Deoxyguanosine Adducts of t-4-Hydroxy-2-nonenal Are Endogenous DNA Lesions in Rodents and Humans: Detection and Potential Sources

Fung-Lung Chung, Raghu G. Nath, Joseph Ocando, Akiyoshi Nishikawa, and Lei Zhang

Division of Carcinogenesis and Molecular Epidemiology, American Health Foundation, Valhalla, New York 10595 [F-L. C., R. G. N., J. O., L. Z.], and Division of Pathology, National Institute of Health Sciences, Tokyo 158, Japan [A. N.]

Abstract

4-Hydroxy-2-nonenal (HNE) is a free radical-mediated oxidation product of polyunsaturated fatty acids. As an electrophile, HNE readily binds to proteins and yields diastereomeric cyclic $1,\alpha_2\beta_2$-propano adducts with deoxyguanosine (dG). Here, we report the detection and identification of the HNE-derived cyclic $1,\alpha_2\beta_2$-propano-dG adducts as endogenous DNA lesions in tissues of untreated rats and humans using a highly sensitive $32\text{P}$-postlabeling method in conjunction with high-performance liquid chromatography. These adducts were first verified by their comigration with the synthetic UV standards of HNE-dG adducts. Subsequently, their identities were unequivocally established by two independent reactions. An ~37-fold increase in the levels of HNE-dG adducts was observed in the liver DNA of F344 rats after treatment with CCl$_4$, suggesting that tissue lipid peroxidation is a likely source of their formation. Our studies in vitro further indicate that $\omega_6$-polyunsaturated fatty acids are likely a unique class of fatty acids involved in HNE-dG adduct formation.

Introduction

A prevailing thesis on the causes of aging-related diseases, such as cancer and brain disorders, has been the generation of reactive oxygen radicals by cellular oxidative pathways. The reactive oxygen species can directly or indirectly, through the generation of reactive compounds, cause damage to proteins and genetic materials in cells (1). A potentially important family of such compounds comprises the $\alpha,\beta$-unsaturated aldehydes (enals) formed by peroxidation of unsaturated fatty acids (2, 3). Enals are reactive bifunctional chemicals that modify proteins and nucleic acid bases via Michael addition (4–8). Enals react with DNA bases, yielding cyclic adducts with a new propano ring moiety (6–8). We previously have reported that the cyclic DNA adducts derived from the environmentally ubiquitous short-chain enals, Acr and Cro, are detected in tissues of rodents and humans (9, 10). Although studies have shown that endogenous lipid peroxidation appears to be a major source (11), exposure to environmental Acr and Cro also is likely to contribute to the formation of these cyclic adducts. Unlike Acr and Cro, HNE is a long-chain enal that appears to be produced specifically by peroxidation of $\omega_6$ PUFA's (2, 3). As a major lipid peroxidation product, HNE has been widely studied for its biochemical and physiological activities (12).

HNE generated from low-density lipoprotein and its subsequent binding to apolipoprotein have been implicated in the pathogenesis of atherosclerosis (13–15). It can modify proteins by cross-linking via thiol conjugation and Schiff's base formation (16–20) and can inactivate P450 cytochromes, particularly 2E1 and 1A1 (21). Immunochemical assays have demonstrated that HNE-bound proteins are present in cells and tissues (22, 23) and that their levels are increased under oxidative conditions, as found in the neurodegenerative brain of Parkinson's and Alzheimer's patients (24–28).

HNE was shown to have antiproliferative activity on tumor cells (29, 30). It modulates the expression of genes that are involved in cell cycle and apoptosis; however, the underlying molecular mechanisms are not understood (31, 32). Upon reaction with dG, HNE yields two pairs of diastereoisomers, HNE-dG 1,2 and 3,4 (Fig. 1; Ref. 8). Until now there has been no evidence for the presence of HNE-derived cyclic DNA adducts in vivo. In this study, we report the detection of four isomeric $1,\alpha_2\beta_2$-propano-dG adducts of HNE in the liver and colon DNA of rats without treatment with a carcinogen and in the liver and colon DNA of humans, using a highly sensitive $32\text{P}$-postlabeling/HPLC method. We also show that these adducts are formed as a result of peroxidation of lipids and that $\omega_6$ PUFA's are likely to be an important source for their formation.

