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COMMENTARY

Lipid peroxidation as a potential endogenous source for the formation of exocyclic DNA adducts

Fung-Lung Chung[1], Hauh-Jyun Candy Chen and Raghu G.Nath

Division of Carcinogenesis and Molecular Epidemiology, American Health Foundation, 1 Dana Road, Valhalla, NY 10595, USA

[1]To whom correspondence should be addressed

A number of promutagenic exocyclic DNA adducts have recently been detected in both humans and rodents without carcinogen treatment. These observations raised questions about their origins and potential significance in carcinogenesis. In this commentary, we present our views pertaining to the *in vivo* sources of these cyclic adducts, specifically the cyclic propano and etheno adducts. The basis for our discussion comes mainly from the information generated through a span of more than a decade from several laboratories, including ours. This commentary summarizes the data from the chemical and biochemical studies that provide support for the hypothesis that lipid peroxidation is involved in the endogenous formation of these exocyclic adducts.

Introduction

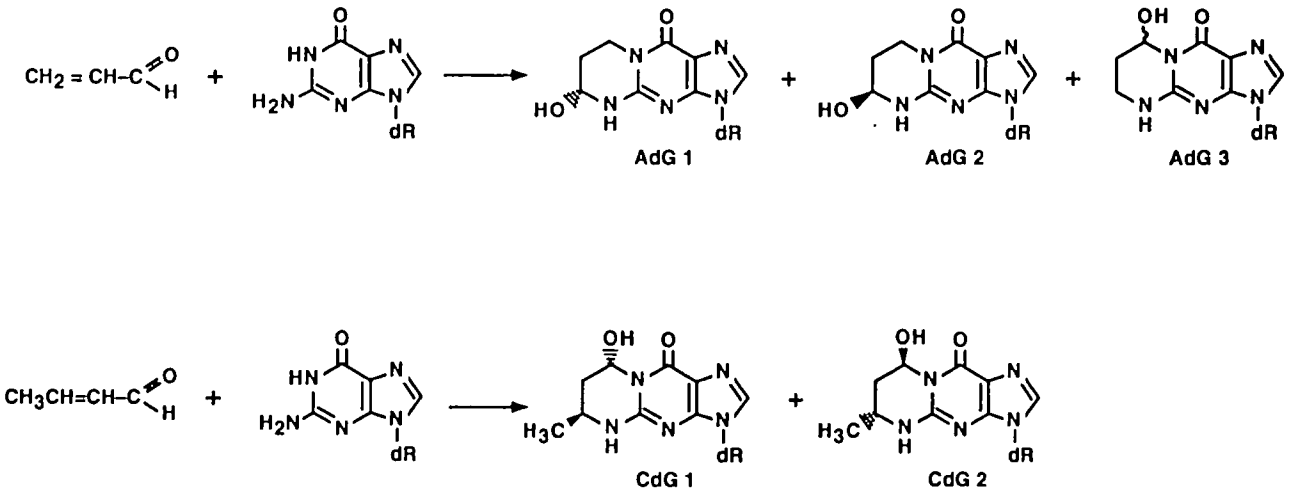
Recently, several studies reported the detection of exocyclic propano, etheno and malondialdehyde adducts in DNA from various tissues of rodents without carcinogen treatment and from humans (1–5). These findings raised questions about the origin of their formation. Since malondialdehyde is a distinct product of lipid peroxidation, our discussions focus only on the propano and etheno adducts (3). 1,N^2-Propanodeoxyguanosine adducts have been detected in a variety of tissues of untreated rats and mice and in human liver, mammary glands and leukocytes (1,2). The etheno adducts of guanine, adenine and cytosine are present as background DNA lesions in liver and other tissues obtained from rodents and humans (4,5). The ubiquity of these exocyclic adducts in tissues of different species suggests that these adducts are potential endogenous DNA lesions. Their potential significance in mutagenesis and carcinogenesis has been studied. Immunochemical studies have shown that 1,N^2-propanodeoxyguanosine adducts are formed in *Salmonella* tester strains TA100 and TA104 upon incubation with acrolein under conditions that induce revertants (6). Evidence for the mutagenicity of these DNA adducts has also been provided by site-specific mutagenesis studies in which exocyclic propano and etheno adducts were shown to induce various types of mutation, including base substitutions and frameshift mutations in bacterial and mammalian hosts (7–11). Studies have shown that etheno adducts in DNA can be repaired in cultured bacterial and mammalian cells, however, little is known about repair of the propano adducts (12,13). The etheno and propano adducts have been detected in target tissues of rodents treated with carcinogens such as vinyl chloride, ethyl carbamate, crotonaldehyde and *N*-nitrosopyrrolidine (14–19). These results suggest that these cyclic adducts are involved in carcinogenesis. In this commentary, our aim is to address the potential endogenous sources for the formation of these adducts. Our discussions, based on the evidence presented, have led us to conclude that lipid peroxidation is involved in the endogenous formation of these exocyclic adducts.

