Formation of Cyclic Deoxyguanosine Adducts from ω-3 and ω-6 Polyunsaturated Fatty Acids under Oxidative Conditions

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The discovery of the cyclic 1,N^2-propanodeoxyguanosine adducts of acrolein (Acr), crotonaldehyde (Cro), and *t*-4-hydroxy-2-nonenal (HNE) as endogenous DNA lesions from lipid peroxidation has raised questions regarding the role of different types of fatty acids as sources for their formation. In this study, we carried out reactions at pH 7 and 37 °C with deoxyguanosine 5′-monophosphate and ω-3 polyunsaturated fatty acids (PUFAs), including docosahexaenoic acid (DHA), linolenic acid (LNA), and eicosapentaenoic acid (EPA); or ω-6 PUFAs, including linoleic acid (LA) and arachidonic acid (AA), each in the presence of ferrous sulfate. The formation of Acr, Cro, and HNE-derived 1,N^2-propanodeoxyguanosine adducts (Acr-, Cro-, and HNE-dG) in the incubation mixture was determined by reversed-phase HPLC analysis. The results showed that Acr and Cro adducts are primarily derived from ω-3 PUFAs, although Acr adducts are also formed, to a lesser extent, from oxidized AA and LA. HNE-dG adducts were detected exclusively in incubations with AA. The kinetics of the formation of these adducts was determined during incubations for 2 weeks and 5 days. The rate of Acr adduct formation was about 5−10-fold that of Cro adducts, depending on the type of PUFAs, and the rate of formation of HNE adducts from AA was also considerably slower than that of Acr adducts. Unlike other cyclic adducts, the formation of Acr adducts was independent of types of PUFAs, but its yield was proportional to the number of double bonds in the fatty acid. Only one of the isomeric Acr adducts was detected, and its stereoselective formation is consistent with that observed previously in vivo. Two previously unknown cyclic adducts, one derived from pentenal and the other from heptenal, were also detected as products from ω-3 and ω-6 fatty acids, respectively. This study demonstrated the specificity for the formation of the cyclic adducts of Acr, Cro, and HNE and other related enals by oxidation of ω-3 and ω-6 PUFAs. These results may be important for the understanding of the specific roles of different types of fatty acids in tumorigenesis.

Introduction

The detection of cyclic DNA adducts in tissues as background lesions has raised questions regarding the origin of these modified bases (*1*−*3*). Cumulative evidence supports the hypothesis that the reactions of DNA bases with α,β-unsaturated aldehydes (enals) produced by oxidation of unsaturated fatty acids constitute an important endogenous pathway for the formation of this type of DNA damage (*3*−*5*). 1,N^2-Propanodeoxyguanosine (1,N^2-propanodG) adducts are reaction products of deoxyguanosine (dG)[1] and enals, such as acrolein (Acr), crotonaldehyde (Cro), and t-4-hydroxy-2-nonenal (HNE), and etheno adducts are products of the epoxides of enals (*4*). Studies in rodents have demonstrated that the formation of the cyclic DNA adducts is increased in the livers of rats administered CCl_4 or in the livers of Long Evans Cinnamon rats which are inflicted with increased lipid peroxidation as a result of genetically predisposed copper accumulation in that tissue (*5*−*7*). Furthermore, depletion of glutathione in the liver of rats treated with buthione sulfoximine results in a significant increase of 1,N^2-propanodG adducts (*8*). Data from studies in rodents, although limited, also suggest that a higher level of 1,N^2-propanodG adducts occur in tissues of high fat content, such as brain, liver, and colon, compared with other tissues (*9*). Studies in humans showed that intake of dietary fats increases the levels of these adducts in lymphocyte DNA (*10, 11*). Perhaps, the most direct evidence comes from studies in vitro demonstrating that the cyclic DNA adducts, both propano and etheno, are formed upon incubation with microsomes or unsaturated fatty acids under oxidative conditions (*6, 12*).