Materials and Methods

Detection and Identification of HNE-dG Adducts in Vivo

DNA was isolated by a modified Marmur's procedure (33). The purity of the DNA was monitored by the 260/280 nm absorbance ratio (>1.8), and DNA was stored at $-80^\circ$C until analysis by a $32\text{P}$-postlabeling method reported previously with some modifications (9, 10). Briefly, 7.5–60 mg of DNA were hydrolyzed by micrococcal nuclease and phosphodiesterase (Sigma, St. Louis, MO). The hydrolysate was diluted in 0.9 ml of 0.23 M ammonium formate (pH 7.0). An aliquot was taken for quantifying dG 3'-monophosphate by HPLC system 1 (see below). A blank sample, consisting of 50 mg of poly(dA·dC):poly(dG·dT), was used as control to ensure that the assay is free of contamination and artifacts. To remove the normal nucleotides, the hydrolysate was applied to a preconditioned (washed with 10 ml of methanol, then 10 ml of H$_2$O) C$_{18}$ solid-phase extraction cartridge (Sep-Pak Plus; Waters Corporation, Milford, MA) and washed with 5 ml of 50 mm ammonium formate (pH 7.0), followed by 5 ml of 10% methanol in 50 mm ammonium formate (pH 7.0). The fraction containing the 3'-monophosphates of HNE-dG adducts was eluted with 5 ml of water:methanol (1:1, v/v). The eluent was dried under vacuum, reconstituted in water, and treated with nuclease P1. The sample was $32\text{P}$-postlabeled with [γ-$32\text{P}$]ATP and T4 PNK (40 min at 37°C), followed by treatment with apyrase (0.06 units for 20 min at 37°C). To determine recovery, 20 fmol of HNE 3'-dGMP adducts were diluted in 0.9 ml of ammonium formate, mixed with normal nucleotides, and processed in an identical manner. The labeled mixture was subjected to one-dimensional polyethyleneimine
cellulose TLC (20 × 20 cm sheets with a wick), developed with 2.25 M NaH₂PO₄ (pH 3.5) for 16–18 h, and autoradiographed. Each adduct spot, identified by comparison to simultaneously labeled HNE-dG 3'-monophosphate standard, was excised and extracted, mixed with synthetic HNE-dG 3',5'-bisphosphate UV standard, purified by reversed-phase HPLC systems 2 and 3, and finally analyzed on HPLC system 4. The adduct levels were calculated from the radioactivity of comigrating peaks after adjusting for recovery from the simultaneously labeled standards. The identities of HNE-dG adducts were further confirmed by comigration of the radioactive peaks with the UV markers served as an indication of their presence (Fig. 2). For confirmation of identities of the comigrating peaks as HNE-dG adducts, they were subjected to either ring-opening/reduction with NaBH₄ or T₄ PNK treatment to remove 3'-phosphate. The resulting products from these reactions, N²-[1-(2-hydroxyethyl)-2-hydroxyheptyl]dG 3',5'-bisphosphates (Ring-opened adducts) and HNE-dG 3'-monophosphates, respectively, again comigrate on HPLC with each of the corresponding UV markers (Fig. 3). The structures of the N²-alkyl substituted ring-opened products were characterized by proton NMR and mass spectrometry (see “Materials and Methods”).

Enzymatic Hydrolysis. The fraction containing HNE-dG 3',5'-bisphosphates collected before final analysis was purified using HPLC system 5 to remove the phosphate in the buffer. The collected fraction was then reconstituted in 100 μl of H₂O and treated with 7 μl of T₄ PNK in 200 μl of 1 M sodium acetate buffer (pH 5) with 60 μl of the kinase buffer (9, 10). The mixture was incubated at 37°C for 1 h. After incubation, the mixture was analyzed by HPLC system 4.

Chemical Ring-Opening Reduction. HNE-dG adducts 1,2 and 3,4 (a total of 100 μg) were dissolved in 200 μl of aqueous NaOH (0.01N). An excess of NaBH₄ was added to the solution. The reaction mixture was incubated for 1 h at 37°C, followed by adjustment to pH 7.0 with 1N HCl. Reversed-phase HPLC analysis (system 5; see above) of the reaction mixture showed three new peaks eluted before HNE-dG adducts. The UV λ₂₅₄ 256 and 283nm of these products are characteristic of an N²-alkyl substituted dG. The structures were determined by mass spectra (electrospray ionization/positive ion, [M + 1]; m/z 426) and by ¹H-NMR spectra as N²-[1-(2-hydroxyethyl)-2-hydroxyheptyl]dG. After acid hydrolysis with 1N HCl at 90°C for 45 min, the corresponding ring-opened guanine derivatives were identified by their mass (electrospray ionization/positive ion, [M + 1]; m/z 310) and ¹H-NMR. The ¹H-NMR results for the guanine derivatives from HNE-dG 1 and 2 were as follows (DMSO-d₆, δ): 12.50 (d, 1, N-9-H); 10.50 (d, 1, N-1-H); 7.68 (s, 1, C-8-H); 6.36 (d, 1, N²-H); 4.92 (d, 1, CHO₂); 4.57 (s, 1, CH₂OH); 3.91 (d, 1, NIH); 3.44–3.60 (m, 2, CH₂OH); 3.00 (m, 1, CHO₂H); 1.77 (m, 1, HOCH₂CH₂); 1.51 (m, 1, HOCH₂CH₂); 1.99–1.48 (m, 8, CH₂CH₂CH₂CH₂); 0.89 (t, 3, CH₃).