***Abbreviations:** εdC, 3N^4-ethenodeoxycytidine; N^2,3-εG, N^2,3-ethenoguanine; CdG, crotonaldehyde-derived 1,N^2-propanodeoxyguanosine; AdG, acrolein-derived 1,N^2-propanodeoxyguanosine; εdA, 1,N^6-ethenodeoxyadenosine; PUFAs, polyunsaturated fatty acids; HNE, *trans*-4-hydroxy-2-nonenal; EH, 2,3-epoxy-4-hydroxynonanal.

In vivo detection of cyclic adducts

The detection of an etheno adduct, 1,N^6-ethenoadenosine, was first reported about two decades ago in a study of RNA adduction in rats treated with ^{14}C-labeled vinyl chloride (14). Subsequently, 3,N^4-ethenodeoxycytidine (εdC*) was detected in rat liver DNA after treatment with vinyl chloride (17). Following these reports, several studies have shown that both 1,N^6-ethenoadenine and 3,N^4-ethenocytosine were present in the liver DNA or RNA of vinyl chloride- and ethyl carbamate (urethan)-treated rats (15,16). More recently, N^2,3-ethenoguanine (N^2,3-εG) has been detected in the liver DNA of vinyl chloride-treated rats (19,20). Both *in vitro* and *in vivo* studies indicate that the intermediacy of chloroethylene oxide, produced by epoxidation of vinyl chloride, is involved in formation of these etheno adducts (21,22). We reported the detection of crotonaldehyde-derived 1,N^2-propanodeoxyguanosine (CdG) in the liver DNA of rats treated with crotonaldehyde and *N*-nitrosopyrrolidine; both are hepatocarcinogens and the latter produces crotonaldehyde upon metabolic activation (18).

It was not realized until recently that cyclic adducts are present in tissue DNA from rodents even without carcinogen treatment. Our earlier study of DNA adducts with *N*-nitrosopyrrolidine and crotonaldehyde, using a ^{32}P-postlabeling method, showed that an adduct chromatographically similar to CdG was found in the tissue DNA of untreated rats (18). Later, using a negative ion chemical ionization gas chromatography mass–spectrometry method, Fedtke *et al.* reported a background level of N^2,3-ethenoguanine in tissue DNA of rats (19). In both cases, however, the identities of the adducts were not rigorously confirmed. Prompted by these observations, we and others have developed more sensitive and specific detection methods to unambiguously detect these adducts and prove their identities. Using a ^{32}P-postlabeling method combined with HPLC designed specifically for the cyclic adducts, we have shown that both acrolein-derived 1,N^2-propanodeoxyguanosine (AdG) and CdG are present in the DNA of a wide variety of tissues, including liver, lung, brain, mammary gland,



Scheme 1. Stereoisomers of 1,N^2-propanodeoxyguanosine adducts formed from reactions with acrolein and crotonaldehyde. The absolute configurations of these adducts are not established, therefore, the structures are arbitrarily assigned. AdG 3 may consist of a pair of diastereomers which are not separated under the HPLC conditions used and the stereochemistry at the carbon bearing hydroxyl groups in AdG 3 is not specified.

kidney, colon and prostate of rats, and liver and skin of mice (1,2). More importantly, these adducts have also been detected in the DNA of human mammary gland, liver, and leukocytes. Co-migration of the radiolabeled adducts with synthetic standards in reverse phase HPLC provided the proof of identity of the adducts. Incorporating HPLC in this method enabled us to detect stereoisomers of AdG and CdG (structures in Scheme 1) and the use of synthetic AdG and CdG adducts as external standards permitted better quantification (23). Among the isomers of AdG, AdG 3 has been consistently detected as the predominant adduct in the DNA of most tissues. Stereoselective detection and the tissue-specific distribution of AdG and CdG indicate that AdG and CdG adduct isomers may be formed and/or repaired at different rates in different tissues. The total level of these adducts in tissue DNA has been estimated in the range of 1 adduct per 10^6–10^7 guanines, which is of the same order of magnitude as that in carcinogen-treated animals. These levels are conceivably unattainable by exposure to exogenous chemicals, which are usually present in the environment at low levels, although it is possible that adduct levels could accumulate due to inefficient repair. Since AdG and CdG have been detected consistently in all tissues studied so far, it is entirely possible that they will be found in other tissues not yet examined.

