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Detection in vivo of a Novel Endogenous Etheno DNA Adduct Derived from Arachidonic Acid and the Effects of Antioxidants on Its Formation

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Abstract

Previous studies showed that the 7-(1',2'-dihydroxyheptyl) substituted etheno DNA adducts are products from reactions with epoxide of (*E*)-4-hydroxy-2-nonenal (HNE), an oxidation product of ω -6 polyunsaturated fatty acids (PUFAs). In this work, we report the detection of 7-(1',2'-dihydroxyheptyl)-1,*N*⁶-ethenodeoxyadenosine (DHHedA) in rodent and human tissues by two independent methods: a ³²P-postlabeling/HPLC method and an isotope dilution liquid chromatography electrospray ionization tandem mass spectrometry method (ID-LC-ESI-MS/MS), demonstrating for the first time that DHHedA is a background DNA lesion *in vivo*. We showed that DHHedA can be formed upon incubation of arachidonic acid (AA) with deoxyadenosine (dA), supporting the notion that ω -6 PUFAs are the endogenous source of DHHedA formation. Because cyclic adducts are derived from the oxidation of PUFAs, we subsequently examined the effects of antioxidants, α -lipoic acid, Polyphenon E and vitamin E, on the formation of DHHedA and γ -hydroxy-1,*N*²-propanodeoxyguanosine (γ -OHPdG), a widely studied acrolein-derived

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Supplementary material

Supplementary experimental part and tables (S1-S3), Figure (S1, S2) can be found at doi:10.1016/j.freeradbiomed.2014.XX.XXX

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

adduct arising from oxidized PUFAs, in the livers of Long Evans Cinnamon (LEC) rats. LEC rats are inflicted with elevated lipid peroxidation and prone to the development of hepatocellular carcinomas. The results showed that while the survival of LEC rats increased significantly by α -lipoic acid, none of the antioxidants inhibited the formation of DHHedA and only Polyphenon E decreased the formation of γ -OHPdG. In contrast, vitamin E caused a significant increase in the formation of both γ -OHPdG and DHHedA in the livers of LEC rats.

Introduction

Lipid peroxidation (LPO), a consequence of oxidative stress from exposure to environmental pollutants or chronic inflammation, is an endogenous cause of protein and DNA damage (1,2). LPO has been implicated in the mechanisms for aging, cancers, neural disorders and other degenerative diseases. Upon oxidation by reactive oxygen species (ROS) or cyclooxygenases (COXs)/lipoxygenases (LOXs), ω -3 and ω -6 polyunsaturated fatty acids (PUFAs) generate highly reactive α,β -unsaturated aldehydes (enals) which can modify DNA bases by forming a variety of exocyclic DNA adducts (3-5).

Dietary ω -6 PUFAs have been strongly implicated in promoting cancers in experimental animals (6), although the evidence from epidemiological studies is less clear (7). The mechanisms underlying their tumor promoting effects are not yet fully understood. Arachidonic acid (AA) is one of the most abundant ω -6 PUFAs present in cellular phospholipid membranes. During oxidative stress and inflammation, AA is released from the membrane by phospholipases (8). AA can be oxidized by COXs and LOXs to yield eicosanoids that are believed to play a role in cancer development due to their growth stimulating activities (9). In addition, enzymatic and non-enzymatic oxidation of AA produces acrolein (Acr) and (*E*)-4-hydroxy-2-nonenal (HNE) as major end products which are capable of modifying DNA bases and cause mutations, therefore, may contribute to carcinogenesis. 1,*N*²-Propanodeoxyguanosine adducts of Acr and HNE (Acr-dG or OHPdG and HNE-dG) have been detected as background lesions in tissue DNA of rodents and humans (10,11). However, the *in vivo* levels of HNE-dG are often too low to be quantitatively detected by current methods (12). This limitation precludes its use as a specific biomarker of DNA damage caused by ω -6 PUFAs.

HNE is known to be epoxidized by various agents and enzymes, including *tert*-butyl hydroperoxide, hydrogen peroxide, fatty acid hydroperoxide and lipoxygenase, to 2,3-epoxy-4-hydroxynonanal (EH). EH is considerably more reactive than HNE toward DNA bases (13-15). Earlier chemical studies showed that EH reacts with deoxyadenosine (dA) and deoxyguanosine (dG) to form both the unsubstituted and substituted etheno adducts, like 1,*N*⁶-ethenodeoxyadenosine (edA) and 7-(1',2'-dihydroxyheptyl)-1,*N*⁶-ethenodeoxyadenosine (DHHedA), respectively (13,14). Although edA is a widely studied *in vivo* adduct, there has been no report on the detection *in vivo* of DHHedA as endogenous DNA lesion. In this study, we demonstrated the detection of DHHedA in tissues of rodents and humans and showed that its levels are considerably higher than that of HNE-dG, making it a potential DNA damage biomarker specific for ω -6 PUFAs. We also showed its

formation increases significantly in the presence of AA, suggesting AA is a possible endogenous source (Figure 1).

