Formation of N^{ε}-(succinyl)lysine in vivo: a novel marker for docosahexaenoic acid-derived protein modification

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Abstract Free radical-catalyzed peroxidation of docosahexaenoic acid (DHA, C22:6/ω-3) generates various lipid peroxidation products that covalently modify biomolecules such as proteins. Under a free radical-generating system, DHA significantly modified lysine residues in bovine serum albumin. Upon incubation of oxidized DHA with an amino-compound pyridoxamine or a lysine-containing peptide, *N*-propanoyl and *N*-succinyl adducts were determined to be the major modification products. The hydroperoxide levels in the oxidized DHA closely reflected the formation of the N^{ε}-(succinyl)lysine (SUL) upon reaction with the peptide, indicating that the hydroperoxides of DHA represent a potential pathway for the formation of SUL. To detect the DHA-derived protein modification in vivo, we developed a monoclonal antibody (mAb2B12) specific to SUL and found that the antibody specifically reacts with the SUL moiety. The formation of SUL was then immunochemically demonstrated in the liver of mice fed with DHA followed by intraperitoneal injection of carbon tetrachloride (CCl_4), a hepatic lipid peroxidation model. Immunoreactive materials with mAb2B12 were observed in the DHA + CCl_4 group, but were not significant in the control, DHA-alone, and CCl_4-alone groups. These data suggest that the formation of DHA-derived adducts such as SUL may be implicated in the oxidative damage observed in DHA-enriched tissues.—Kawai, Y., H. Fujii, M. Okada, Y. Tsuchie, K. Uchida, and T. Osawa. **Formation of N^{ε}-(succinyl)lysine in vivo: a novel marker for docosahexaenoic acid-derived protein modification. *J. Lipid Res.* 2006.** 47: **1386–1398.**

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Several lines of evidence suggest that lipid peroxidation products represent potential intermediates for the oxidative modification of proteins under oxidative stress (1–3). During the lipid peroxidation reaction, lipid hydroperoxides are formed as primary products; subsequent decomposition leads to the formation of reactive intermediates that covalently modify biomolecules, including proteins. It has been reported that the protein modification products derived from lipid hydroperoxides (4–9), short chain aldehydes (10–12), and cholesteryl ester-core aldehydes (13) are formed in vivo samples associated with oxidative stress and the related diseases. Docosahexaenoic acid (C22:6, ω3) (DHA) is enriched in the central nervous system, particularly the brain. DHA is involved in memory function (14), excitable membrane function (15), photoreceptor cell biogenesis and function (16), and neuronal signaling (17) and neuroprotection (18, 19). Beneficial physiological effects of DHA, including hypolipidemic and antihypertensve effects, learning ability, and brain/retinal functions have been reported (20–24). Although the precise mechanism for the function of DHA in vivo is not well understood, a deficiency of DHA is associated with abnormalities in brain function (25). DHA is a highly unsaturated fatty acid and, therefore, is particularly susceptible to peroxidation. It has been suggested that lipid peroxidation reactions in the brain might be implicated in its deficiency and in neural injury (26–28). In addition, many studies have been conducted to explore the relationship between high intake of DHA and oxidative stress (29–33). During the peroxidation of DHA, unique DHA-derived peroxidation products such as isoprostane, termed "neuroprostane," have been identified, and in vivo formation has been revealed in brain samples, including human brain; therefore, they are suggested to be a useful marker of oxidative injury in DHA-enriched tissues such as brain (34–36).

We have recently found that *N*-acyl-type (amide-linkage) adducts are universally formed in the reaction of primary

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Abbreviations: BGL, N^{α}-benzoyl-glycyl-L-lysine; CID, collision-induced dissociation; DHA, docosahexaenoic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HNE, 4-hydroxy-2-nonenal; KLH, keyhole limpet hemocyanin; LC-MS, liquid chromatography-mass spectrometry; MDA, malondialdehyde; PI, peroxidizability index; PM, pyridoxamine; SUL, N^{ε}-(succinyl)lysine; sulfo-NHS, *N*-hydroxysulfosuccinimide; TBARS, thiobarbituric acid-reactive substances.

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amino groups with polyunsaturated fatty acid hydroperoxides (7–9). During this type of reaction, a set of fatty acid CH_3- and COOH-terminus *N*-acyl adducts can be formed. For example, *N*-hexanoyl and *N*-azelayl adducts are formed upon reaction with linoleic acid hydroperoxides (7–9). It has been shown that N^{ε}-(azelayl)lysine is a major antigenic structure formed in the linoleic acid hydroperoxide-modified protein (5, 8). In addition, N^{ε}-(hexanoyl)-lysine has been purified as one of the major products in the reaction of lysine residue with linoleic acid hydroperoxide (7). These observations suggest that *N*-acyl adducts may be the major class of modification products formed during lipid peroxidation. Although the precise reaction mechanism still remains unknown, we have revealed the in vivo formation of *N*-acyl adducts using immunochemical analysis with specific antibodies (7–9, 37, 38). Similarly, upon reaction with oxidized DHA, the formation of *N*-propanoyl and *N*-succinyl adducts in vitro has recently been suggested (8). In this study, we present evidence that DHA-derived *N*-acyl lysine adducts, especially N^{ε}-(succinyl)-lysine (SUL), are formed in significant amounts in vitro and in vivo during the peroxidation of DHA.

EXPERIMENTAL PROCEDURES

Materials

DHA, arachidonic acid, α-linolenic acid, pyridoxamine (PM) dihydrochloride, dicarboxylic acid monomethyl esters, bovine serum albumin (BSA), Freund's adjuvants (complete and incomplete), 2-alkenals, and phospholipase A_2 (EC 3.1.1.4, from bee venom) were obtained from Sigma-Aldrich Co. (St. Louis, MO). Linoleic acid and propionic anhydride were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Keyhole limpet hemocyanin (KLH), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and *N*-hydroxysulfosuccinimide (sulfo-NHS) were obtained from Pierce Chemical Co. (Rockford, IL). The sodium salt of malondialdehyde (MDA) was prepared by Dowex hydrolysis of MDA-bis(diethyl acetyl) as previously described (39). 4-Hydroxy-2-nonenal (HNE) was prepared by the acid treatment (1 mM HCl) of HNE dimethylacetyl synthesized according to the procedure of De Montarby, Mosset, and Gree (40). N^{α}-benzoyl-glycyl-L-lysine (BGL) was obtained from Peptide, Inc. (Osaka, Japan). DHA-ethyl ester and eicosapentaenoic acid were kindly provided by NOF Co. Tsukuba research laboratory (Tsukuba, Japan).

Fatty acid peroxidation reaction

Fatty acids (10 mM) were incubated with 50 μM $FeSO_4$ and 1 mM ascorbate at 37°C for 0–3 days in phosphate-buffered saline (PBS). After reaction, lipid peroxidation levels were determined as thiobarbituric acid-reactive substances (TBARS) (5) and/or lipid hydroperoxide levels (monitored by Lipid Hydroperoxide assay kit, Cayman).

In vitro modification of protein, PM, or peptide

The oxidized fatty acid solution described above was incubated with an equal volume of BSA (2 mg/ml), PM (2 mM), or BGL (10 mM) at 37°C in PBS. After incubation, the modification of BSA was evaluated by SDS-PAGE or amino acid analysis. Briefly, protein samples were incubated with SDS sample buffer at 100°C for 5 min, and the samples were then separated by 10% SDS-PAGE, followed by Coomassie Brilliant Blue staining. Upon amino acid analysis, the protein samples 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. The PM adducts were analyzed by high-performance liquid chromatography (HPLC) using a Develosil ODS HG-5 column (4.6 × 250 mm; Nomura Chemicals, Aichi, Japan) with ultraviolet (UV) (294 nm) detection at a flow rate of 0.8 ml/min. The gradient programs (solvent A, 5% CH_3CN containing 0.01% acetic acid; solvent B, 100% CH_3CN containing 0.01% acetic acid) were as follows. Program 1: 0–40 min, linear gradient to 65% B; 40–41 min, linear gradient to 100% B; hold 10 min; 51–52 min, linear gradient to 100% A. Program 2: 0–5 min, linear gradient to 100% A; 5–50 min, linear gradient to 50% B; 50–55 min, linear gradient to 100% B; hold 10 min; 65–70 min, linear gradient to 100% A. The modified BGL was analyzed by liquid chromatography-mass spectrometry (LC-MS; Micromass VG Platform II, Micromass, Manchester, UK) in electrospray ionization-positive mode. The sample was injected into a Develosil ODS HG-5 column (4.6 × 250 mm) and eluted with a gradient (Program 1) at 0.8 ml/min. For the quantitation of SUL, *tert*-butoxycarbonyl (Boc) 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* 408 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.