While all of these studies support the notion that fatty acids in tissue are important sources for the formation of endogenous cyclic DNA adducts, the relative contribution of different types of fatty acids in the formation of these cyclic adducts has not yet been examined. In this study, we determined the specific role of different types of fatty acids, ω-3 and ω-6 polyunsaturated fatty acids (PUFAs), in the formation of 1,N^2-propanodG adducts of

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[1] Abbreviations: dG, deoxyguanosine; Acr, acrolein; Cro, crotonaldehyde; HNE, *trans*-4-hydroxy-2-nonenal; EH, 2,3-epoxy-4-hydroxy nonenal; Pen, *trans*-2-pentenal; Hep, *trans*-2-heptenal; Hex, *trans*-2-hexenal; Oct, *trans*-2-octenal; PUFA, polyunsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LNA, linolenic acid; AA, arachidonic acid; LA, linoleic acid; SA, stearic acid.

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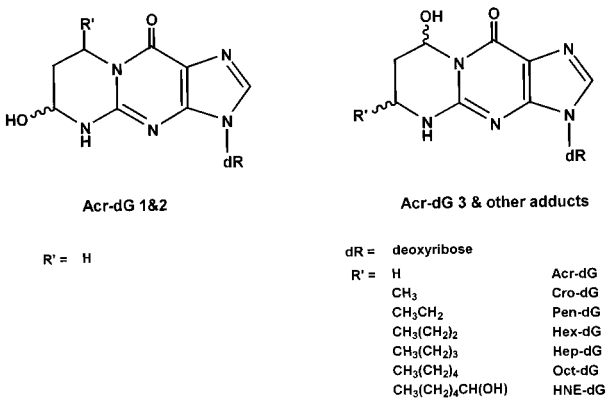


Figure 1. Structures of the cyclic 1,N^2-propanodG adducts studied. Acr-dG 1 and 2 and Acr-dG 3 are regio-isomers as a result of opposite ring-closure previously characterized (*14*). The OH and R′ on the propano ring are in trans configuration (*4*).

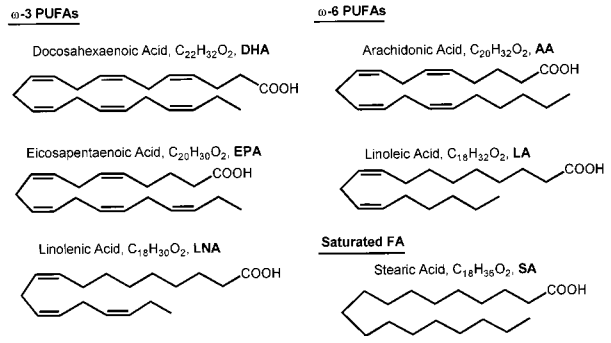


Figure 2. Structures of ω-3 and ω-6 PUFAs and stearic acid.

Acr, Cro, and HNE (Acr-, Cro-, and HNE-dG, Figure 1). These cyclic adducts have been found in vivo in untreated rodents and in humans as background DNA lesions (*6*, *9*). Fatty acids were incubated with dG 5′-monophosphate in a pH 7 buffer at 37 °C in the presence of Fe(II). The fatty acids studied include docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and linolenic acid (LNA) as ω-3 PUFAs, arachidonic acid (AA) and linoleic acid (LA) as ω-6 PUFAs, and stearic acid (SA) as saturated fatty acid (Figure 2). The Acr, Cro, and HNE adducts formed were identified and quantified; the kinetics of the formation of each adduct was also determined. Two new cyclic 1,N^2-propanodG adducts were also detected and their structures were characterized.

Materials and Methods

Chemicals. DHA, EPA, LNA, AA, and LA were purchased from Cayman Chemical Company (Ann Arbor, MI). SA, dG 5′-monophosphate, Acr, Cro, *trans*-2-pentenal (Pen), *trans*-2-hexenal (Hex), *trans*-2-heptenal (Hep), and *trans*-2-octenal (Oct) were obtained from Sigma-Aldrich Co. (St. Louis, MO), and HNE was synthesized by a previously described method (*13*). All other reagents, otherwise stated, are from Sigma-Aldrich Co. (St. Louis, MO) and Fisher Chemical (Fair Lawn, NJ).