With an immunoaffinity enrichment and ^{32}P-postlabeling method, Nair *et al.* have detected 1,N^6-ethenodeoxyadenosine (εdA) and εdC in the liver DNA of humans and untreated rodents (4). These adducts are detected by autoradiography using two-dimensional thin layer chromatography. For quantification, both internal and external standards of deoxyuridine 3′-monophosphate were used and the levels of εdA and εdC in liver DNA were in the range of 1–2 adducts per 10^8–10^9 corresponding bases, approximately two orders of magnitude lower than those of AdG and CdG. Another etheno adduct, N^2,3-ethenodeoxyguanosine (N^2,3-εdG), has been detected by a gas chromatography/high resolution mass spectrometry method in DNA of rat and human tissues (5). The N^2,3-εdG levels in the liver DNA were similar to the levels of the propano adducts, approximately two orders of magnitude higher than εdA and εdC (5). The large difference in the levels of the etheno adducts in DNA is intriguing. This difference may result from different chemical reactivity toward bases, rates of formation and repair and/or method of detection. Table I summarizes tissues from various species so far examined that contain the cyclic adducts as background DNA lesions and the range of adduct levels detected by various methods in each species. In general, adduct levels in human tissues show the largest inter-individual variability. The levels of these adducts detected could represent the basal levels in each tissue as a result of continuous formation and repair. The ubiquity and significant levels of the cyclic adducts in tissue DNA suggest that endogenous compounds are likely to be involved in their formation. The following discussions provide the current evidence from chemical and biochemical studies which supports the role of α,β-unsaturated aldehydes (enals), from lipid peroxidation, in the formation of these cyclic adducts.

Chemical and biochemical studies

Lipid peroxidation is a normal biochemical process which involves the oxidation of polyunsaturated fatty acids (PUFAs) as components of cell membranes. Elevated lipid peroxidation in tissues has been associated with tissue injury, due to generation of reactive species which causes damage to cellular macromolecules, including DNA. Because of this, it is also believed that lipid peroxidation may play a role in carcinogenesis. Both *in vitro* and *in vivo* studies have shown that oxidation of PUFAs produces, among other reactive compounds, a host of aldehydes (24,25). Enals with different alkyl chain lengths, ranging from 3 to 10 carbons, are among the products which have been identified. These aldehydes are formed in different quantities from fatty acids under different oxidation conditions. For example, *trans*-4-hydroxy-2-nonenal (HNE) arises from oxidizing ω-6 fatty acids, such as arachidonic acid and linoleic acid, and has been detected in various tissues in rodents and humans at estimated levels from 0.3 to 8 nmol/g tissue, depending on the tissue and pathological conditions (24,26). Among the lipid peroxidation-generated enals, acrolein, crotonaldehdye, malondialdehyde and HNE have been most widely studied for their chemical and biological activities and have been shown to be capable of modifying proteins and DNA.

Studies from this and other laboratories have shown that 1,N^2-propano adducts are common products of reactions of deoxyguanosine with enals (27–29). These reactions could

Table I. Tissues of untreated mouse and rat and tissues of human in which exocyclic adducts are detected in DNA and ranges of adduct levels in each species

Adduct	Species	Tissue	Range of adducts detected (μmol/mol parent base)[a]
AdG and CdG[b]	Mouse	Liver, skin	0.31–1.69
	Rat	Liver, lung, colon, brain, prostate, mammary gland, kidney	0.11–1.12
	Human	Liver, mammary gland, leukocytes	0.01–2.11
εdA and εdC[c]	Mouse	Liver	0.0028–0.0031
	Rat	Liver	0.0010–0.0012
	Human	Liver	0.000–0.040
N^2,3-εdG[d]	Mouse	Liver	0.25–0.70
	Rat	Liver, kidney, brain, spleen, lung	0.18–0.23
	Human	Liver	0.07–0.70