The aldehyde-modified protein was prepared by incubating BSA (1 mg/ml) with 5 mM aldehyde in phosphate buffer, pH 7.4, for 3 days at 37°C.

Synthesis of *N*-acyl adducts

N-acyl adducts were chemically synthesized by incubating amino substrates (PM, BGL, or N^{α}-acetyl-lysine, 25 mmol) with carboxylic anhydride (0.5 mmol) in 5 ml of 100 mM sodium phosphate buffer (pH 7.4)-saturated sodium acetate (1:1, v/v) for 60 min at room temperature. Propionic anhydride and succinyl anhydride were utilized for preparing *N*-propanoyl and *N*-succinyl adducts, respectively. The synthesized adducts were purified by reverse-phase HPLC using a Develosil ODS-HG-5 column (20 × 250 mm) in an isocratic system of 15% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 6 ml/min. The elution profiles were monitored by absorbance at 240 nm. The proteins that contained *N*-acyl carboxylic adducts were chemically synthesized as previously reported (8).

Free amino acid SUL was also synthesized as follows. The *tert*-Boc lysine was succinylated as described above. The formed Boc-SULs were semipurified by reverse-phase HPLC using a Develosil ODS-HG-5 column (20 × 250 mm) in an isocratic system of 25% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 6 ml/min. The elution profiles were monitored by absorbance at 210 nm. The obtained Boc-SUL was dried in vacuo and then treated with trifluoroacetic acid at room temperature for 1 h. After incubation, the reaction mixture was neutralized with sodium hydroxide. The formed free amino acid SUL was purified by reverse-phase HPLC using a Develosil ODS-HG-5 column (20 × 250 mm) in an isocratic system of 5% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 6 ml/min. The elution profiles were monitored by absorbance at 210 nm. The synthesized adducts were identified by ^{1}H-NMR using a Bruker AMX400 (400 MHz; Bruker, Karlsruhe, Germany).

Preparation of a monoclonal antibody to SUL

The succinylated KLH was prepared by a carbodiimide (EDC/sufo-NHS system) method as previously described (8) and used as the immunogen. Balb/c mice (female, 7 weeks) were injected with 60 μg of the immunogen with complete Freund's adjuvant. At 2 week intervals, they were boosted with 20 μg of the immunogen with incomplete Freund's adjuvant. Three days after the final boost (50 μg of the conjugate in PBS), spleen cells from one of the mice were fused with P3/U1 murine myeloma cells. Aliquots of the hybridoma supernatants were tested for the presence of anti-succinyl-BSA by enzyme-linked immunosorbent assay (ELISA). The ELISA procedure was performed as previously described (5). For the hydrolysis of ester bonds, prior to blocking, wells were treated with 0.25 N NaOH or phospholipase A_2 (1.0 U/μl) at 37°C for 1 h.

Animal experiments

Three-week-old male ICR mice were purchased from CLEA Japan, Inc. (Tokyo, Japan) and housed under standard experimental conditions (23°C, 55% humidity, 12 h light and 12 h dark cycle). After the prefeeding with control diet (CLEA Rodent Diet CE-2) for a week, mice were randomly divided into four groups and fed with the diet of each group (groups 1 and 3, control diet; groups 2 and 4, control diet mixed with 3% DHA-ethyl ester) for 19 days ad libitum. Mice of groups 3 and 4 were intraperitoneally injected with carbon tetrachloride (15% in olive oil, 200 μl/mouse) at 24 h before euthanization. Mice of groups 1 and 2 were injected with vehicle (olive oil, 200 μl) only. A portion of a liver was homogenized with PBS containing 0.1% 2,6-di-*t*-butyl-*p*-cresol or fixed with 10% formalin neutral buffer solution (Wako) for immunohistochemical analysis. The protein contents of the homogenates were determined by using a BCA protein assay reagent kit (Pierce).

Fatty acid analysis

The lipids in liver homogenates were extracted with chloroform-methanol (2:1). The chloroform layers were evaporated under a nitrogen stream and dissolved in 100 μl methanol. The solution (25 μl) was mixed with an equal volume of 1 N NaOH and hydrolyzed at 37°C for 1 h. After incubation, samples were neutralized with 0.1 N HCl, and mixed with 75 μl of 0.1% 9-anthryldiazomethane (Funakoshi; Tokyo, Japan) methanol solution. After a 10 min incubation at room temperature, the derivatized fatty acids were analyzed by reverse-phase HPLC using a Develosil C8-5 column (4.6 × 150 mm) and 90% aqueous acetonitrile at a flow rate of 1.1 ml/min with fluorescence detection (excitation at 365 nm, emission at 412 nm). The peroxidizability index (PI) of the lipids was calculated according to the following equation (41): PI = (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 3) + (% pentaenoic × 4) + (% hexaenoic × 5).

Lipid peroxidation assay

Lipid peroxidation levels were determined by thiobarbituric acid (TBA) assay. The liver homogenates (200 μl) were mixed with 8.1% SDS aqueous solution (50 μl), 20% acetic acid buffer, pH 3.5 (225 μl), and 0.8% TBA aqueous solution (225 μl) and boiled at 100°C for 1 h. After cooling in an ice bath, the absorbance of the supernatants was measured at 532 nm. MDA, prepared by acid hydrolysis of tetramethoxypropane, was used as the standard. Data were calculated as MDA equivalent and expressed as thiobarbituric acid-reactive substances (TBARS).

Determination of SUL by ELISA

Prior to ELISA, the homogenates (protein concentration, 2 mg/ml) were mixed with an equal volume of 0.25 N NaOH and incubated at 37°C for 1 h. After incubation, the mixtures were coated onto wells and the immunoreactivities with mAb2B12 were determined by ELISA.

Immunohistochemistry

Paraffin-embedded sections (5 μm thickness) were deparaffinized, incubated with normal serum for 30 min to block the nonspecific binding of the secondary antibody, and then with mAb2B12 at 4°C overnight. For ester bond hydrolysis, prior to blocking, the sections were incubated with 0.25 N NaOH for 1 h or 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).

HPLC-tandem mass spectrometry

HPLC-tandem mass spectrometry (MS/MS) analyses were carried out on the API 2000 triple quadrupole mass spectrometer (Applied Biosystems) through a TurboIonSpray source. Chromatography was carried out on a Develosil ODS-HG-3 column (2.0 × 250 mm) using an Agilent 1100 HPLC system. The chromatographic separation was performed by a gradient elution as follows: 0–10 min, linear gradient from 0.1% formic acid to 50% aqueous acetonitrile containing 0.1% formic acid; 10–15 min, hold; 15–20 min, linear gradient to 0.1% formic acid; flow rate = 0.2 ml/min. The instrument response was optimized by infusion experiments on the standard compounds using a syringe pump at a flow rate of 5 μl/min. The *N*-acyl adducts were detected using electrospray ionization MS/MS in the multiple reaction monitoring mode.

Enzymatic digestion of the protein samples was performed as previously reported (36). Briefly, samples were heated at 98°C for 5 min. After cooling, Pronase (3 mg/mg of starting protein weight) was added, and the mixture was incubated overnight at 37°C. Samples were then heated at 98°C for 5 min to inactivate the Pronase, and after cooling, aminopeptidase M (0.11 U/mg of starting protein weight) was added, and the digest was incubated at 37°C for 18 h. The digest was filtrated thorough Microcon YM-10 (Millipore) and injected to HPLC-MS/MS.