Synthesis of the Adduct Standards. The 5′-monophosphates of Acr-dG, Cro-dG, HNE-dG, and the substituted 1,N^2-ethenodG from the epoxide of HNE were prepared as previously described (*14*−*16*). A similar method was used to prepare the Pen-dG, Hex-dG, Hep-dG, and Oct-dG 5′-monophosphate standards. Briefly, 200 μg of the enal were added to a 5 mL solution containing 2 mg/mL dG 5′-monophosphate in 100 mM Tris-HCl (pH 7.0). The reaction mixture was placed in a shaker at 37 °C for 2 days, followed by extraction twice with 5 mL of $CHCl_3$ to remove the unreacted aldehyde. Adducts were purified using HPLC Systems 5 and 4 sequentially. The identities of these adduct standards were established by their characteristic UV spectrum and further confirmed by their mass spectra (electrospray ionization/positive ion). The mass spectrometry was performed on a Finnegan Mat TSQ-700 spectrometer connected with a Shimadzu SCL-10A VP HPLC solvent delivery system. The samples were analyzed by direct infusion with 70% acetonitrile as the mobile phase and at a flow rate of 0.2 mL/min [M + Na]: *m*/*z* 374, 388, 402, and 416 for nucleoside of Pen-, Hex-, Hep-, and Oct-dG, respectively.

Incubation of dG 5′-Monophosphate with Fatty Acids in the Presence of $FeSO_4$. Fatty acids were dissolved in 100 μL ethanol before they were added to a reaction mixture containing 1 mg/mL (3.0−3.5 mM) PUFA, 14.4 mM dG 5′-monophosphate, 5 mM $FeSO_4$ in 10 mL of 0.1 M Tris-HCl (pH 7.1) buffer. The reaction mixture was incubated for 19 days at 37 °C. The control experiments involve incubations carried out under nitrogen. At different time intervals, an aliquot (1 mL) was taken and filtered through an Acrodisc syringe filter. The filtrate was then extracted with 2 mL of $CHCl_3$ by shaking vigorously followed by centrifugation at 1000*g*, and 0.7 mL of the aqueous layer was carefully removed by pipetting for HPLC analysis.

Quantification of Adducts by HPLC. The aqueous sample obtained above was concentrated to 0.1−0.2 mL and applied to the HPLC System 1 for purification of adduct peaks. Fractions corresponding to all of the adducts studied were collected according to the retention times and UV spectra of the synthetic standards. The collected fractions were dried and reconstituted in 100 μL of H_2O before being reanalyzed on the HPLC System 2 for quantification of Acr-, Cro-, and Pen-dG, and System 3 for Hex-, Hep-, Oct-, and HNE-dG. For quantification, 20 μL of 3.0 mM Cro-dG was used as a standard by applying to the same sequential HPLC analysis, and the peaks from the second HPLC were integrated for quantification.

Confirmation of Identities of Adducts. The identity of adducts in the incubation mixture was confirmed by co-injecting with the adduct standard. Identical UV and comigration of the adduct with the standard were taken as the initial evidence of identity. The identity of the adduct was further confirmed using the ring-opening/reduction reaction, a characteristic of 1,N^2-propanodG adducts (*6*). Briefly, the pH of the collected adduct fraction from the second HPLC was adjusted to 11−12 by adding 50 μL of 0.1 N NaOH, followed by adding an excess of $NaBH_4$. The reaction mixture was incubated at room temperature for 15 min and then neutralized to pH ∼7 with 0.1 N HCl and followed by analysis using the same HPLC system for quantification. The ring-opened standards of Pen-, Hex, Hep-, and Oct-dG adducts were established by their UV and mass spectra as described above [M + Na]: *m*/*z* 376, 390, 404, and 418, respectively. Comigration and the UV spectra identical to those of the standards were taken as final confirmation of identities.