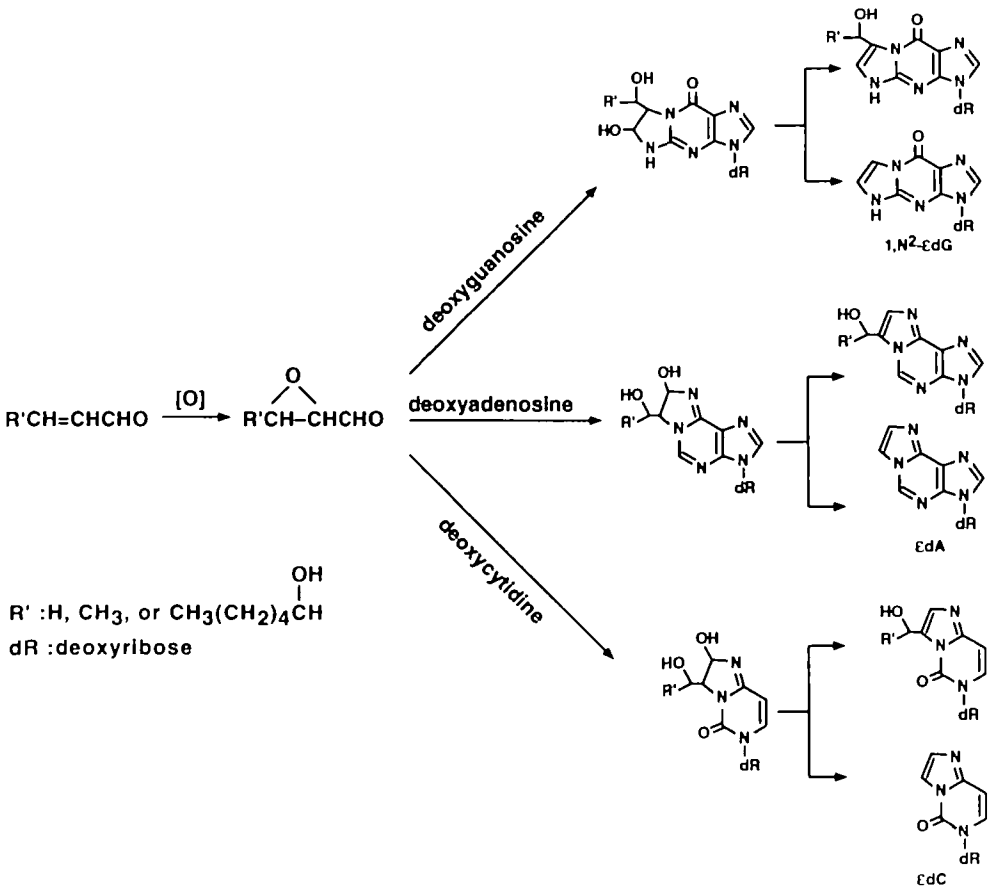
[a]These values are compiled based on data given in Nath and Chung (1), Nath *et al.* (2), Nair *et al.* (4), Swenberg *et al.* (5) and Fedtke *et al.* (19).
[b]Detection by the ^{32}P-postlabeling/HPLC method in Nath and Chung (1) and Nath *et al.* (2).
[c]Detection by the immunoaffinity column/^{32}P-postlabeling method in Nair *et al.* (4).
[d]Detection by the negative ion GC–MS method in Swenberg *et al.* (5) and Fedtke *et al.* (19).

occur at 37°C and pH 7 via an initial Michael addition followed by ring closure. Acrolein, the simplest enal, reacts readily with deoxyguanosine, yielding at least three stereoisomeric adducts namely, AdG 1, 2 and 3 (Scheme 1). AdG 1 and 2 are a pair of diastereomers. AdG 3, presumably consisting of two diastereomers, is a regio-isomer formed from ring closure in the opposite direction. As a homolog of acrolein, crotonaldehyde reacts with deoxyguanosine in a similar manner, yielding cyclic 1,N^2-propano adducts CdG 1 and CdG 2 (Scheme 1). However, due to steric hindrance between the methyl group and the oxygen at C6, Michael addition by N1 to the olefinic carbon is prohibited, consequently only one pair of diastereomers is formed. On the basis of the proton NMR coupling constants, the OH and methyl groups of CdG isomers are assigned the *trans* configuration. The formation of the 1,N^2-propano adducts of deoxyguanosine from HNE has also been demonstrated (29). However, the rate of formation is considerably slower than that of the shorter chain enals. Similar reactions have been observed between enals and other DNA bases (30). Despite differences in stereochemistry and the rates of reaction, the formation of cyclic propano adducts appears to be a general reaction for enals. Acrolein and crotonaldehyde have also been shown to form 7,8-propano adducts with deoxyguanosine that could lead to depurination (31,32).