Because DHHedA and OHPdG are arisen from oxidation products of PUFAs, we further examined the effects of antioxidants, including α -lipoic acid, Polyphenon E, and vitamin E, on their level in the livers of Long Evans Cinnamon (LEC) rats that are inflicted with increased LPO in the livers due to abnormal copper accumulation, mimicking that of human Wilson's disease. As a result, LEC rats develop acute hepatitis, followed by chronic hepatitis, and eventually hepatocellular carcinomas (HCC) (16). α -Lipoic acid is an organosulfur antioxidant that accumulates in tissues upon dietary administration and is converted to dihydrolipoic acid (17). α -Lipoic acid and dihydrolipoic acid exhibit direct free radical scavenging properties (18,19). They also show antioxidant effects through chelating metal ions (e.g. Fe^{2+} and Fe^{3+}) and regenerating endogenous and exogenous antioxidants such as ubiquinone, glutathione (GSH) and ascorbic acid (20,21). Furthermore, various studies have shown that α -lipoic acid decreases LPO *ex vivo* (22) and *in vivo* (23). α -Lipoic acid has been used in treating several diseases including hepatic disorder (e.g. mushroom poisoning and alcoholic liver disease) and diabetes (18). α -Lipoic acid was recommended as one of the promising antioxidants for chemoprevention studies in a NCI sponsored screening report (24). Green tea has been shown to inhibit chemical-induced hepatocarcinogenesis *in vivo* (25,26). A cohort study showed that the consumption of green tea was associated with a reduced risk of human liver cancer incidence (27), although the collective evidence from epidemiological studies on the protective effect of green tea consumption against liver cancer is not conclusive (28). Polyphenon E is a well-defined mixture of decaffeinated green tea polyphenols including epigallocatechin gallate (EGCG), the most abundant and potent antioxidative catechin. Because of its highly controlled and reproducible formulation, it is a suitable form for clinical prevention trials. It has been shown that Polyphenon E can scavenge the stable free radicals generated from lipid peroxidation, it can inhibit tobacco carcinogen-induced lung tumor formation in mice (29) and chemically-induced bladder cancer (30). However, it is not known whether it can inhibit spontaneous hepatocarcinogenesis. Vitamin E is fat-soluble antioxidant and it inhibits carcinogenesis *in vivo* (31). It protects liver tissues against oxidative stress-induced chromosomal damage in transgenic mice with over-expressed *c-myc* gene and transforming growth factor- α (32). Vitamin E can inhibit liver dysplasia and adenomas and prevent malignant formation in these mice. However, it did not inhibit LPO in healthy humans (33). In fact, dietary supplementation of vitamin E has been shown to be associated with an increased risk of prostate cancer in healthy men, raising public health concerns about vitamin E supplementation (34-36). Nevertheless, a more recent work suggested that the different forms and mixture of tocopherols may play a key role in the cancer preventive effect of vitamin E (37).

Materials and methods

Chemicals and enzymes

Calf intestine alkaline phosphatase grade I was bought from Roche Diagnostic (Indianapolis, IN). Phosphodiesterase I from *Crotalus adamanteus* venom, deoxyribonuclease I type II

from bovine pancreas, dA, dA 3'-monophosphate (dA-3'-P), dA 5'-monophosphate (dA-5'-P), dG, dG 3'-monophosphate, micrococcal nuclease (MN), RNase A, RNase T1, protease, human placental DNA, calf thymus DNA, glycylaldehyde diethyl acetal, Amberlyst 15 ion exchange resin, dietary vitamin E (α -tocopherol), α -lipoic acid were obtained from Sigma-Aldrich Co. (St. Louis, MO). Polyphenon E was a generous gift from Dr. Hara (Mitsui Norin, Japan). [$^{15}\text{N}_5$]-dA was from Spectra Stable Isotopes (Columbia, MD). Spleen phosphodiesterase (SPD) was obtained from Boehringer Mannheim (Indianapolis, IN), T4 polynucleotide kinase (T4 PNK) was from U.S. Biochemicals (Cleveland, OH). [γ - ^{32}P]-ATP (specific activity 3000 Ci/mmol) was from Amersham (Arlington Heights, IL) and mung bean nuclease (MBN) was from Thermo Fisher Scientific (Fair Lawn, NJ). All other chemicals, solvents and reagents were from Sigma-Aldrich Co. (St. Louis, MO) or Thermo Fisher Scientific (Fair Lawn, NJ).

Animals

LEC rats at the age of 4 weeks were obtained from Charles River Japan, Inc. (3-19-5 Shinyokohama, Kouhoku-ku, Yokohama 222, Japan.). They were housed in a temperature-controlled light-regulated space with 12-hour light and dark cycles and were given unrestricted access to food and water throughout the experiments. The protocol used in this study was approved by Georgetown University Animal Care and Use Committee. The animals were fed with AIN-76A powder diets obtained from Dyets, Inc. (Bethlehem, PA) at the age of 4 weeks; four types of diets (control, α -lipoic acid, Polyphenon E and vitamin-E) were used in this study. The only difference between the diets is the sucrose content to be replaced by the antioxidant (Supplementary materials Table S1: the doses of the antioxidants, α -lipoic acid (2 g/kg), Polyphenon E (20 g/kg) and vitamin E (1.8 g/kg)), were selected according to recent studies respectively (25,26,33,38). The nutrition for all the four diets was calculated within the range of 3694 to 3766 kcal/kg. The body weights of LEC rats were measured weekly. LEC rats ($n = 3 - 5$) were sacrificed after 12 and 20 weeks. The liver tissues were dissected and kept at -80°C until usage for DNA isolation.