HPLC. The HPLC system consists of two LC-10AD VP pumps, a SCL-10A VP controller, and a SPD-M10A VP photodiode array detector (Shimadzu, Kyoto, Japan) with Prodigy ODS 3 C18 reversed-phase column (5 μm, 250 mm × 4.6 mm) from Phenomenex (Torrance, CA), eluted with a linear gradient program at a flow rate of 1 mL/min. The solvent systems used are as follows: System 1: (A) 50 mM NaH_2PO_4 (pH 7.4), (B) 50% methanol; the solvent gradient was 0 to 25% B in 25 min, followed by 25 to 100% B in 20 min, and finally at 100% B for 10 min. Systems 2, 3, and 4: (A) 50 mM NaH_2PO_4 (pH 5.8), (B) 50% methanol. The solvent gradients were 0 to 45% B in 45 min for System 2, 50 to 100% B in 50 min for System 3, and 0 to 100% B in 50 min for System 4. In System 5, A was 25 mM triethylamine phosphate (pH 6.5), B was 50% methanol, and the gradient was 0 to 100% B in 100 min.

Results

Figure 3 shows typical HPLC chromatograms obtained from analysis of adduct standards (upper panel) and the

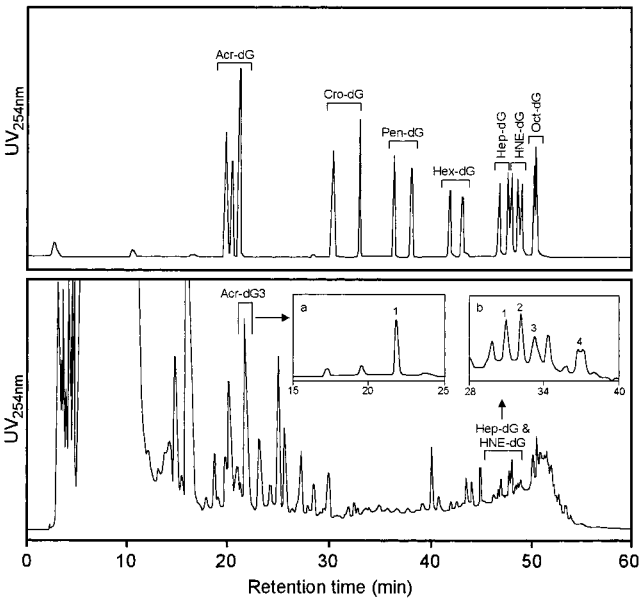


Figure 3. HPLC chromatograms obtained using HPLC System 1 from analysis of synthetic adduct standards (upper panel) the reaction mixture with AA after incubation for two and a half weeks at pH 7.1 and 37 °C (bottom panel). On the basis of their retention times and UV spectra, fractions corresponding to the synthetic standards of all the adducts were collected and reanalyzed by a second HPLC (see Materials and Methods). Inset a shows the detection of Acr-dG 3 (peak 1) using HPLC System 2; and inset b shows the detection of Hep-dG 1 and 2 adducts (peaks 1 and 2, respectively) and HNE-dG adducts 1, 2, and 3, 4 (peaks 3 and 4, respectively), using HPLC System 3.

incubation mixture of dG 5′-monophosphate with AA (bottom panel). The fractions corresponding to the retention times of Acr-dG (19.7, 20.2, and 20.9 min), Cro-dG (30.4 and 32.9 min), Pen-dG (36.4 and 38.0 min), Hex-dG (41.9 and 43.1 min), Hep-dG (46.8 and 47.7 min), HNE-dG (48.0, 48.8 and 49.1 min), and Oct-dG (50.2 and 50.4 min) in HPLC System 1 were collected and reanalyzed using a second HPLC system for identification and quantification. Acr-dG, Hep-dG, and HNE-dG adducts were detected in the corresponding fractions (inset, Figure 3). In addition to their comigration and identical UV with the synthetic standards, the identities of these adducts were further verified by a chemical conversion involving a ring-opening reaction followed by $NaBH_4$ reduction (*6*). Figure 4 shows the HPLC comigration with the standard of the ring-opened product of Acr-dG collected from incubation with AA. Among the isomers of Acr-dG adducts, only Acr-dG 3 was detected. However, no stereoselective formation of Cro-dG, Hep-dG, or HNE-dG adducts was noted.