Upon incubation with calf thymus DNA at 37°C and pH 7, acrolein and crotonaldehyde modify deoxyguanosine, forming AdG and CdG (28). Acrolein is considerably more reactive than crotonaldehyde in these reactions. The reactivity of enals toward deoxyguanosine in DNA for forming propano adducts appears to decrease with increasing alkyl chain and the relative reactivity is acrolein > crotonaldehyde > HNE (28,33). The propano adducts of DNA bases other than guanine are also formed upon incubation with acrolein (34). It is expected that crotonaldehyde or HNE can modify other bases in DNA, although it has yet to be demonstrated. It should also be noted that because cyclic adduction requires bonding of the two nitrogens in the bases which are normally involved in hydrogen bonding of α-helical DNA, the levels of modification are lower for double-stranded DNA than for single-stranded DNA.

Alternatively, enals can be converted to epoxyaldehydes by hydroperoxides (35–37). Epoxy aldehydes are more reactive toward DNA than the parent enals, especially the long chain enals. Reactions of the epoxyaldehydes of acrolein, crotonaldehyde and HNE with purine and pyrimidine nucleosides have been studied. These studies have shown that reactions of epoxyaldehydes yield primarily guanine adducts with 1,N^2-ethano or -etheno modifications, adenine adducts with 1,N^6-etheno modification, and cytosine adducts with 3,N^4-etheno modification (Scheme 2). Glycidaldehyde, the epoxide of acrolein, yields the substituted 1,N^2-ethano and 1,N^2-etheno adducts and the unsubstituted 1,N^2-etheno adduct of guanine (38). 2,3-Epoxybutanal from crotonaldehyde gives 1,N^2-ethenoguanosine, 1,N^6-ethenoadenosine and 3,N^4-ethenocytidine (35). 2,3-Epoxy-4-hydroxynonanal (EH), formed as two diastereomers I and II on reaction of HNE with t-butyl hydroperoxide, modifies deoxyguanosine yielding, in addition to the substituted and unsubstituted 1,N^2-etheno adducts, a unique tetracyclic 1,N^2-ethano adduct as two pairs of diastereomers (33,36). The latter adducts are readily converted to the unsubstituted 1,N^2-etheno adduct, 1,N^2-ethenodeoxyguanosine (1,N^2-εdG) in basic medium, with a $t_{1/2}$ of 9 min at 37°C and pH 10 (39). Upon reaction with deoxyadenosine, EH gives εdA and a pair of the substituted εdA isomers (33). EH isomers I and II react with deoxyadenosine and deoxyguanosine in a stereoselective manner (33). The relative yields of the substituted and the unsubstituted etheno adducts vary depending on the epoxy aldehydes and the pH of the reactions. Consistent with base-catalyzed conversion, unsubstituted 1,N^2-εdG is formed in greater yield upon reaction with EH at basic pH than at neutral pH (36). A pH-dependent formation of the substituted and unsubstituted etheno adducts has also been observed with glycidaldehyde and 2,3-epoxybutanal (35,38). So far, no evidence shows that reactions of deoxyguanosine with EH or glycidaldehyde produce N^2,3-εdG, a major adduct of chloroethylene oxide or other 1-halooxiranes (22,40). This is interesting because this would suggest that N^2,3-εdG detected *in vivo* either originates from sources other than enals or that the chemistry for its *in vivo* formation with DNA is different from that in nucleosides. More studies are needed to answer this question.

