis and other oxidative stress-related diseases, and 13Ab may be a good tool for the detection of these lesions.

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