The double bonds in fatty acids are essential for the formation of Acr-, Cro-, and HNE-dG, as no adducts were detected upon incubation with SA under the same conditions. The ω-3 PUFAs, DHA, EPA, and LNA, are the main sources for Acr-dG adducts, although AA and LA can also form Acr-dG adducts. The order of formation of Acr adducts from the fatty acids studied is DHA > EPA > AA > LNA > LA (Figure 5a). Cro-dG adducts are detected only from ω-3 fatty acids, and their yields are several-fold lower than those of Acr-dG. In contrast to the short-chain enals, the long-chain HNE-derived dG adducts are exclusively produced via oxidation of ω-6 PUFAs, especially AA. Since HNE is also a known oxidation product of LA, it is surprising that HNE-dG adducts were not detected from LA. It is possible that

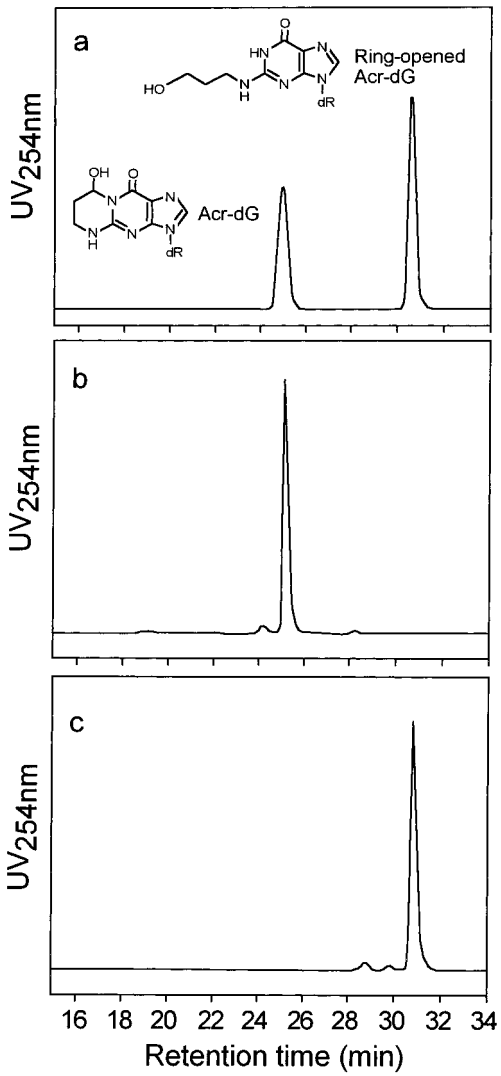


Figure 4. Confirmation of identities of Acr-dG adducts by ring-opening/reduction with $NaBH_4$ at pH 11−12. (a) UV standards of Acr-dG 3 and its ring-opened product. (b) Acr-dG 3 collected from incubation with AA as analyzed using HPLC System 2. (c) Conversion to a product that comigrated with the ring-opened product after reaction with $NaBH_4$ at pH 11−12.

they were formed at yields too low to be detected by the method used.

Two previously unidentified 1,N^2-propanodG adducts, Pen- and Hep-dG adducts, were detected in these reactions. The former is found in the incubation with ω-3 PUFAs, whereas the latter from AA. The identities of these newly detected 1,N^2-propanodG adducts were initially confirmed by comparing their UV spectra and retention times with those of the synthetic standards previously characterized by their UV and mass spectra and were further verified by the ring-opening/reduction reaction with $NaBH_4$. The yields of Pen- and Hep-dG were comparable to those of Cro-dG and HNE-dG adducts from ω-3 and ω-6 PUFAs, respectively, and were considerably lower than that of Acr-dG. The only adducts detected from LA were Acr-dG adducts, and the yields were considerably lower than those from other fatty acids, including AA. The dG adducts with characteristic UV spectra of 1,N^2-ethenodG adducts were also detected. The major adducts were from AA eluted near HNE-dG adducts at 48 min and from ω-3 PUFAs eluted slightly later than Cro-dG adducts at 35.8 min in HPLC System