Statistical analysis

All data were analyzed using Bonferroni/Dunn's multiple comparison procedure.

RESULTS

DHA-derived protein modification

The susceptibility of polyunsaturated fatty acids to the lipid peroxidation reaction is generally thought to be dependent on the number of methylene groups between double bonds. Thus, DHA (22:6, ω-3) might be the most susceptible to lipid peroxidation among the endogenous polyunsaturated fatty acids. As shown in **Fig. 1A**, in fact, DHA was extremely susceptible to the free radical-catalyzed peroxidation reaction at 37°C in phosphate buffer (pH

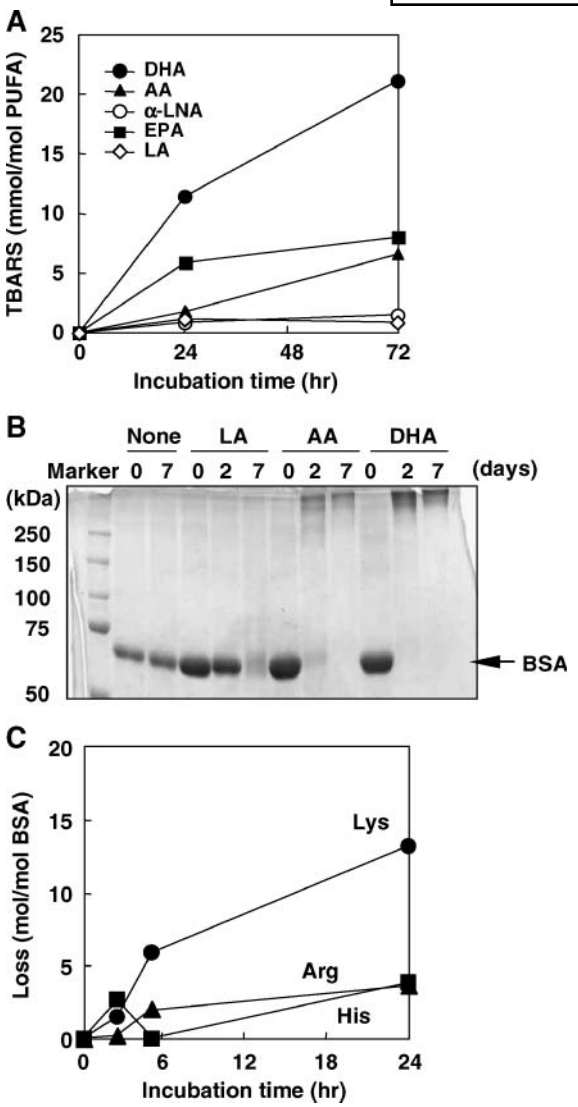


Fig. 1. Peroxidation of polyunsaturated fatty acids and the modification of protein. Polyunsaturated fatty acids (10 mM) were incubated in the presence of Fe^{2+}/ascorbic acid (50 μM/1 mM) in 0.1 M phosphate buffer (pH 7.4) at 37°C. A: Lipid peroxidation levels of various polyunsaturated fatty acids were monitored and expressed as thiobarbituric acid-reactive substances (TBARS). B: The oxidized (for 24 h) polyunsaturated fatty acids were further incubated with bovine serum albumin (BSA) (1 mg/ml) at 37°C for 0–7 days. After incubation, samples were analyzed by SDS-PAGE. C: The loss of lysine (Lys), arginine (Arg), and histidine (His) in the oxidized DHA-modified BSA (after 7 day incubation) determined by amino acid analysis. Other amino acids were scarcely modified in the protein. LA, linoleic acid; AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicospentaenoic acid; LNA, linolenic acid.

7.4). The peroxidation levels (expressed as TBARS) were clearly dependent on the number of double bonds; DHA > eicosapentaenoic acid (20:5, ω-3) > arachidonic acid (20:4, ω-6) > α-linolenic acid (18:3, ω-3) > linoleic acid (18:2, ω-6). When linoleic acid, arachidonic acid, or DHA was oxidized in the presence of BSA and analyzed with SDS-PAGE, the bands of BSA (67 kDa) were time-dependently decreased (Fig. 1B). In the absence of fatty acids, the disappearance of BSA bands was scarcely observed, suggesting that this modification may be derived from lipid peroxidation products. In addition, the appearance of protein bands with higher molecular mass was observed in the oxidation of arachidonic acid and DHA, whereas this was scarcely observed in the oxidation of linoleic acid. These results showed that highly unsaturated fatty acids such as DHA significantly lead to the formation of lipid peroxidation products and subsequent protein modification/aggregations. Amino acid analysis of oxidized DHA-modified BSA showed that the lysine residues in the protein represent a potential target of the modification reaction by oxidized DHA (Fig. 1C).

Characterization of lysine adducts with oxidized DHA

To characterize the DHA-derived adducts upon reaction with protein lysine groups, we first examined the reaction of oxidized DHA with PM. Here, we utilized PM as an amino probe because of its high reactivity with lipid peroxidation products (42). Upon reaction of oxidized DHA with PM, three major peaks (peaks 1–3) were successfully detected in the HPLC-UV (at 294 nm derived from PM) analysis (**Fig. 2A, B**). These peaks were not observed in the incubation of oxidized DHA or PM alone (data not shown), suggesting that these peaks could be the reaction products of oxidized DHA and PM. Peak 1 was also detected as a major product in the reaction of MDA with PM (data not shown). A previous report that *N*-propenal adduct is a major product in the reaction of MDA with a primary amino group (43) suggests that peak 1 may be the *N*-propenal PM adduct. Compared with synthetic compounds, peaks 2 and 3 were then identified to be *N*-succinyl-PM and *N*-propanoyl-PM, respectively. The identified chemical structures are shown in Fig. 2C. These results showed that *N*-acyl-type adducts as well as the MDA-derived adduct could be the major modification products derived from oxidized DHA. The formation of these two *N*-acyl adducts was also confirmed using the lysine-containing model peptide, BGL. LC-MS analysis of the reaction mixture of oxidized DHA and BGL gave molecular ion peaks at *m/z* 408 and 364, which corresponded to the authentic N^{ε}-succinyl and N^{ε}-propanoyl BGL adducts, respectively (**Fig. 3**). Chemical structures of the authentic adducts were identified by ^{1}H-NMR (supplemental Fig. S1A, B). The formation of these two *N*-acyl adducts was further confirmed by HPLC-MS/MS analysis. Collision-induced dissociation (CID) of authentic N^{ε}-succinyl (*m/z* 408) and N^{ε}-propanoyl (*m/z* 364) BGL adducts produced the same daughter ions at *m/z* 77, 84, and 105 (**Fig. 4A**, left). These ions can be assigned as the structures shown in Fig. 4A (right). The ions at *m/z* 77 and 105 were identified to be derived from the benzoyl moiety of BGL. The ions at *m/z* 84 were identified to be derived from the lysine side chain of BGL. The detection of this ion has been reported in the CID spectra of isoketal-lysyl adducts (36). On the basis of the information in the CID spectra, the reaction mixture of oxidized DHA and BGL was analyzed by HPLC-MS/MS. As shown in Fig. 4B, the peaks indicating N^{ε}–succinyl and *N*-propanoyl BGL adducts were