HPLC systems

Ten HPLC systems were used and the details are listed in the supporting information. Preparation of DHHedA 3'- and 5'-monophosphate for ^{32}P -postlabeling. HNE and EH were synthesized by previously described methods (39,40). For the preparation of DHHedA 3'-monophosphate (DHHedA 3'-P) and of DHHedA 5'-monophosphate (DHHedA 5'-P), 10 mg EH was dissolved in 100 μL DMSO and mixed with 10 mg of the nucleotides (dA-3'-P or dA-5'-P) in 100 mM sodium phosphate buffer (pH 7.2, total volume of 1.5 mL). The mixture was shaken at 37°C for 72 h and extracted with 3 mL chloroform to remove unreacted EH. Portions of the aqueous phase were analyzed by HPLC System 1 and adduct peaks were identified by retention times and UV spectra of standards. The peaks were collected, dried in SpeedVac and repurified using HPLC System 2. The purified DHHedA 3'-P standards were desalted using same SPE System as in "Detection and Quantitation of DHHedA by a HPLC-based ^{32}P -postlabeling assay in tissue DNA" section. Alternatively, the reactions can be performed with EH generated *in situ* from HNE and hydrogen peroxide (see "Synthesis of DHHedA and [$^{15}\text{N}_5$]-DHHedA for LC-MS/MS-MRM" section.), yielding the same two pairs of DHHedA isomers which were separated by HPLC System 1. Under

the conditions used, the yields of the second pair of isomers were 4 – 5 times higher than the first pair. By mixing approximately equal amounts of each pairs of isomers collected, the DHHedA 3'-P and DHHedA 5'-P were then used as standards in ³²P-postlabeling for labeling and UV reference, respectively.

Synthesis of the 5'-monophosphate of 7-hydroxymethyl substituted 1,N⁶-edA (HMedA)

Glycidialdehyde diethyl acetal (90% purity, 375 µL) was dissolved in tetrahydrofuran (THF):water (3:1, 2.25 mL) and mixed with Amberlyst 15 ion exchange resin (400 mg). The suspension was gently mixed overnight, filtered and the filtrate was added to dA-5'-P (15 mg) in sodium phosphate buffer (100 mM, pH 7.2, 3 mL). The resulting solution was shaken at 37 °C for 48 h. The reaction mixture was extracted with chloroform (3 × 3 mL) to remove the excess of glyceraldehyde and the aqueous phase was concentrated on SpeedVac and purified using HPLC System 4 yielding 20% of the product. The identity of the product was confirm by characteristic UV spectrum and co-elution with previously synthesized compound (39,41) using HPLC System 4. HMedA was then used as a standard for the detection of DHHedA in tissue DNA by the ³²P-postlabeling assay (see below).

Detection and quantitation of DHHedA by a HPLC-based ³²P-postlabeling assay in tissue DNA

DNA was isolated from tissues by a modified Marmur's procedure using chloroform-isomyl alcohol extraction and RNase A and T1 and protease treatment as previously described (42). DNA was dissolved in water, the purity was monitored by the 260/280 nm absorbance ratio (1.8-2.0) and stored at -80 °C until analysis. 50 to 100 µg DNA samples were digested by MN and SPD as previously described (10,11). DHHedA 3'-monophosphate (20 fmoles) together with 100 µg calf thymus DNA in duplicate) and one blank (100 µL water) sample were included in the assay of each set of DNA samples. Solid phase extraction (SPE) method was used to remove majority of the unmodified nucleotides in the MN/SPD digest before postlabeling. The Bond Elut C18, 100 mg, 1 mL volume SPE column (Agilent Technologies, Inc., Agilent, Santa Clara, CA formerly Varian, Harbor City, CA) was preconditioned with 3 mL methanol followed by 3 mL deionized water before loading the sample. Samples were loaded and the cartridges were washed with 3 mL 15% methanol/50 mM ammonium formate (pH 7.5). The adduct fraction eluted with 3 mL 1:1 water: methanol was collected and dried in a SpeedVac. The samples were treated with 10 units of MBN in 10 µL dilution buffer provided by the manufacturer and incubated for 60 min at 37 °C. The reaction was terminated by the addition of 3 µL Tris base (0.5 M). The samples were ³²P-postlabeled in the presence of 30 units T4 PNK and 20 µCi [γ-³²P]-ATP (60 min at 37 °C) as previously reported (10,11). To convert the labeled adduct bisphosphate to 5'-P, the labeled digest was mixed with 20 µL sodium acetate (0.5 M, pH 5) and 150 units of T4 PNK and incubated for 60 min at 37 °C. Majority of the radioactivity was removed by preliminary purification using the SPE method as outlined above. The fractions were dried, mixed with 5'-P UV standard and further purified sequentially by HPLC systems 1 and 2. A portion of the purified adduct was analyzed as DHHedA 5'-P on HPLC System 3. To confirm its identity, the purified adduct was converted to HMedA following a previously reported procedure (39,41). Briefly, purified ³²P-labeled DHHedA-5'-P from tissue DNA was dried, reconstituted in 0.5 mL water, mixed with 100 µL sodium metaperiodate (40

mg/mL) and stirred for 16 – 18 h at room temperature. To the reaction mixture, 100 μ L sodium borohydride (60 mg/mL) was added and stirred at room temperature for 60 min. The resulting mixture was neutralized by treating with 10 μ L phosphoric acid, mixed with authentic UV standard and analyzed using HPLC System 4. To determine the levels of modification, the radioactivity was adjusted for decay and procedural loss (recovery) and converted to fmol (10^4 dpm = 1 fmol) as previously described (10,11). To avoid contamination from radioactivity carried over to subsequent analysis, the system was washed and tested with blank and/or adduct UV standard before next analysis.