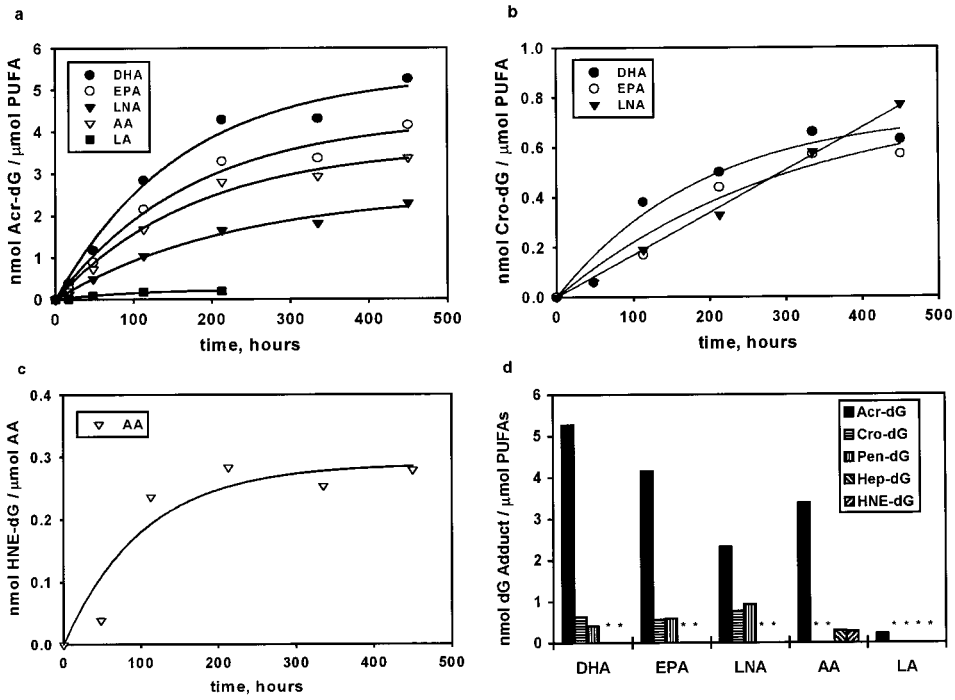


Figure 5. Kinetics of formation of (a) Acr-dG, (b) Cro-dG, and (c) HNE-dG adducts upon incubation with ω-3 and ω-6 PUFAs and (d) total yields of each type of adducts from PUFAs after incubation was completed. The asterisks indicate that the adducts were not detected.

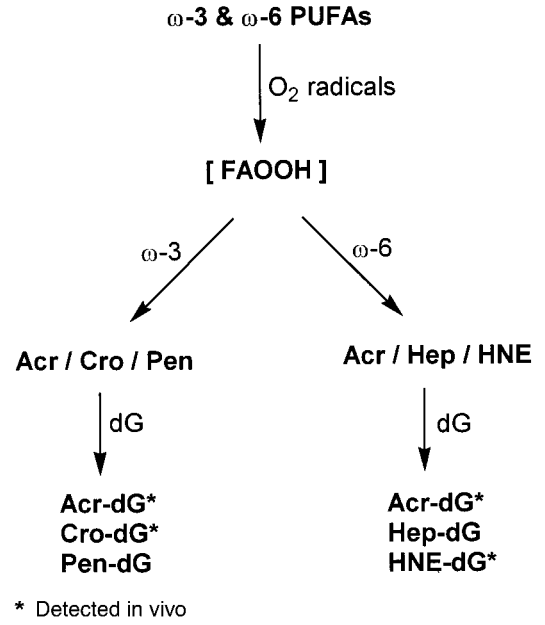
1. These adducts were collected and reanalyzed and their retention times in HPLC Systems 2 and 3, respectively, indicated that they are not the unsubstituted and dihydroxyheptyl substituted 1,N^2-ethenodG from reaction with the epoxides of Acr, Cro, or HNE characterized previously (*17*). The structures of these etheno adducts were not further elucidated in this study.

During the 19-day incubation, the kinetics of the formation of adducts from each fatty acid was determined. Both the rates and yields of the formation of Acr adducts were much greater than those of Cro and HNE. Figure 5 shows the kinetics of formation of Acr, Cro-dG, and HNE adducts. In general, the rates and yields showed a near linear increase for Acr-dG and Cro-dG for the first 100 h of incubation, followed by a reduced rate of formation. The formation of HNE-dG appeared to plateau after day 6.