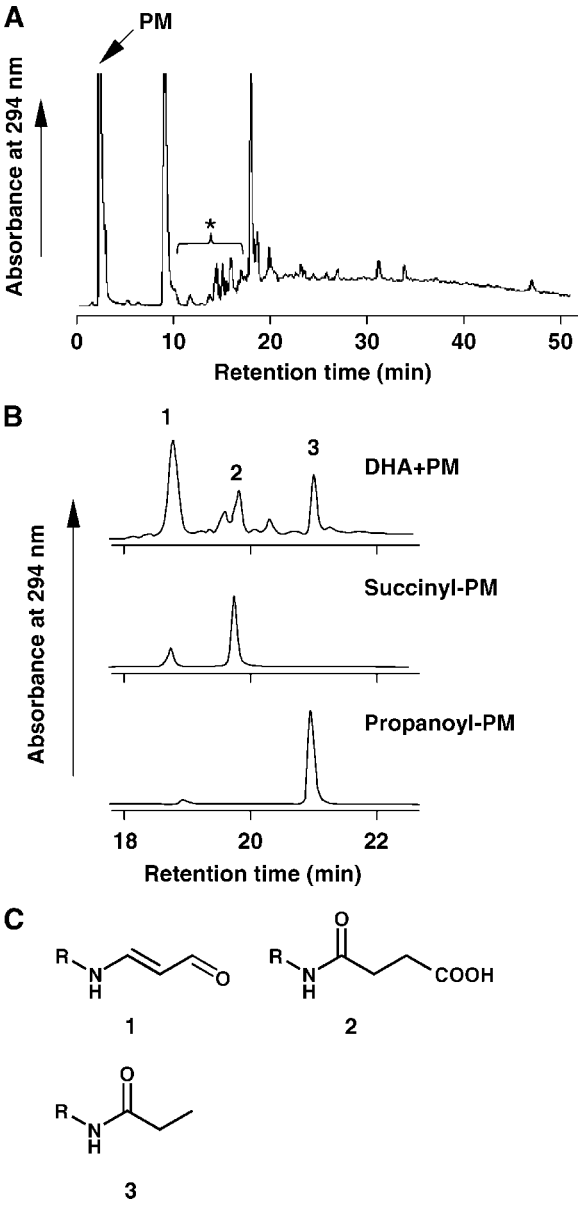


Fig. 2. High-performance liquid chromatography (HPLC) analysis of the reaction mixture of oxidized DHA and pyridoxamine (PM). DHA (10 mM) was oxidized in the presence of Fe^{2+}/ascorbic acid at 37°C for 24 h, and further incubated with PM (1 mM) at 37°C for 72 h. A: The HPLC profile (gradient elution, Program 1) of the reaction mixture was monitored by absorbance at 295 nm. The asterisk indicates a period of time that contains the reaction products. Other peaks were also observed in the analysis of the incubation mixture of oxidized DHA alone. B: The major reaction products (peaks 1–3) observed in Fig. 2A were further characterized (gradient elution, Program 2) by comparison with the HPLC profiles of presumed DHA-derived *N*-acyl adducts. *N*-propanoyl and *N*-succinyl derivatives were chemically synthesized as described in Experimental Procedures. C: The proposed structures of peaks 1–3. Peak 1, malondialdehyde-derived propenal adduct; peak 2, *N*-succinyl adduct; peak 3, *N*-propanoyl adduct. R-NH_2 indicates PM).

successfully detected at *m/z* 408 → 84 and *m/z* 364 → 84, respectively. The retention times were consistent with those of authentic adducts. These chromatographic characterizations showed the formation of N^{ε}-succinyl and N^{ε}-propanoyl adducts in the reaction of lysine ε-amino groups or other amino compounds with oxidized DHA.

In addition to the formation of N^{ε}-succinyl and N^{ε}-propanoyl adducts with low-molecular-weight compounds, the formation of these adducts in protein was also examined using HPLC-MS/MS. Authentic free amino acid SUL adduct was also chemically synthesized and identified by ^{1}H-NMR (supplemental Fig. S2). CID of authentic SUL (*m/z* 247) produced the daughter ions at *m/z* 56, 84, and 184 (**Fig. 5A**, upper). These ions can be assigned as the structures shown in Fig. 5A (lower). The ion at *m/z* 56 was identified to be derived from the lysine aliphatic moiety. The relatively weak fragment ion at *m/z* 184 was identified to be derived from the succinyl moiety. On the basis of this information, HPLC-MS/MS analysis of the oxidized DHA-modified BSA was performed. Because general methods for acid hydrolysis of protein (e.g., in 6 N HCl at 105°C for 24 h) may result in cleavage of the amide linkage in the *N*-acyl adducts, proteins were enzymatically hydrolyzed with Pronase and aminopeptidase M. As shown in Fig. 5B (top), authentic SUL was detected by HPLC-MS/MS analysis at *m/z* 247 → 84. We confirmed the successful hydrolysis of the modified protein by the detection of SUL in the hydrolysate of chemically synthesized succinylated BSA (Fig. 5B, middle). As shown in Fig. 5B (bottom), the enzymatic hydrolysis of oxidized DHA-modified BSA resulted in the appearance of the peak comigrated with SUL. The formation of SUL was also detected as a weak peak at *m/z* 247 → 184, a specific fragment of SUL (data not shown). In addition, we confirmed a time-dependent increase in this peak (data not shown). After 72 h incubation, the amount of SUL was approximately 30 mmol/mol BSA. These results showed the formation of SUL in proteins modified with oxidized DHA.

These results and the fact that *i*) MDA is known to be formed during the peroxidation of various polyunsaturated fatty acids and *ii*) N^{ε}-propanoyl adduct could be commonly formed in the reaction with ω-3 polyunsaturated fatty acids showed that the SUL adduct may be the major and specific product formed in the reaction of lysine residues and oxidized DHA. Indeed, the N^{ε}-propanoyl adduct could be formed not only by oxidized DHA but also by oxidized α-linolenic acid and eicosapentaenoic acid; however, the N^{ε}-succinyl adduct was formed only by oxidized DHA (data not shown). Although the precise reaction mechanism for the formation of *N*-acyl lysine adducts is still unknown, we have suggested that the contribution of the lipid hydroperoxide-derived initially formed intermediate(s), but not of aldehydic end-products, to the formation of linoleic acid-derived *N*-acyl adducts (7, 8). As shown in **Fig. 6**, the formation of SUL was dependent on the content of lipid hydroperoxides in oxidized DHA formed during the preincubation prior to the reaction with BGL. Longer preincubation (e.g., for 3–5 days) of DHA significantly attenuated the ability to generate SUL. This result suggests that initially generated DHA peroxidation product(s) may be the intermediate(s) for the formation of SUL.

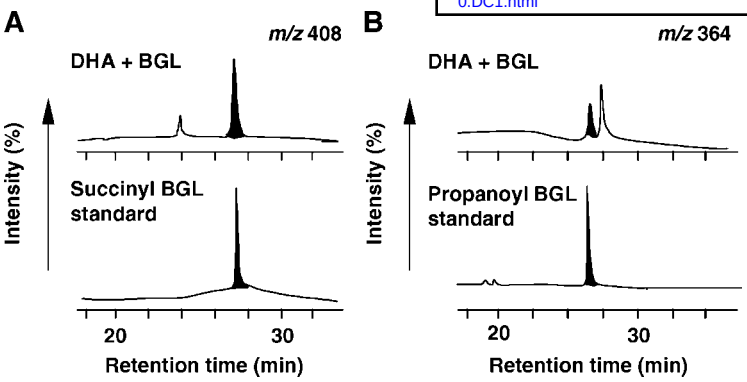


Fig. 3. Formation of *N*-acyl lysine adducts upon reaction with oxidized DHA. The oxidized DHA was incubated with N^{α}-benzoyl-glycyl-L-lysine (BGL, 5 mM) in 0.1 M phosphate buffer (pH 7.4) at 37°C for 72 h. The reaction mixture was analyzed by liquid chromatography-mass spectrometry (LC-MS). A: Chromatograms of selected ion monitoring at *m/z* 408. Upper, reaction mixture of oxidized DHA/BGL; lower, chemically synthesized N^{ε}-succinyl-BGL adduct. B: Chromatograms of selected ion monitoring at *m/z* 364. Upper, reaction mixture of oxidized DHA/BGL; lower, chemically synthesized N^{ε}-propanoyl-BGL adduct.