Like their reactions with nucleosides, epoxides of enals modify DNA or RNA with the formation of similar adducts. Again, the levels of modification are much higher with single-stranded DNA or RNA than with double-stranded DNA (33). EH isomers I and II exhibit a clear stereoselective reaction with adenine and guanine in RNA, yielding the etheno adducts as major products. Furthermore, isomer I preferentially modifies adenines over guanines, whereas isomer II shows comparable reactivity toward both purine bases (33). Consistent with the greater reactivity of EH isomer II toward nucleic acids,



Scheme 2. Formation of various etheno adducts from reactions of deoxyribonucleosides with epoxy aldehydes. For all three reactions, the substituted etheno adducts are formed by a loss of H_2O from the intermediate ethano adducts and the unsubstituted etheno adducts are formed by base-catalyzed losses of R'CHO and H_2O.

EH isomer II was more mutagenic than isomer I in both TA 100 and TA 104 (41). The parent aldehyde is inactive in both tester strains, though it induces chromosomal changes in cell culture (42). Furthermore, EH has been shown to be a weak tumorigen, whereas HNE is devoid of tumorigenic activity (41). These results suggest that epoxidation may constitute an important activation pathway for long-chain, less reactive enals.

In addition to the chemical studies described, evidence for the involvement of enals from lipid peroxidation in exocyclic adduct formation is provided by *in vitro* and *in vivo* biochemical studies. We have shown that epoxidation of HNE could occur with H_2O_2 and fatty acid hydroperoxides or under the conditions in which these oxidants are generated, and that the EH produced in these reactions can be trapped by deoxyadenosine as its 1,N^6-etheno adducts (37,43). After incubating HNE with 9- or 13-lineoleic acid hydroperoxide at 37°C for 24 h in the presence of deoxyadenosine, εdA is formed in 1.66 or 1.78% yield respectively. Under similar conditions, H_2O_2 gives almost 3% of the etheno adduct. Consistent with this finding, the xanthine/xanthine oxidase/superoxide dismutase system, which generates H_2O_2, also produces εdA. In the presence of lipoxygenase, fatty acid such as lineolic acid, γ-lineolic acid and arachidonic acid converts HNE to its epoxide, which modifies deoxyadenosine forming an etheno adduct: These results demonstrate that cellular oxidants are capable of epoxidation of enals leading to the formation of etheno adducts. El-Ghissassi *et al.* have reported that 1,N^6-ethenoadenine (εA) and 3,N^4-ethenocytosine are formed by lipid peroxidation mediated by iron or cumene hydroperoxide in a rat liver microsome incubation mixture containing the nucleosides or nucleotides of these bases (44). These adducts are also formed upon incubation of nucleosides with arachidonic acid in the presence of iron (44). The yields of the etheno adducts decrease sharply upon incubation with denatured microsomes. These observations suggest that either reactive compounds, readily scavenged by nucleophilic sites in proteins, are responsible for etheno adduct formation and/or that the epoxidation enzyme is deactivated.

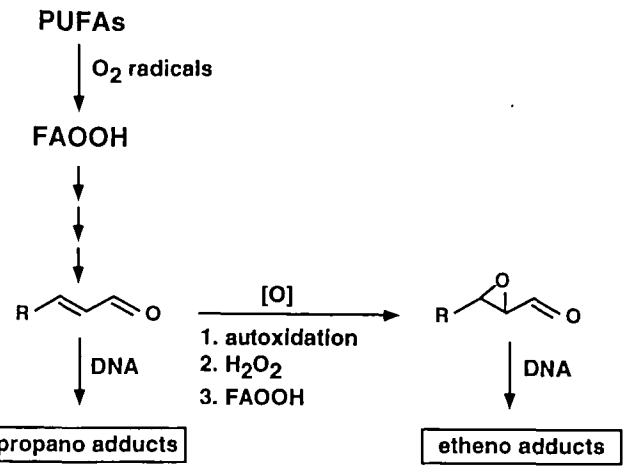
The repair of etheno adducts has been well documented and is mainly mediated by the action of glycosylase, which results in apurinic/apyrimidinic sites. Oesch *et al.* demonstrated that rat brain homogenate removes εA and N^2,3-εG in chloroacetaldehyde-modified DNA (45). Singer and co-workers reported that human cells contain a protein with high binding affinity for etheno adducts in duplex DNA, which functions as a repair enzyme (46,47). The repair activity for εA (in human cells and tissues) has been attributed to 3-methyladenine-DNA glycosylase, since both purified human εA binding protein and 3-methyladenine-DNA glycosylase are capable of removing εA and 3-methyladenine (48). In fact, all four etheno adducts, εdA, N^2,3-εdG, 1,N^2-dG and εdC, have been shown to be repaired by human glycosylase and by *Escherichia coli* expressing cloned human 3-methyladenine-DNA glycosylase, although the etheno adducts of adenine and cytosine appear to be repaired much more efficiently than the guanine adduct (13). It should be noted that bacteria also express glycosylase

activity to remove N^2,3-εG (12). The demonstration of repair of etheno adducts in DNA by ubiquitous and non-specific DNA glycosylases is suggestive of the endogenous origin of these adducts. Although the repair of cyclic propano adducts is much less understood, it is plausible that these lesions, like etheno adducts, are highly amenable to repair.