Synthesis of DHHedA and [$^{15}\text{N}_5$]-DHHedA for LC-MS/MS-MRM

HNE (24.4 mg) dissolved in 400 μ L of THF was mixed with 25 μ L of 30% hydrogen peroxide and stirred at room temperature for 1 h. The reaction mixture was then poured into 10 mg of [$^{15}\text{N}_5$]-dA or dA solubilized in 1:1 mixture of THF and 50 mM phosphate buffer pH 7.3. The resulting mixture was incubated with shaking at 50 $^{\circ}\text{C}$ for 24 h and the solvents were evaporated to dryness using SpeedVac. The crude material was re-dissolved in 50% aqueous methanol and analyzed on HPLC system 6. Adduct (two peaks) was identified based on retention times and characteristic UV spectra. DHHedA was purified on HPLC system 5 to yield two HPLC separable pairs of DHHedA stereoisomers (DHHedA 1,2 and 3,4). Standards for mass spectrometry were dissolved in water and quantified by UV spectroscopy using molar extinction coefficient $\epsilon = 5200 \text{ M}^{-1} \times \text{cm}^{-1}$ 278 (43). The identity of synthetic standard and internal standard was confirmed by high resolution mass spectrometry using QSTAR Elite time of flight mass spectrometer (Applied Biosystems, Foster City, CA). Expected masses for DHHedA: $[\text{M}+\text{H}]^+ = 406.20902 \text{ m/z}$, $[\text{M}-\text{dA}+2\text{H}]^+ = 290.1617 \text{ m/z}$; masses found: 406.2079 m/z and 290.1605 m/z). Expected masses for [^{15}N]-DHHedA: $[\text{M}+\text{H}]^+ = 411.1942 \text{ m/z}$, $[\text{M}-\text{dA}+2\text{H}]^+ = 295.1469 \text{ m/z}$; masses found: 411.1930 m/z and 295.1456 m/z).

Synthesis of α and γ -OHPdG, [$^{13}\text{C}_{10},^{15}\text{N}_5$]- α -OHPdG and [$^{13}\text{C}_{10},^{15}\text{N}_5$]- γ -OHPdG for LC-MS/MS-MRM

The synthesis of both OHPdG standards and the stable-isotope labelled internal standards was described in our earlier publication.(44)

DNA isolation and hydrolysis for LC-MS/MS-MRM

DNA samples from human and rat livers were isolated by a QIAGEN Blood and cell culture DNA Maxi Kit (QIAGEN, Hilden, Germany) using the tissue protocol as recommended by the manufacturer. For hydrolysis dry DNA (0.4 to 1 mg) was dissolved in 5 mM magnesium chloride and 0.5 mM GSH solution (1 mL per 1 mg of DNA) then 100 fmol of [$^{13}\text{C}_{10},^{15}\text{N}_5$]- α -OHPdG, 50 fmol of [$^{13}\text{C}_{10},^{15}\text{N}_5$]- γ -OHPdG, 50 fmol of [$^{15}\text{N}_5$]-DHHedA 1,2 and 50 fmol of [$^{15}\text{N}_5$]-DHHedA 3,4 were added as internal standards. DNA was hydrolyzed by incubation with DNase I (1300 units per mg of DNA) for 30 min at 37 $^{\circ}\text{C}$ followed by second addition of DNase I (1300 units per mg of DNA) and incubation for additional 10 min at 37 $^{\circ}\text{C}$. Finally phosphodiesterase I (0.06 units per mg DNA), alkaline phosphatase (380 units per mg DNA) and adenosine deaminase (0.5 units) were added and the sample was incubated for 60 min at 37 $^{\circ}\text{C}$. After hydrolysis small portion of hydrolysate was saved for further dG quantification and the remaining sample was purified using Phenomenex

Strata-X 33 μ 30 mg/1 mL polymeric reversed phase solid phase extraction columns (Phenomenex, Torrance, CA). Before loading samples columns were washed by ACN (3 $\times$ 1mL) and stabilized by 25 mM ammonium formate pH = 4.00 (3 $\times$ 1mL). After loading DNA hydrolysate columns were washed by 2.5% ACN in 25 mM ammonium formate pH = 4.00 (1 $\times$ 1mL) then OHPdG was collected by 5% ACN in 25 mM ammonium formate pH = 4.00 (1 $\times$ 1mL) followed by DHHedA collection by 30% ACN in 25 mM ammonium formate pH = 4.00 (1 $\times$ 1mL). OHPdG and DHHedA fractions were dried using SpeedVac rotary concentrator, re-dissolved in 400 μ L 1:1 water:ACN, transferred to HPLC vials, dried and kept at -20 °C. Before quantification samples were dissolved in 60 μ L of water and 37 μ L was injected on LC-MS.