Monoclonal antibody specific to SUL

To evaluate the endogenous generation of the SUL adduct, we prepared a novel monoclonal antibody to SUL. The synthetic succinylated KLH was used as the immunogen (**Fig. 7A**). The positive clones that significantly recognized succinylated BSA were selected, and a clone (clone No. 2B12) was finally obtained. The specificity of the obtained antibody (named "mAb2B12") was then characterized. As shown in Fig. 7B, in contrast to the significant recognition of the antibody to the succinylated protein, unmodified protein and lipid aldehyde-modified proteins were not recognized. Among the various oxidized fatty acid-modified proteins, mAb2B12 could specifically react with oxidized DHA-modified protein. A competitive ELISA

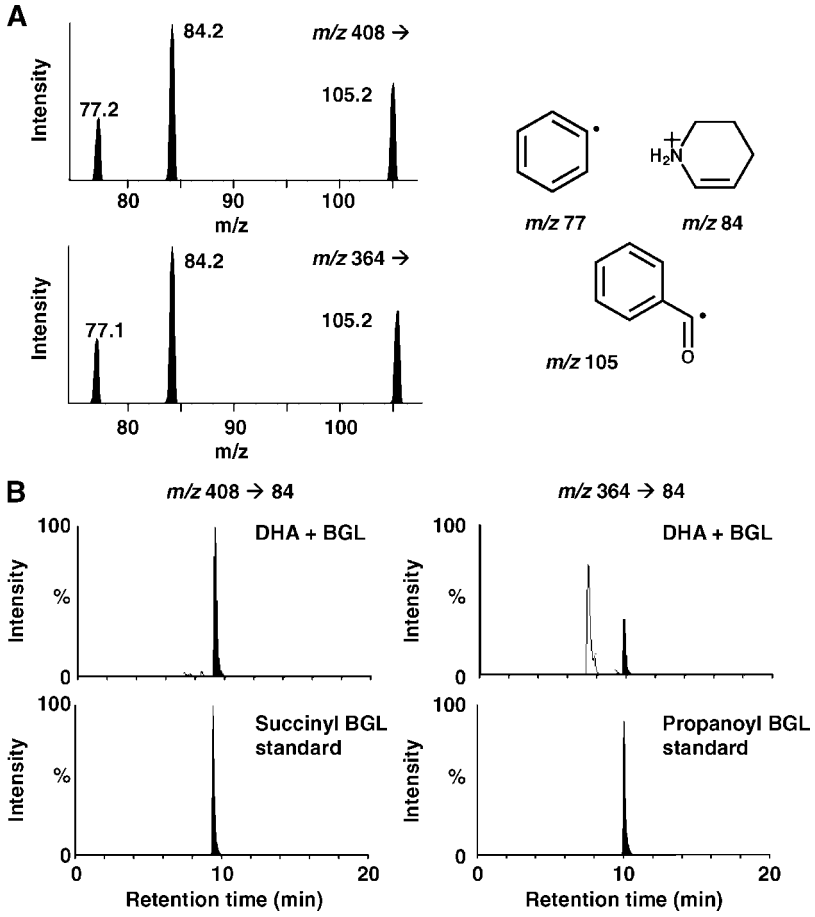


Fig. 4. HPLC-tandem mass spectrometry (MS/MS) analysis of *N*-acyl adducts formed during oxidation of DHA in the presence of a lysine derivative. A: The $[MH]^+$ ions *m/z* 408 of the N^{ε}-succinyl- (upper) and *m/z* 364 of the N^{ε}-propanoyl- (lower) BGL adducts were subjected to collision-induced dissociation (CID), and daughter ions were scanned. The proposed structures of individual ions are shown (right). B: Selected ion monitoring of the transitions from *m/z* 408 to *m/z* 84 (left) and *m/z* 364 to *m/z* 84 (right) for N^{ε}-succinyl adduct and N^{ε}-propanoyl adduct, respectively. Upper, oxidized DHA-modified BGL; lower, authentic *N*-acyl BGL adducts.

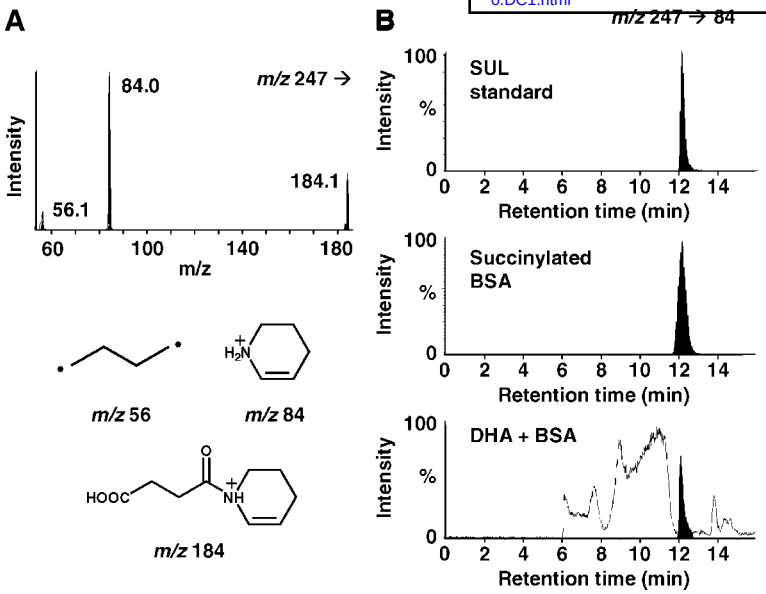


Fig. 5. HPLC-MS/MS analysis of N^{ε}-succinyl-lysine (SUL) formed during oxidation of DHA in the presence of BSA. A: The $[MH]^+$ ion m/z 247 of the SUL was subjected to CID, and daughter ions were scanned (upper). The proposed structures of individual ions are shown (lower). B: Selected ion monitoring of the transitions from m/z 247 to m/z 84 for SUL. Top, authentic SUL; middle, enzymatic hydrolysate of succinylated BSA; bottom, enzymatic hydrolysate of oxidized DHA-modified BSA.

experiment with the succinylated adduct of N^{α}-acetyl-lysine showed that mAb2B12 did indeed react with the SUL moiety (Fig. 7C). Furthermore, when the cross-reactivities of mAb2B12 with structurally analogous *N*-acyl carboxylic adducts were examined, the antibody could distinguish SUL from various alkyl-length *N*-acyl carboxylic adducts (Fig. 7D). In addition, the antibody could not react with *N*-butanoyl (four carbon, noncarboxylic adduct) (data not shown), suggesting that the carboxylic group is essential for the recognition of mAb2B12. Collectively, these characterizations clearly showed that the obtained mAb2B12 is highly specific to the SUL adduct. Using the antibody, the antigenic materials in oxidized DHA-modified protein were assessed by immunoblot analysis. As shown in Fig. 7E, the incubation with oxidized DHA resulted in a time-dependent increase in the antigenicity of BSA, whereas the incubation without DHA did not generate any antigenic materials. Interestingly, the high-molecular-weight protein exhibited a strong immunoreactivity with mAb2B12.

Immunochemical detection of SUL in vivo

To detect the generation of the SUL adduct in vivo and clarify its implication in oxidative stress in the DHA-rich tissues, we conducted an animal experiment using DHA-fed mice combined with a hepatic oxidative stress model induced by carbon tetrachloride. During the experimental period, changes in the body weights of mice between the experimental groups could not be observed (data not shown). As shown in **Table 1**, significant accumulation of DHA was observed in the liver of mice fed with DHA. In the DHA-fed groups, the composition of arachidonic acid was decreased, resulting in an increased n-3/n-6 ratio. In addition, the calculated PI was elevated in the DHA-fed groups. **Figure 8A** shows lipid peroxidation levels (evaluated as TBARS) in the liver. DHA supplementation itself did not influence the TBARS values. Under our experimental conditions, only the injection of carbon tetrachloride in the normal-diet group did not influence the TBARS. However, the injection of carbon tetrachloride in the DHA-fed group significantly increased the TBARS values. These results showed that accumulation of DHA increased the susceptibility to oxidative stress. Figure 8B shows the formation of SUL analyzed by ELISA with mAb2B12. The DHA supplementation with carbon tetrachloride injection significantly increased the concentration of SUL. A slight but not statistically significant increase in SUL was also observed in the group with carbon tetrachloride injection without DHA supplementation. This may be explained by the observation that basal DHA levels in the groups without DHA supplementation were relatively high, approximately 8–9% (Table 1). Increases in SUL were not observed in the two groups without carbon