Studies with Long-Evans cinnamon rats provide additional evidence supporting lipid peroxidation in cyclic adduct formation. Long-Evans cinnamon rats, an inbred strain which develops high incidences of hepatitis and spontaneous hepatocellular carcinoma, are considered a useful model for the study of hepatitis, liver cancer and human Wilson's disease, due to high levels of copper accumulation in the liver resulting from a genetically predisposed abnormality in copper metabolism (49). Studies have shown that the high copper concentration in the liver of these rats causes increased lipid peroxidation (50). Nair *et al.* have recently observed that εdA and εdC levels in the liver DNA of Long-Evans cinnamon rats are significantly higher than Long-Evans agouti (LEA) rats, a strain with normal copper metabolism (51). The highest levels of εdA and εdC seen in 18-week-old Long-Evans cinnamon rats, corresponding to 28- and 5-fold increases respectively, coincide with the levels of copper accumulation and the manifestation of hepatitis in rats of this age. The increases in εdA and εdC levels also correlate with other markers of oxidative damage, such as 8-hydroxydeoxyguanosine. Our preliminary study, using the liver DNA of 18-week-old Long-Evans cinnamon rats, has shown that AdG and CdG levels, as compared with those of LEA rats, are ~26-fold higher for AdG 3 and 7 and 20-fold higher for CdG 1 and CdG 2.

Conclusion

We could not rule out exogenous compounds as sources of cyclic propano and etheno adduct formation. Some enals, such as acrolein and crotonaldehyde, are ubiquitous environmental chemicals and are metabolites of environmental carcinogens and small amounts of HNE have been found in certain foods. Urethane and other small chlorinated compounds in beverages and drinking water are also possible sources. While the contribution of these exogenous chemicals to cyclic adduct formation is uncertain, the studies discussed here suggest that lipid peroxidation is an important endogenous source for the formation of the cyclic propano and etheno adducts in tissue DNA and that enals such as acrolein, crotonaldehyde and HNE generated by lipid peroxidation are likely to be involved. On this basis, we propose a pathway for *in vivo* exocyclic adduct formation, as outlined in Scheme 3. Oxidation of PUFAs produces fatty acid hydroperoxides that subsequently decompose to enals. The metabolic fates of enals involve oxidation and reduction by aldehyde and alcohol dehydrogenase and conjugation with cellular sulfhydryls (52–55). Our recent studies have shown that treatment of rats with L-buthionine sulfoximine, which depletes tissue glutathione, leads to a significant increase in AdG and CdG levels in liver DNA. Enals could also react directly with DNA bases forming propano adducts. As an alternative pathway, enals could be epoxidized by enzymes such as cytochrome P450s and/or prostaglandin synthase, by cellular H_2O_2 and/or fatty acid hydroperoxides and by autoxidation. The resulting epoxyaldehydes, as reactive electrophiles, could modify DNA bases yielding the etheno adducts. The *in vivo* propano and etheno adducts detected to date probably represent only a fraction of



Scheme 3. The outline of the proposed pathway for *in vivo* formation of the propano and etheno DNA adducts via fatty acid hydroperoxides (FAOOH) generated by peroxidation of polyunsaturated fatty acids (PUFAs)

the exocyclic adducts that could exist in tissue DNA. The well-characterized chemical reactions of enals and their epoxides with DNA bases support this possibility. Obviously, more *in vitro* and *in vivo* studies will be needed to verify this pathway. For example, the detection of other adducts, such as substituted εdA and εdG, will provide more insight into the role of lipid peroxidation and the specific enals in adduct formation. In addition, when one considers the potential mutagenic and carcinogenic activity of these cyclic adducts, it is imperative to study the kinetics of their formation and repair, as well as their mutation characteristics, in order to understand their roles in cancer.

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