Quantification of OHPdG in DNA by LC-MS/MS-MRM

Quantification was carried out on Applied Biosystems/MDS SCIEX 4000 QTRAP triple quadrupole mass spectrometer (Life Technologies Corporation, Carlsbad, CA) interfaced with an Waters ACQUITY UPLC liquid chromatography system equipped with Waters Acquity UPLC BEH C18 50 $\times$ 2.1 mm, 1.7 μ m particle size column (Waters Corporation, Milford, MA). The separation of adducts was performed isocratically by eluting with 3% ACN, 1 mM ammonium formate buffer over 3.5 min using 0.5 mL/min flow rate at 40 °C, followed by 100% ACN wash. The ESI source operated in positive mode. The MRM experiment was performed using ion transitions of 324.2 $\rightarrow$ 208.1 m/z (OHPdG) and 339.2 $\rightarrow$ 218.1 m/z ($[^{13}\text{C}_{10}, ^{15}\text{N}_5]$ -OHPdG) with a collision energy (CE) of 20 eV for quantification, and those of 324.2 $\rightarrow$ 190.1 m/z (OHPdG) and 339.2 $\rightarrow$ 200.1 m/z ($[^{13}\text{C}_{10}, ^{15}\text{N}_5]$ -OHPdG) with a CE of 47 eV were used for structural confirmation. All other parameters were optimized to achieve maximum signal intensity. Calibration curves were constructed for all three HPLC resolved isomers before each analysis using standard solutions of α - and γ -OHPdG and α - and γ - $[^{13}\text{C}, ^{15}\text{N}]$ -OHPdG. A constant concentration of $[^{13}\text{C}_{10}, ^{15}\text{N}_5]$ -OHPdG (1 fmol/ μ L) was used with different concentrations of OHPdG (1.68 amol/ μ L – 220 fmol/ μ L) and analyzed using 37 μ L injections by LC-MS/MS-MRM. The standard curves were linear in the range from 0.41 to 900 fmols of OHPdG on column for all three HPLC resolved isomers (1/x weighting; $r^2 = 0.9987$, $r^2 = 0.9988$ and $r^2 = 0.9994$ for OHPdG 1, 2 and 3 respectively). Measured limit of quantification (LOQ) was 0.41 fmol/column and limit of detection (LOD) was 0.14 fmol/column. The overall method detection limit (MDL) for DNA samples was calculated to be 5-10 fmol of each HPLC resolved isomers/sample.

Quantification of DHHedA in DNA by LC-MS/MS-MRM

Quantification was carried out using same instrument as for OHPdG adducts. The separation of adducts was performed isocratically by eluting with 13.5% ACN, 1 mM ammonium formate buffer over 6.5 min using 0.5 mL/min flow rate at 40 °C, followed by 100% ACN wash. The ESI source operated in positive mode. The MRM experiment was performed using ion transitions of 406.2 $\rightarrow$ 290.2 m/z (DHHedA) and 411.2 $\rightarrow$ 295.1 m/z ($[^{15}\text{N}_5]$ -DHHedA) with CE of 28 eV for quantification, and those of 406.2 $\rightarrow$ 160.1 m/z (DHHedA) and 411.2 $\rightarrow$ 165.0 m/z ($[^{15}\text{N}_5]$ -DHHedA) with a CE of 80 eV were used for structural confirmation. All other parameters were optimized to achieve maximum signal intensity. Calibration curves were constructed for two HPLC resolved peaks before each analysis using standard solutions of DHHedA and $[^{15}\text{N}_5]$ -DHHedA. A constant concentration of

[¹⁵N₅]-DHHedA (1 fmol/μL) was used with different concentrations of DHHedA (3.3 amol/μL – 65 fmol/μL) and analyzed using 37 μL injections by LC-MS/MS-MRM. The standard curves were linear in the range from 0.37 to 800 fmols of DHHedA on column for both peaks (1/x weighting; $r^2 = 0.9997$ and $r^2 = 0.9981$ for DHHedA 1,2 and 3,4 respectively). Measured LOQ was 0.37 fmol/column and LOD was 0.1 fmol/column for both peaks. The overall MDL for DNA samples was calculated to be 2-5 fmol of each HPLC resolved isomers/sample.

Quantification of dG in DNA hydrolysate

dG was quantified using HPLC System 10 with detection at 254 nm. Standard curve (from 5 nmol to 5 pmol of dG on column) was constructed using UV quantified dG standard ($\epsilon = 13700 \text{ M}^{-1} \times \text{cm}^{-1}$ 254 in water (45)).

Incubation of dA with AA

dA (10 mM) was incubated with AA (10 mM) and without AA (control) in 500 μL of Tris buffer (100 mM, pH 7.4) at 37 °C. After 16 h, the mixtures were extracted with 2 mL of chloroform and the aqueous phase was purified using SPE System as described above. After SPE, samples were dried on SpeedVac and finally DHHedA was quantified by the LC-MS/MS-MRM method as described above.

Statistical analysis

The results obtained in rats are expressed as means $\pm$ standard deviation throughout the article. The differences in adduct levels were analyzed for statistical significance using Student's *t* test, differences were considered significant when 2-tailed tests indicated values of $p < 0.05$. The *p* values in the survival data were calculated by using MedCalc software (MedCalc Software bvba, Ostend, Belgium).