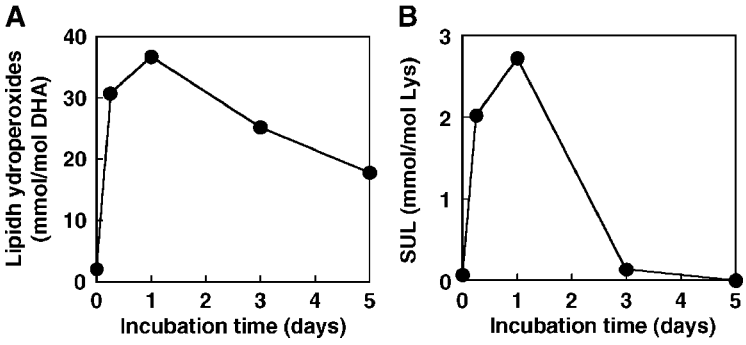


Fig. 6. Lipid hydroperoxide-dependent formation of SUL. DHA (10 mM) was oxidized in the presence of Fe^{2+}/ascorbic acid in 0.1 M phosphate buffer (pH 7.4) at 37°C. After incubation (0–5 days), the content of lipid hydroperoxides (A) in the oxidized DHA samples was measured as described in Experimental Procedures. Alternatively, the oxidized DHA was further incubated with BGL (5 mM) at 37°C for 24 h. The formation of SUL was quantitated by using LC-MS as described in Experimental Procedures (B).

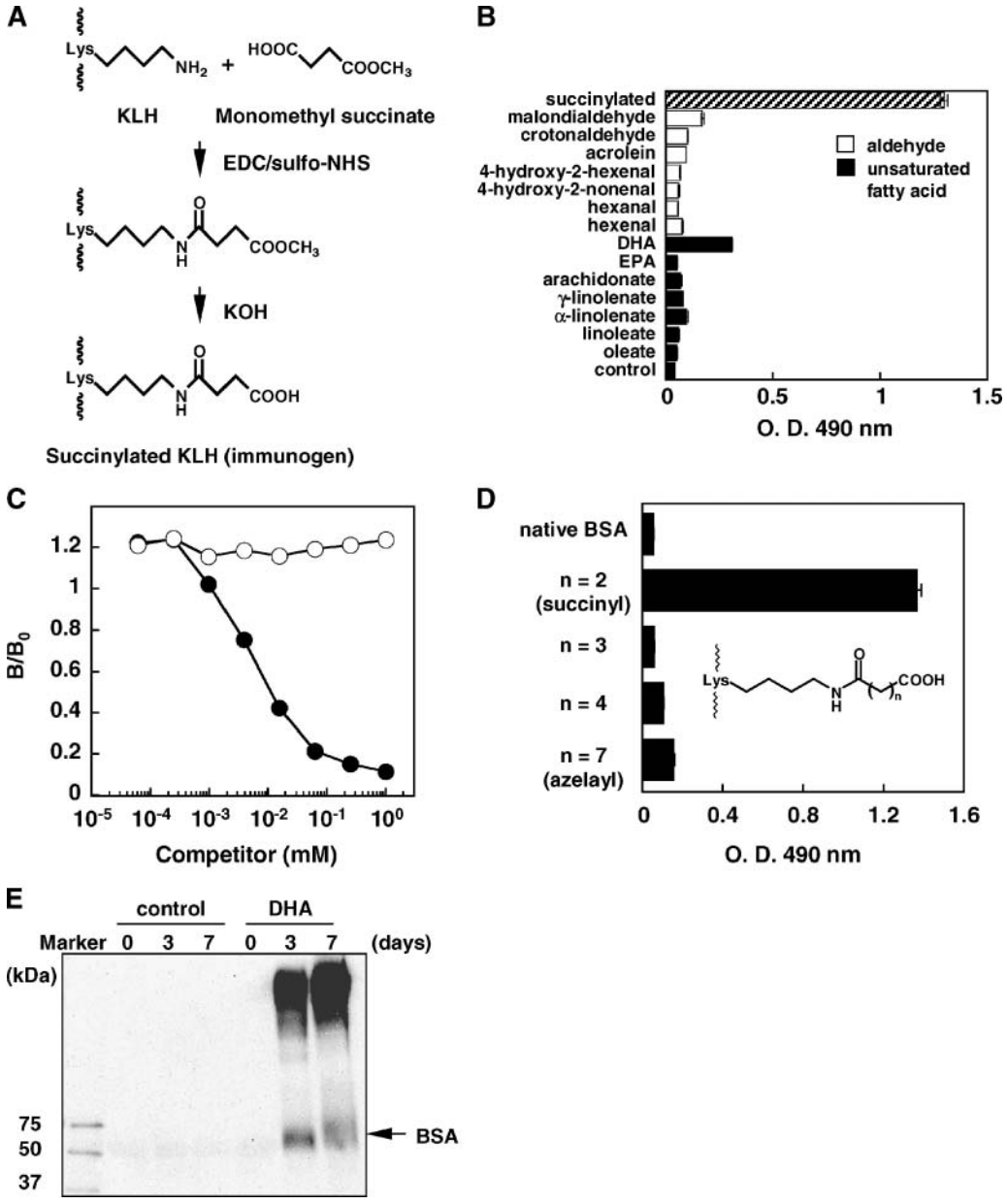


Fig. 7. Preparation and characterization of the monoclonal antibody to SUL. A: A scheme for the preparation of succinylated keyhole limpet hemocyanin (KLH) as immunogen. B: Immunoreactivities of mAb2B12 to the proteins modified by various lipid peroxidation products (aldehydes and oxidized unsaturated fatty acids) determined by enzyme-linked immunosorbent assay (ELISA). Coating antigen was prepared by incubating BSA (1 mg/ml) with aldehydes (5 mM) or oxidized fatty acids in phosphate buffer (pH 7.4) at 37°C for 3 days. C: Competitive ELISA analysis with N^{α}-acetyl-lysine (open circles) and N^{α}-acetyl-SUL (closed circles). B/B_0, absorbance in the presence of competitor/absorbance in the absence of competitor (absorbance at 450 nm). D: Immunoreactivities of mAb2B12 to various *N*-acyl carboxylic adducts in protein. The proteins that contain *N*-acyl carboxylic adducts were chemically synthesized by conjugating BSA with a monomethyl dicarboxylic acid using carbodiimide, followed by treatment with alkali that hydrolyzes the methyl esters to free carboxyl groups. E: Immunoblot analysis of oxidized DHA-modified BSA with mAb2B12.

tetrachloride injection. These results showed that DHA-derived lipid peroxidation closely reflected the SUL in vivo. In this ELISA experiment, samples were hydrolyzed with alkali prior to application of primary antibody. The immunoreactivity with mAb2B12 was scarcely observed in nonhydrolyzed samples (data not shown), suggesting the abundance of esterified SUL adduct rather than free carboxylic SUL in vivo.

The endogenous formation of SUL in the liver of DHA-fed mice was further characterized by immunohistochemical staining (**Fig. 9**). The positive staining localized around the portal vein was observed in the DHA/carbon

TABLE 1. Fatty acid composition in the liver of rats

Fatty Acid (%)	Normal	DHA	Normal + CCl_4	DHA + CCl_4
16:0	10.6	12.7	11.1	11.2
16:1	1.9	0.1	2.5	1.8
18:0	17.3	18.4	18.6	15.3
18:1	32.6	30.1	35.3	34.9
18:2	11.8	14.5	11.4	13.2
20:4	15.1	2.0	10.9	4.0
20:5	1.8	5.9	1.5	4.4
22:6	9.1	15.8	8.7	15.2
n-3/n-6	0.4	1.3	0.5	1.1
PI	109.4	122.9	93.6	119.0

Fatty acids were derivatized with 9-anthryldiazomethane and then analyzed by reverse-phase HPLC as described in Experimantal Procedures. PI, peroxidizability index.

tetrachloride group (Fig. 9D), whereas the staining was scarcely observed in other groups (Fig. 9A–C). When the immunostaining with mAb2B12 was performed after the pretreatment of the sections with phospholipase A_2, which cleaves the ester bonds in the *sn*-2 position of phospholipids, enhanced positive staining was observed in the two carbon tetrachloride-injected groups (Fig. 9G, H), and was especially significant in the DHA/carbon tetrachloride group. The result showed the abundance of phospholipid-esterified SUL adducts in vivo. Such enhancement by the treatment with phospholipase A_2 could not be observed in control and DHA groups (Fig. 9E, F). The immunostaining with mAb2B12 clearly correlated with the results in the ELISA experiments (Fig. 8B). Similar results were also observed in the sections hydrolyzed with alkali prior to staining (data not shown).