The 3',5'-bisphosphates of HNE-dG adducts 1,2 and 3,4 were ring-opened under the same conditions to give four peaks by HPLC system 4 (see below). The sequence of elution of the ring-opened adducts, however, was different.

5 Peaks disappeared after D₂O treatment.
6 bs, broad singlet; s, singlet; m, multiplet; t, triplet.
7 Collapsed to doublet doublet when irradiated at 1.77 ppm.
8 Collapsed to doublet when irradiated at 1.48 ppm.
from that of the parent adducts. At the bisphosphate level, HNE-dG 1 and 2 were eluted as the second and third peaks after ring-opening, respectively, and HNE-dG 3 and 4 were eluted as the first and fourth peaks, respectively. The identities of the ring-opened adducts were confirmed by treatment with alkaline phosphatase to convert to the corresponding nucleosides.

**Treatment with CCl₄** Fifteen 13-week-old male F344 rats were divided into three groups of 5 animals. CCl₄ was administered i.p. at a dose of 3.2 g/kg body weight. Rats in the control group were treated with vehicle (olive oil) only. Animals in the treated groups were sacrificed 24 and 72 h after dosing; animals in the control group were sacrificed 24 h after dosing. A portion of the liver was harvested for DNA isolation for the analysis of HNE-dG adducts as described above.

**Incubation with Fatty Acids in Vitro.** The incubations were carried out with a mixture of 1.5 mM fatty acids and 25 mM dG 5'-monophosphate in the presence of 0.75 mM FeSO₄ in 1 ml of 0.1 M Tris-HCl buffer (pH 7.1). Fatty acids were dissolved in methanol before addition to the reaction mixture. The mixture was incubated at 37°C for 30 min and then 80°C for another 30 min. After extraction with CH₂Cl₂, the mixture was analyzed using a sequential reversed-phase HPLC method (system 7 for initial purification, and then system 8 for identification and quantification).

**HPLC Systems.** HPLC systems were as follows: The HPLC was performed on a Waters system, equipped with two Model 510 pumps, a Model 660 solvent programmer, and a Waters 994 Photodiode array detector or a Waters system 8 for identification and quantification.

**Results and Discussion**

The ³²P-postlabeling/HPLC method, developed by modifying the previous assay (9), entails the following key steps: (a) the diphosphate is hydrolyzed enzymatically to nucleoside 3'-monophosphates; (b) the hydrolysate is purified on a C₁₈ solid-phase extraction column to obtain the fraction enriched with HNE-dG; (c) the collected fraction is ³²P-postlabeled, followed by a one-dimensional TLC; (d) the area corresponding to the adduct spot on TLC is excised and the radioactivity extracted; (e) the extract is purified by two separate HPLC methods; and (f) the purified HNE-dG fraction is analyzed by a reversed-phase HPLC for identification by comigration with the HNE-dG UV standards. For analysis of each set of samples, a sample of poly(dA·dC):poly(dG·dT) was used as a blank control. Using this method, we detected HNE-dG adducts in the DNA of liver and colon of rats and humans as indicated by the comigration of radiolabeled peaks with the UV standards of HNE-dG adducts (Fig. 2). Additional experiments were performed to verify their identities. In the first experiment, the ³²P-labeled HNE-dG 3',5'-diphosphate adducts were collected from the HPLC and hydrolyzed to the corresponding 5'-monophosphates by T₄ PNK; and in the second experiment, the HPLC-collected HNE-dG 3',5'-bisphosphates were treated with alkali followed by sodium borohydride to yield the ring-opened derivatives of N²-[1-(2-hydroxyethyl)-2-hydroxyethyl]dG. These conversion reactions are illustrated in Fig. 1. The HPLC analyses show that the products of both of these reactions again comigrated with the corresponding UV reference compounds, as shown in Fig. 3. Because the ring-opening reaction could occur only with the HNE-dG adducts, the comigration of the products with the corresponding synthetic standards unequivocally established their identities.