Results

Detection of DHHedA in rodent and human tissues

HPLC-based ³²P-postlabeling method—Although DHHedA is a known product of the reaction of EH with dA, its formation in tissue DNA from rodents and human has not yet been reported. For the detection of DHHedA, DNA samples after enzymatic digestion and enriched by SPE were subjected to a HPLC-based ³²P-postlabeling assay. Although the recovery of the assay based on the adduct standard was only ~5%, this assay can detect as low as one fmol of DHHedA (Figure 2a-d). Two pairs of stereoisomers of DHHedA are formed due to two chiral carbons in the side chain. However, unlike DHHedG (46), the stereochemistry of DHHedA isomers has yet to be fully assigned. The HPLC chromatogram (Figure 2d) shows two major radioactive peaks from the colonic mucosa DNA of a Fischer rat. Although not completely resolved, they have the same retention times as the UV and ³²P-labeled standards of DHHedA 3 and 4 (Figure 2a and b, respectively). A small unresolved but discernible, radioactive peak was also detected in the DNA that co-migrated with DHHedA 1 and 2. No cross-contamination was noted as the blank sample (Figure 2c) showed no radioactive peaks. These results indicate that DHHedA adducts are present as endogenous lesions. Interestingly, there is stereo-selective formation of DHHedA 3 and 4 *in*

vivo as the recoveries of all 4 isomers based on the standards are comparable in this assay. To further confirm the identity of DHHedA detected *in vivo*, the adduct fractions were collected by HPLC and chemically converted to the corresponding short-chain 7-hydroxymethyl substituted edA (HMedA) with sodium metaperiodate followed by sodium borohydride addition. The HPLC chromatograms showed a single peak, due to the loss of the chiral carbons, having the same retention time as the UV standard of HMedA (Figure 2e, f, h). Using the recovery rate of simultaneously labeled DHHedA standards, the levels of modification by DHHedA in Sprague Dawley rat liver, Fischer rat colonic mucosa, human placenta and calf thymus DNA are estimated to be in the range of 3 to 960 adducts per 10^9 dG, (Table 1). These results indicate that DHHedA is present at a relatively high level in tissue DNA as a background modification (47).

LC-MS/MS method—To confirm the results from ^{32}P -postlabeling assay, a quantitative LC-MS/MS-MRM method was developed for detecting DHHedA level. Calibration curves for both chromatographically resolved pairs of DHHedA stereoisomers were constructed using synthetic standards and $^{15}\text{N}_5$ -DHHedA internal standards. Standard curves were linear between 0.37 and 800 fmols per injection ($r^2 = 0.9997$ and $r^2 = 0.9981$ for DHHedA 1,2 and 3,4, respectively). The method LOD was as low as 0.1 fmol/column with good LOQ around 0.37 fmol/column. DHHedA was detected as set of two peaks with retention time around 3.6 and 4.3 min respectively (Figure 3). The detection of the latter-eluting peak as the predominant adduct isomers is in agreement with its stereoselective formation observed using the ^{32}P -postlabeling method. Five human liver samples were analyzed. The levels of two resolved pairs of isomers, DHHedA 1,2 and DHHedA 3,4 were combined and reported as the total amount of DHHedA; the level in human livers and LEC rat livers are 27-63 adducts and 20-75 adducts per 10^9 dG respectively. Although the sensitivity of the LC-MS/MS method is not as high as that of the ^{32}P -postlabeling method, the high levels of DHHedA modification in tissue DNA permits its detection. Together, these results unequivocally demonstrated the presence of DHHedA *in vivo* as endogenous DNA lesions.

AA as a source of DHHedA

Previously, we reported that HNE-dG is formed by incubating dG with ω -6 PUFAs under oxidative conditions (3). In this work, a milder condition via autoxidation (no oxidizing agent was used) was chosen to investigate whether ω -6 PUFAs are source of DHHedA. We incubated dA with and without AA; the incubation mixtures were purified on SPE and analyzed by the LC/MS/MS method. DHHedA was detected at 507 ± 124 fmol in samples incubated with AA, whereas no DHHedA was found in samples incubated without AA. These results suggest that AA is a likely source of DHHedA *in vitro*.

Effects of antioxidants on DHHedA and γ -OHPdG in liver DNA of LEC rats

Both DHHedA and γ -OHPdG are formed from oxidized PUFAs. In order to examine the effects of antioxidants on the formation of DHHedA and γ -OHPdG *in vivo*, LEC rats were fed with three types of antioxidant (α -lipoic acid, Polyphenon E and vitamin E) diets. The body weights were monitored weekly for 20-week through the entire experiment (Figure S2). There was a 10-20% body weight loss after 10 weeks on α -lipoic acid, although no adverse effects were noted. At the end of the experiment, the weights were: 295.9 ± 24.0 g

(control); 274.3 ± 21.1 g (α -lipoic acid); 278.1 ± 13.4 g (Polyphenon E) and 280.3 ± 32.2 g (vitamin E) (Table S3). The survival curves of the four groups are shown in Figure 4. At termination (24-week-old or 20 weeks on antioxidant diet), the percentages of rats that survived were as follows: 47%, 83%, 56% and 39% for control, α -lipoic acid, Polyphenon E and vitamin E diet, respectively. There is a significant protective effect of α -lipoic acid against the mortality caused by acute hepatitis in LEC rats. The increased survival rate is highly significant compared to the control group ($p < 0.001$).