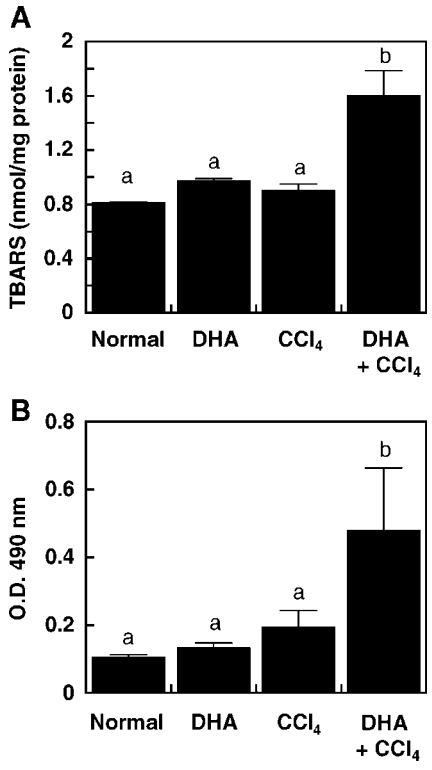


Fig. 8. Formation of SUL in vivo. A: Lipid peroxidation levels in the liver of mice determined and expressed as TBARS. B: The formation of N^{ε}-succinyl lysine in the liver of mice was determined by ELISA. Bars with different letters are significantly different ($P < 0.05$) as determined by the Bonferroni/Dunn multiple comparison test.

DISCUSSION

In this study, we identified the SUL adduct as a novel class of DHA-derived protein modification product and revealed the in vivo formation during the peroxidation of DHA. SUL is an *N*-acyl carboxylic adduct composed of a C_4 unit from the COOH terminus of DHA. The analogous *N*-acyl carboxylic adducts, N^{ε}-(azelayl)lysine (C_9 adduct) and N^{ε}-(glutaryl)lysine (C_5 adduct), were also suggested to be formed in vivo (5, 6, 8). These two *N*-acyl carboxylic adducts were found to originate from multiple polyunsaturated fatty acids; i.e., N^{ε}-(azelayl)lysine is formed by oxidized linoleic acid and α-linolenic acid, and N^{ε}-(glutaryl)lysine is formed by oxidized arachidonic acid and eicospentaenoic acid (8). In our reaction conditions in vitro, N^{ε}-propanoyl and MDA adducts were also identified to be the major adducts by oxidized DHA (Fig. 2), but these two adducts could also be formed by other polyunsaturated fatty acids. The N^{ε}-propanoyl adduct can be formed by all ω-3 polyunsaturated fatty acids, and MDA is known as the product common to all polyunsaturated fatty acids. Because DHA is the only fatty acid source that generates the SUL adduct, as far as we know, SUL could be a specific marker for DHA-derived protein modification in vivo. We identified the formation of SUL by using a mass spectrometric approach (Figs. 3–5). Use of MS/MS spectrometry showed not only the parent ions of SUL but also the daughter ions. We confirmed that the SUL, compared with the mass spectrum for the authentic SUL standard that was identified by ^{1}H-NMR (supplemental Figs. S1, S2), is indeed formed during the oxidation of DHA in the presence of a lysine derivative or protein.

DHA is known as a major unsaturated fatty acid in neural tissues. Several reports have shown that oxidative stress in neural tissues may be implicated in various neurodegenerative diseases (26–28). The formation of isoprostane-like compounds derived from DHA (known as neuroprostanes) has been reported in vitro and in vivo (34). During the similar neuroprostane pathway, the formation of highly reactive γ-ketoaldehydes (neuroketals) and subsequent protein modification have also been reported (36). Neuroketal-derived lysyl-lactam adducts were detected in normal human brain at a level of 9.9 ± 3.7 ng/g of brain tissue (36). These reports show that the neuroprostane pathway products of DHA and the subsequent protein modifications are involved in DHA-derived oxidative injury in tissues such as brain. In our experimental in vitro conditions, we could not detect the neuroprostane pathway-derived lysine modification products. Although further studies are needed for estimating the relative contribution of DHA-derived protein modification products such as SUL adduct to the oxidative injury of DHA-enriched tissues, our results indicate that the for-

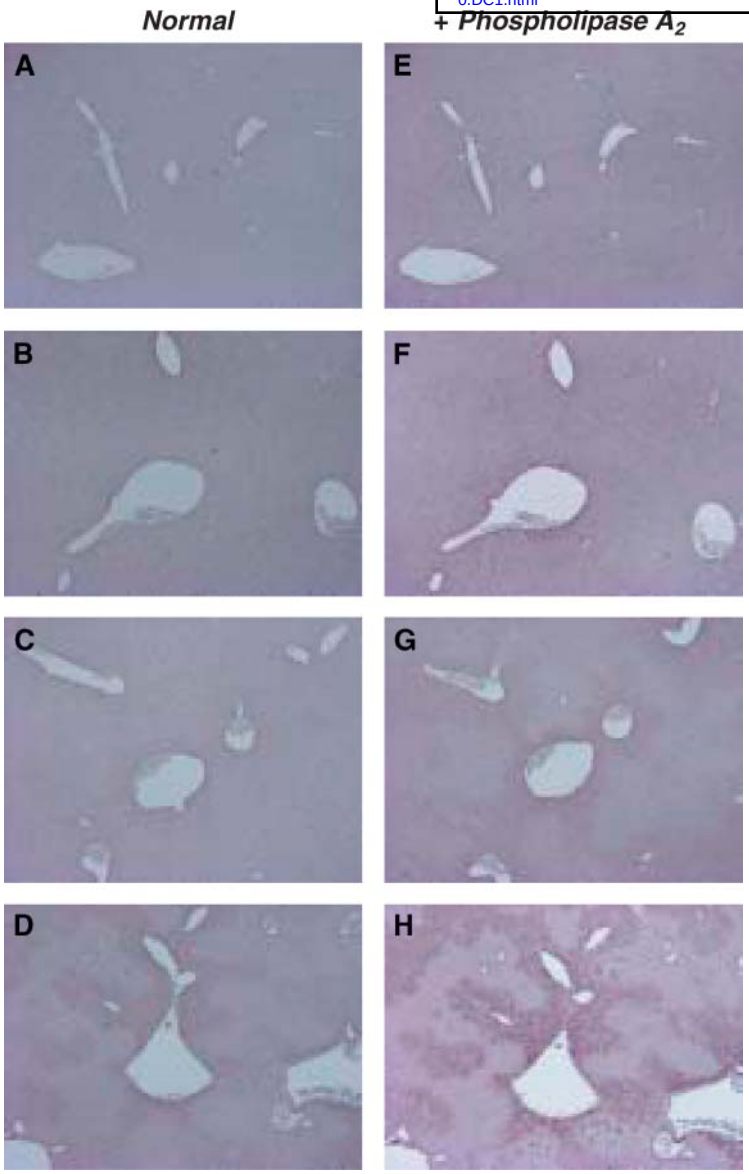


Fig. 9. Immunohistochemical detection of SUL in vivo. The liver sections were immunostained with mAb2B12. A, E: Group 1 (control diet); B, F: group 2 (DHA diet); C, G: group 3 (control diet + CCl_4 injection); D, H: group 4 (DHA diet + CCl_4 injection). A–D: Nonhydrolyzed sections; E–H: hydrolyzed sections with phospholipase A_2.

mation of SUL represents a major protein modification product formed during the peroxidation of DHA.