Discussion

This study demonstrates the formation of cyclic DNA adducts from the oxidation of different types of PUFAs. The short-chain cyclic adducts Acr-, Cro-, and Pen-dG are derived primarily from ω-3 fatty acids, whereas, the long-chain Hep- and HNE-dG adducts are formed exclusively from ω-6 fatty acids. The sources of PUFAs and enals for the formation of these cyclic adducts are outlined in Scheme 1. Enals are oxidation products of PUFAs (*18*). As expected, the enal-derived cyclic adducts were detected only from incubation of fatty acids with multiple double bonds; no adducts were detected from SA, a saturated fatty acid. Consistent with the high reactivity and yield of Acr, Acr-dG is the predominant product from both ω-3 and ω-6 PUFAs. Acr-dG adducts are the only adducts studied which are formed from both ω-3 and ω-6 PUFAs. The formation of Acr-dG adducts appears to be proportional to the number of double bonds in PUFAs, suggesting a pathway involving initial attacks by oxygen

Scheme 1. Sources of PUFAs and Enals for the Formation of Acr-, Cro-, Pen-, Hep-, and HNE-dG



radicals at the methylene carbons between double bonds. The similar patterns of formation of Cro- and Pen-dG adducts from DHA, EPA, and LNA and Hep- and HNE-dG adducts from AA (Figure 5d) also suggest a common pathway for the formation of Cro and Pen by ω-3 PUFAs and Hep and HNE by ω-6 PUFAs. Pentenal is likely formed via an intermediate of hydroperoxide at C18, C16, and C14 carbon in DHA, EPA, and LNA, respectively, whereas the mechanism of the formation of heptenal is less obvious and may involve oxidation at C16 of AA. Little or no adduct was formed in the incubations under anaerobic conditions.

As a unique and major oxidation product of ω-6 PUFAs, HNE has been extensively studied for its chemical and

biological activities (*18*, *19*). In addition to its strong binding affinity to proteins, HNE-dG adducts have been detected recently in the DNA of rodent and human tissues (*6*). Mechanisms for HNE formation from LA and AA via iron-mediated oxidation or by lipoxygenase have been proposed and investigated (*20*, *21*). Esterbauer et al. reported previously that, in the presence of iron, AA yielded 10 times more HNE than LA (*18*). The present study showed that HNE-dG adducts were detected from AA but not from LA. At a higher temperature the incubation of LA did yield detectable amounts of HNE-dG adducts, but the yield from LA was 6-fold less than AA (*6*). The lower yields of HNE and its dG adducts from LA compared with those from AA again indicate that the two additional double bonds in AA may facilitate the production of HNE. We did not detect HNE-dG adducts upon incubation of AA in the presence of soybean lipoxygenase, possibly due to low yields of HNE from AA by enzymatic oxidation. It is likely that adducts formed vary under different oxidative conditions. The qualitative and quantitative differences in the formation of HNE from lipoxygenase or iron-mediated oxidation of AA by lipoxygenase or iron have yet to be elucidated.

The kinetics of the formation of each adduct from PUFAs is dictated by both the yield of enal and its reactivity toward dG. Acr is a major product of both ω-3 and ω-6 and is the most reactive enal; the reactivity of enals toward dG decreases with the increasing chain length Acr > Cro > Pen > Hep > HNE (unpublished results). It was reported previously that, upon oxidation by Fe^{2+} and H_2O_2, ω-6 PUFAs yielded comparable amounts of Acr and HNE as major products (*23*). However, the reactivity of HNE toward dG is much lower than Acr, thus, the yields of HNE-dG adducts were considerably less than those of Acr-dG adducts. The low yields of Cro-, Pen-, and Hep-dG are also consistent with the low yields of the respective enals and their relatively low reactivity. As another example, we did not detect the Oct-derived dG adducts in the incubation mixture, even though it has been reported that Oct is one of the major enals from oxidation of ω-6 PUFAs (*23*). It is conceivable that the reactivity of Oct is too low to form detectable amounts of adducts.