Three to five LEC rats fed with diets containing antioxidants were harvested at two time points; one at onset (16-week-old or 12 weeks on antioxidant diet) and the other during the phase of acute hepatitis (24-week-old or 20 weeks on antioxidant diet) for DNA isolation. Digested DNA samples were analyzed by LC-MS/MS for DHHedA and OHPdG content. As expected, γ -OHPdG was the only isomer detected in all samples (44). In the rats fed with control diet, there is no significant difference in the levels of both adducts between 12 and 20 weeks. Previous studies also showed that there was little difference in adduct levels between 12- and 20-week-old LEC rats in the levels of 8-oxo-dG and edA in the liver DNA (48,49). Among the groups fed with different antioxidant diets, a significant decrease of γ -OHPdG only in rats on Polyphenon E diet was observed. At week 12, the levels of γ -OHPdG were 67 ± 27 compared to 307 ± 133 per 10^9 dG in control group ($p = 0.03$). After 20 weeks, the levels were 106 ± 37 compared to 291 ± 62 per 10^9 dG in control ($p = 0.04$). However, the levels of γ -OHPdG were not affected by α -lipoic acid. Surprisingly, feeding vitamin E for 20 weeks caused a more than two-fold increase in γ -OHPdG from 291 ± 162 to 671 ± 158 per 10^9 dG ($p = 0.02$) (Figure 5A). None of the antioxidants showed significant inhibitory effects on DHHedA formation. Again, dietary vitamin E dramatically increased the levels of DHHedA by almost four-fold at week 20 ($p = 0.02$) from 23 ± 4 per 10^9 dG in control group to 90 ± 25 per 10^9 dG in treatment group (Figure 5B).

Discussion

The detection, formation, repair, mutagenicity, and potential roles in carcinogenesis of edG and edA as endogenous DNA lesions have been extensively studied since the 90s. Earlier chemical studies have demonstrated that both unsubstituted and DHH-substituted etheno adducts are formed in the reactions of EH, the epoxide of HNE, with dA and dG (50,51). These findings have led to the assumption that the formation of EH from epoxidation of HNE may be responsible for the etheno adducts (edA and edG) detected *in vivo* (50,51). In this study, we detected for the first time the DHH-substituted etheno dA in rodent and human tissues and found that they are present in DNA at relatively high levels. Furthermore, we demonstrated that AA is a potential source of its formation. These results provide support that epoxidation of HNE, a unique product of ω -6 PUFAs, may indeed constitute a pathway leading to the formation of the etheno adducts *in vivo*.

The alkyl substituted etheno adducts have emerged in recent years as novel endogenous DNA lesions. The heptanone-substituted etheno adducts, presumably derived from (*E*)-4-oxo-2-nonenal, were detected in the small intestine of Min mice (52). DHHedG and DHHedA were found in cultured human monocytes treated with HNE under peroxidation conditions (53). In that study, their levels were reported to be even lower than that of the

propano adducts formed directly from HNE. It was suggested that one reason for the poor yields of DHH adducts was the inefficient conversion of HNE to EH in cells. In this study, we demonstrated unequivocally that DHHedA is present in rodent and human tissues and found that DHHedA levels in tissues are in the range of one modification per 10^7 - 10^8 bases, comparable to that of γ -OHPdG and edA. The relatively high levels of DHHedA *in vivo* may be attributed to the conversion of HNE to highly reactive EH and/or its slow repair. The increased formation of DHHedA upon incubation with AA suggests that AA is a potential source for DHH adduct *in vivo* upon autoxidation.

Chronic inflammation, a risk factor for cancer, is known to be closely associated with LPO (54). 8-Oxo-dG is regarded as a general marker for oxidative stress, but it is not specifically formed by oxidized PUFAs; In fact, the mechanism of its formation has been disassociated with LPO-induced DNA damage (55). The malondialdehyde-derived cyclic adduct is likely formed primarily from the base propenal, a major enal generated by oxidation of the ribose moiety of DNA, not from lipids (56). While acrolein and crotonaldehyde are known oxidation products of PUFAs, both are ubiquitous environmental pollutants (cigarette smoke, automobile exhaust etc.). The etheno modifications, such as edA and edG, can also be formed from exposure to environmental carcinogens, like vinyl chloride and ethyl carbamate. Therefore, these adducts are not suitable markers of lipid-derived DNA damage. It has been suggested that the cyclic 1,*N*²-propano-dG adduct of HNE, a unique oxidation product of ω -6 PUFAs, would serve as a specific biomarker of LPO-induced DNA damage. However, its application is compromised by the low *in vivo* level. Little is known about the biological role of the DHH adducts, however, the other structurally related etheno adducts, e.g. the heptanone-etheno adduct of deoxycytidine have been shown to be highly mutagenic in both *E. coli* and human cells (57,58). The fact that DHHedA is a unique DNA lesion from ω -6 PUFAs that presents in tissue at relatively high levels renders its usefulness as a specific biomarker of lipid peroxidation and its role in cancer promotion associated with ω -6 PUFAs may be further exploited.