The ³²P-postlabeling/HPLC method used in this study is capable of detecting as low as one adduct in 1 billion DNA bases. Although this method is highly sensitive, quantitation by this method is compromised by relatively high variability due to multiple steps and lack of an internal standard. On the basis of the recovery of external standards, the concentrations of HNE-dG adducts in DNA are estimated to be in the range of 3–9 nmol/mol guanine in rat and human tissues.
glutathione (34). It is also possible that HNE-dG adducts are repaired efficiently. At present, no information is available on the repair of these adducts. The repair of other related cyclic adducts appears to involve base and nucleotide excision (35–37).

The detection of the HNE-dG adducts in rats without carcinogen treatment and in normal human tissues suggests that they are of endogenous origin. To determine whether tissue lipid peroxidation is involved in the formation of HNE-dG adducts, F344 rats were treated with a single dose (3.2 g/kg body weight) of CCl4 known to induce lipid peroxidation (38), and liver DNA obtained 24 and 72 h after dosing was analyzed. We found that the levels of HNE-dG adducts were increased 37-fold in the CCl4-treated rats 24 h after CCl4 dosing was analyzed. We found that the levels of HNE-dG adducts were increased 37-fold in the CCl4-treated rats 24 h after CCl4 treatment compared with those in the control animals (104 ± 12 nmol/mol guanine versus 2.8 ± 1.8 nmol/mol guanine; P = 0.0006). Furthermore, these adducts appeared to be persistent in liver under the treatment conditions because significantly high levels of adducts (88 ± 81 nmol/mol guanine; P = 0.008 compared with control and P = 0.006 compared with 24 h) were still present 72 h after dosing. These data support that lipid peroxidation is an important endogenous source for their formation.

Because it has been reported that HNE appears to be an oxidation product of ω-6 PUFAs (2, 3), the specific roles of ω-6 PUFAs in HNE-dG adduct formation were also investigated. Peroxidation of each fatty acid AA (ω-6), LA (ω-6), DHA (ω-3), and SA (saturated), was initiated by incubating FeSO4 at 37°C in the presence of dG 5′-monophosphates under aerobic conditions; the reaction mixtures were analyzed by reversed-phase HPLC. The HPLC chromatograms obtained from the analysis of the incubation mixture with AA are presented in Fig. 4a, showing the formation of HNE-dG adducts under these conditions. The yields of HNE adducts with different types of fatty acids are shown in Fig. 4b. AA appears to be a major source, producing a total of 20.6 μmol of HNE-dG adducts, whereas LA yielded 3.5 μmol. In contrast, DHA and SA failed to produce any detectable levels of HNE-dG adducts. These results clearly demonstrated the specific role of ω-6 PUFAs in the formation of HNE-dG adducts. Because high intake of dietary ω-6 PUFAs has been implicated in tumor promotion in laboratory animals and has been linked with increased risk of certain human cancers, these results warrant a closer examination of a potential role of HNE-dG adducts in tumor promotion caused by ω-6 PUFAs. In this context, it would be important to know whether the HNE-dG adducts are mutagenic. Circumstantial evidence supporting this possibility include the following: (a) HNE is mutagenic in mammalian cells (39); and (b) site-specific mutagenesis studies have shown that a model 1,3-propano-dG adduct without the alkyl side chain and hydroxyl group on the propano ring causes frame shift or base mispairing (G-to-T and G-to-A mutations; Ref. 40, 41). To date, tumor bioassays in laboratory animals have shown that HNE is at best a weak tumor-initiating agent (42). It is tempting to speculate based on available data that HNE-induced DNA adducts may be involved in tumor promotion because oxidative reactions are believed to be associated with this process (43).
HNE DEOXYGUANOSINE ADDUCTS ARE ENDOGENOUS DNA LESIONS

In summary, our study shows that HNE-DG adducts are present as background DNA lesions in rodent and human tissues, and appear to originate from tissue lipid peroxidation. Evidence supports a role of ω-6 PUFAs in their formation. These results further suggest that cyclic DNA adduction by enals constitutes a common reaction in vivo and that the cyclic propano adducts detected to date may represent only a fraction of this type of DNA damage in tissue DNA.

Acknowledgments

We thank Steve Shiff (Rockefeller University, New York, NY) and Regina Santella (Columbia University, New York, NY) for providing human colon biopsies and liver samples, and Shantu Amin (the Organic Synthesis Facility, American Health Foundation) for providing HNE.

References

Deoxyguanosine Adducts of t-4-Hydroxy-2-nonenal Are Endogenous DNA Lesions in Rodents and Humans: Detection and Potential Sources


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/60/6/1507

Cited articles

This article cites 40 articles, 19 of which you can access for free at:
http://cancerres.aacrjournals.org/content/60/6/1507.full.html#ref-list-1

Citing articles

This article has been cited by 17 HighWire-hosted articles. Access the articles at:
/content/60/6/1507.full.html#related-urls

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.