The preferential formation of Acr-dG 3 over the other isomers, Acr-dG 1 and 2, from oxidized PUFAs is intriguing. Our early studies showed that upon reaction of Acr with dG, three isomeric adducts are formed, designated as Acr-dG 1, 2, and 3 (Figure 1). Adducts 1 and 2 possess a propano ring with the hydroxyl group adjacent to the N^2 of guanine, whereas the Acr-dGuo 3 is a regio-isomer resulting from ring closure of the opposite direction. Interestingly, Acr-dG 3 was also the predominant adduct in DNA detected in vivo using the ^{32}P-postlabeling/HPLC method (*9*). The results of the present study again show the preferential formation of Acr-dG 3, corroborating the role of tissue fatty acids toward the regioselective formation of Acr-dG adducts. Alternatively, acrolein is not directly involved in the formation of Acr-dG 3; instead, a yet unidentified related compound is the actual reactant. The chemical basis for the stereoselectivity needs further investigation. Isomers of Cro-dG and HNE-dG adducts are formed by Michael addition via the same regiochemistry as that for Acr-dG 3; no stereoselectivity was observed in their formation from oxidized PUFAs.

The long incubation time was chosen for two reasons: to mimic in vivo conditions and to increase the yields of adducts for better detection and quantification. The kinetics studies indicate that, in general, there is a linear increase of adduct formation during the first 150 h, followed by an apparent decrease in the rate of formation or a plateau. Since these adducts are relatively stable under the reaction conditions, these observations suggest several other possibilities: degradation of fatty acids, depletion of Fe^{2+}/Fe^{3+}, or hydrolysis of dG 5′-monophosphate during incubation. The latter two possibilities were supported by the observations that there was a slow precipitation of salts and the appearance of dG detected by HPLC during the long incubation. Furthermore, a background level of Acr-dG adducts was detected in the reaction of DHA without the addition of Fe^{2+}, indicating that the autoxidation of PUFAs could also contribute to the formation of these adducts in the presence of trace amounts of transition metals in the incubation mixture.

Our previous studies have shown that HNE can be epoxidized by several pathways, including autoxidation, H_2O_2, or fatty acid hydroperoxides and the epoxides can subsequently react with dG to form 1,N^2-ethenodG and the heptyl substituted 1,N^2-ethenodG (*16*). The 1,N^2-ethenodG adduct detected in the reactions with AA, however, did not comigrate with the synthetic etheno adduct standards. Although the structure of the adduct was not fully characterized in this study, it is plausible that it may be related to the 2-oxo-heptyl substituted 1,N^2-ethenodG adduct from 4-oxo-2-nonenal reported by Rindgen et al. (*22*). The detection of the ethenodG adducts and the newly identified Pen- and Hep-dG from ω-3 and ω-6 PUFAs suggests that these cyclic adducts may also be present in tissue DNA.

Bioassays in animals have shown that ω-6 PUFAs promote tumorigenesis at various sites, including colon and mammary glands, whereas, ω-3 PUFAs are protective (*24*). These results are consistent with the observations from epidemiological studies (*25*). However, the underlying mechanisms for the contrasting effects of the two types of fatty acids are yet to be fully understood. The present study demonstrates distinct patterns of cyclic adduction by short-chain vs long-chain enals from different PUFAs. With the exception of Acr, ω-3 PUFAs give rise to the cyclic adducts of short-chain enals ($C < 5$), whereas ω-6 PUFAs yield the cyclic adducts of long-chain enals ($C > 7$). It is not known whether the specific patterns of adduct formation contribute to the differential effects of these fatty acids. Recent studies reported that Acr-dG 3 in DNA, due to its facile ring opening, does not cause mispairing of bases and is a nonmutagenic lesion in vivo (*26*−*28*). The mutational characteristics of the other cyclic adducts need to be determined in order to fully assess their potential roles in tumorigenesis.

The results of this study emphasize the potential roles of different types of dietary fat toward the formation of cyclic DNA adducts in vivo. Consumption of a diet high in LA or PUFAs has been shown to cause an increase of the etheno and malondialdehyde-derived adducts in human leukocyte DNA (*10*, *11*). These particular adducts, however, do not allow us to distinguish their origins between ω-3 and ω-6 PUFAs, since they are reaction products of aldehydes from both types of fatty acids. The formation of varied cyclic 1,N^2-propanodG adducts from ω-3 and ω-6 PUFAs suggests their application as specific biomarkers in the study of the relative contribution of different types of PUFAs in DNA damage and possibly tumorigenesis.

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