Excessive LPO induced by inflammatory processes can cause DNA damages which may eventually cause cancer (4). It is known that antioxidants can inhibit LPO (59) and carcinogenesis (60-62). We previously demonstrated that EGCG and α -tocopherol have an opposite effect toward γ -OHPdG formation from ω -3 PUFAs under oxidative conditions *in vitro*; the former is an effective blocker, whereas the latter enhances its formation (63). Of the three antioxidants used in this study, only Polyphenon E significantly inhibited the formation of γ -OHPdG in the livers of LEC rats at the onset of and during acute hepatitis. These findings agree with the previous *in vitro* studies, consistent with its potential activity in scavenging ROS, aldehydes, and chelate metal ions, including Cu^{2+} (64,65). The increased survival rate by α -lipoic acid, yet its failure to reduce the adduct levels, is intriguing, suggesting a mechanism other than LPO-induced adducts is likely involved in its survival. These results did confirm a previous report that α -lipoic acid can ameliorate the mortality of copper-induced acute hepatitis in LEC rats (66). Surprisingly, none of the antioxidants showed any inhibitory effects against DHHedA formation in the livers of LEC rats. Similar to the previous *in vitro* studies, vitamin E caused a significant increase of γ -OHPdG and DHHedA in the livers of LEC rats (63). It is known that vitamin E possesses

both antioxidant and prooxidant properties, depending on its concentrations and the reaction conditions (67). Because cyclic DNA adducts may play a role in carcinogenesis (48), the increase of both γ -OHPdG and DHHedA by feeding a diet containing vitamin E raises concerns that underscores the importance of understanding their biological roles in liver cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We demonstrated the detection of DHHedA *in vivo* for the first time, and AA is a likely source of DHHedA.
- DHHedA is a potential DNA damage biomarker specific for ω -6 PUFAs.
- Within the three antioxidants (α -lipoic acid, Polyphenon E and vitamin E) used in this study, only Polyphenon E significantly inhibited the formation of γ -OHPdG in the livers of LEC rats at the onset of and during acute hepatitis. These findings agreed with the previous *in vitro* studies, consistent with its potential activity in scavenging ROS, aldehydes, and chelate metal ions, including Cu^{2+} .

Figure 1.

Proposed mechanisms of the formation of the cyclic adducts studied in this work induced by PUFAs through hydroperoxy fatty acids (FAOOH): α -OH-1, N^2 -propano-2'-deoxyguanosine (α -OHPdG), γ -OH-1, N^2 -propano-2'-deoxyguanosine (γ -OHPdG), 1, N^6 -ethenodeoxyadenosine (edA), 7-(1',2'-dihydroxyheptyl)-1, N^6 -ethenodeoxyadenosine (DHHedA), and trans-4-hydroxy-2-noenal-deoxyguanosine (HNE-dG).

Figure 2.

Detection of DHHedA in Sprague Dawley rat liver DNA by the HPLC-based ^{32}P -postlabeling method: (a) UV standards, (b) ^{32}P -postlabeled standards, (c) blank, and (d) rat liver DNA. Confirmation of DHHedA detected *in vivo* by converting DHHedA to HMedA by sodium metaperiodate/sodium borohydride: (e) UV standards, (f) ^{32}P -postlabeled standards, (g) blank, and (h) rat liver DNA. Similar results are obtained with DNA from Fischer rat colonic mucosa, human placenta and calf thymus.

Figure 3.

Detection of DHHedA in LEC rat liver and human liver DNA by the LC-MS/MS method. Three double panels show mass chromatograms from three experiments. Top one shows mass chromatograms for the synthetic DHHedA standard ($406.2 \rightarrow 290.2$ m/z) and [$^{15}\text{N}_5$]-DHHedA internal standard ($411.2 \rightarrow 295.1$ m/z); 29.7 fmol of each DHHedA 1,2 and 3,4 standards and 37 fmol for each [$^{15}\text{N}_5$]-DHHedA 1,2 and 3,4 internal standards. The middle panel presents mass chromatograms obtained from DNA of normal human liver (4.8 fmol of DHHedA 1,2 and 12.1 fmol of DHHedA 3,4 detected). The bottom panel presents mass

chromatograms for DNA from LEC rat liver (12.1 fmol of DHHedA 1,2 and 21.1 fmol of DHHedA 3,4 detected).

Figure 4.

The survival curves of LEC rats with different antioxidant diets (α -lipoic acid, Polyphenon E and vitamin E) and control diet throughout 20 weeks of bioassay.

(A)

(B)

Figure 5.

γ -OHPdG (A) and DHHedA (B) levels detected by the LC-MS/MS method in LEC rats liver after feeding different antioxidant diets (α -lipoic acid, Polyphenon E and vitamin E) and control diet for 12-week and 20-week, * $p < 0.05$.

Table 1

Tissue (n = 3 – 6)^a	DHHedA/dG × 10⁹
Sprague Dawley rat liver ^b	3 – 150
Fischer rat colonic mucosa ^b	10 – 960
Human placenta ^b	20 – 30
Calf thymus ^b	10 – 370
Human Liver ^c	27 – 63
LEC rat liver ^c	20 – 75

^a n is sample number;

^b ³²P-postlabeling-HPLC method;

^c LC-ESI-MS/MS-MRM method