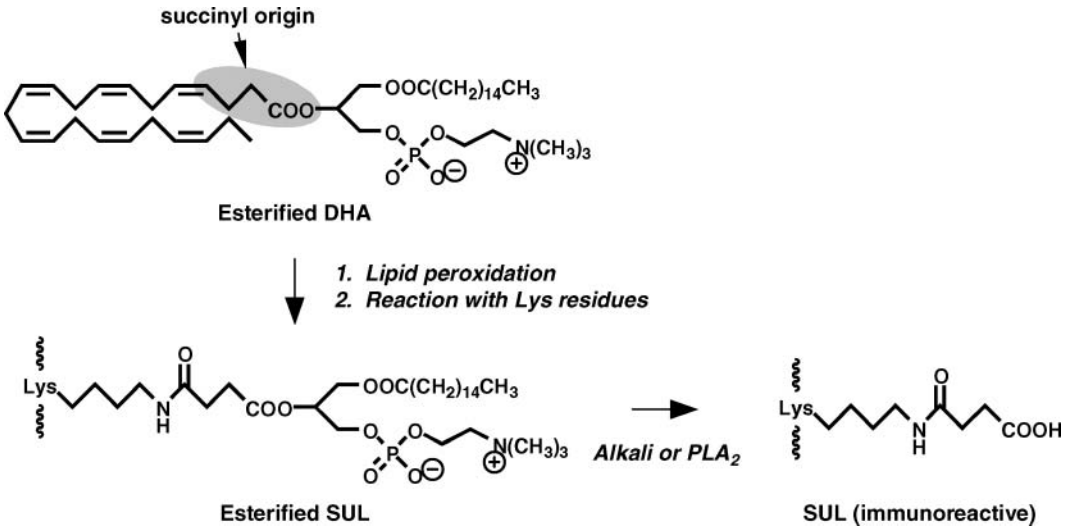
We have identified several *N*-acyl-type (amide-linkage-type) lysine adducts as the major products upon reaction with lipid hydroperoxides (5–9). The abundance of *N*-acyl adduct formation may be explained by our previous finding that the formation of *N*-acyl adducts is dependent on the lipid hydroperoxide levels rather than on aldehydic end-products in the reaction mixture (7). Indeed, the SUL formation paralleled the hydroperoxide levels in oxidized DHA in the reaction mixture (Fig. 6). The formation of *N*-acyl adducts was significantly enhanced in the presence of transition metals in the reaction mixture (7). In addition, *N*-acyl adducts could not be formed in an organic solvent such as chloroform and diethyl ether (Y. Kawai, K. Tsuji, T. Osawa, unpublished observations). These observations suggest that oxidative decomposition of lipid hydroperoxides may also be essential for the SUL formation. Although the precise reaction mechanism is still unknown, the formation of *N*-acyl adducts may be occurring in the initial stage in the decomposition pathway of lipid hydroperoxide to aldehydic/carbonyl end-products. Metz et al. (44) proposed a possible mechanism for the formation of *N*-acyl adduct involving α-dicarbonyl intermediates upon the reaction with PM, whereas the mechanism upon reaction with lysine remains unknown. To determine the reaction mechanism for the formation of *N*-acyl lysine adducts, we are now purifying the intermediates that generate *N*-acyl adducts in the decomposition mixture of a linoleic acid hydroperoxide.

We clearly revealed the formation of SUL during the peroxidation of DHA in vivo by using an animal experiment model for dietary enrichment of tissue DHA combined with a carbon tetracholoride-induced hepatic oxidative stress model. For in vivo experiments, we used an immunochemical technique using a novel monoclonal antibody (mAb2B12) raised against the SUL adduct. The antibody could distinguish the SUL adduct from other

oxidatively formed lipid-protein adducts, including the analogous *N*-acyl carboxylic adducts such as N^{ε}-(azelayl)-lysine and N^{ε}-(glutaryl)lysine (Fig. 7). We have reported the preparation of similar monoclonal antibodies that recognize similar *N*-acyl lysine adducts, *N*-hexanoyl and *N*-azelayl lysine (9, 37). Anti-hexanoyl monoclonal antibody, prepared by immunizing hexanoylated (six-carbon amide-linked) protein as immunogen, could not recognize hexanal (six-carbon aldehyde)-modified protein, showing that anti-hexanoyl antibody can differentiate amide-linkage adducts from imine (Schiff's base) adducts. Anti-SUL monoclonal antibody was prepared by immunizing succinylated protein (see Experimental Procedures). The results of these studies and our current characterization suggested that the mAb2B12 is highly specific to the SUL structure, including the amide-linkage moiety. Using the specific antibody, we showed the in vivo formation of the SUL adduct in the liver of mice fed with a DHA-enriched diet followed by oxidative stress induction with carbon tetrachloride. The results obtained from the animal experiment showed that *i*) DHA-enriched liver tissues increased the sensitivity to the lipid peroxidation reaction initiated by carbon tetrachloride injection, *ii*) DHA-enrichment alone could not initiate the lipid peroxidation reaction, and *iii*) the formation of SUL was observed paralleled with the lipid peroxidation levels in DHA-enriched tissues (Fig. 8). As stated above, the SUL adduct is the product specific to DHA-derived protein modification. Thus, these observations suggest that the formation of SUL may serve as one mechanism for the DHA-derived oxidative injury in DHA-enriched tissues. We have also shown the formation of analogous *N*-acyl (amide-linkage) adducts, such as N^{ε}-hexanoyl and N^{ε}-(azelayl)lysine (linoleic acid-derived adducts), in human urine (45) and oxidized LDL (Y. Kawai, S. Suzuki, T. Osawa, unpublished observations) by using HPLC-MS/MS. These observations indicate that in addition to immunochemical analysis using the specific antibodies, HPLC-MS/MS analysis is also a powerful tool for quantitative analysis of *N*-acyl adducts. The detection of the SUL adduct in tissues by using HPLC-MS/MS is now in progress in our laboratory.

For detecting the SUL adduct in vivo, we pretreated samples with phospholipase A_2 or alkali, which hydrolyze the fatty acid ester bonds, showing that SUL is formed as the esterified form linked with phospholipids in vivo (**Scheme 1**). It has been reported that oxidatively modified fatty acid esters in phospholipids are excluded by phospholipase(s), resulting in the release of free oxidized fatty acids (46, 47). However, the formation of a free carboxylic SUL adduct (i.e., immunoreactivity of nonhydrolyzed samples) was scarcely observed in our in vivo studies, suggesting that the adduct formation reaction occurred in the reaction of protein lysine residues with phospholipid intermediate(s) but not with free fatty acid intermediate(s). We have also previously shown similar results on the formation of N^{ε}-(azelayl)lysine and N^{ε}-(glutaryl)lysine adducts esterified with phospholipids/cholesteryl esters in oxidized LDL and/or atherosclerotic lesions using specific antibodies (5, 6, 8). Although the pathophysiological consequences of the formation of esterified *N*-acyl carboxylic adducts are still unknown, the formation of phospholipid-esterified peroxidation products and the subsequent protein modification during the oxidation of LDL have been suggested to be involved in the recognition by macrophages associated with atherosclerosis (48). In addition, the formation of 4-hydroxy-2-hexenal, a marker for the peroxidation of ω-3 polyunsaturated fatty acids, including DHA, has been reported in oxidized LDL and human atherosclerotic lesions (49). Although the formation of SUL in oxidized LDL has not yet been examined, these observations suggest that the peroxidation of DHA and subsequent protein modifications may be implicated, at least in part, in the pathogenesis of atherosclerosis. Examination for the implication of the SUL adduct in brain function is needed in future investigations.

In conclusion, we here demonstrated that SUL is a major DHA-derived protein modification product in vitro and characterized the in vivo formation using a specific



Scheme 1. Scheme for the endogenous formation of N^{ε}-(succinyl)lysine.

monoclonal antibody. SUL is a product specific to the peroxidation of DHA; therefore, the formation may be a useful marker for the peroxidation of DHA, modification of protein, and related dysfunction that occurs in DHA-enriched tissues.

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