P-075

Mutagenicity of polyunsaturated fatty acid peroxidation products in the standard Ames assay

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Abstract

Polyunsaturated fatty acids (PUFAs) represent the main building blocks of cellular membranes and their composition impacts the animal lifespan as well as susceptibility to cancer. Increased intake of ω-3 fatty acids (*n*-3) is taught to compensate for the abundance of ω-6 fatty acids (*n*-6) in modern diet, resulting in a higher *n*-3/*n*-6 ratio and preventing cardiocirculatory diseases. The increased exposure of general population to the PUFAs of marine and seed origin, which easily oxidize to aldehydic products known to form DNA adducts, warrants further investigation of their potential genotoxic risks.

We have used the standard bacterial assay (the Ames test) to examine the mutagenicity of 2 lipid peroxidation products of the common *n*-3 in human tissues and diet, i.e. 4-hydroxyhexenal (4-HHE) and 2-hexenal (2-HE) derived from docosahexaenoic acid (DHA) and alpha-linolenic acid (ALA), respectively. Both 4-HHE and 2-HE induced base substitutions in the TA104 and TA100 enterobacterial strains in a dose dependent manner. At the highest usable doses, the spontaneous mutation counts doubled in the TA100 but not in TA104 strain. The mutagenicity was dependent on the presence of Y-family DNA polymerase RI. We have not observed the promotion of frameshift mutagenesis such as the -2 and -1 frameshifts in the TA98 and TA97 strains.

Our data further extend the previous findings that the related *n*-3 lipid peroxidation product 4-oxohexenal, which DNA footprint constitutes a significant part of the human adductome, is mutagenic in the Ames test. The mechanisms of lipid peroxide mutagenicity and implications for human health are discussed.

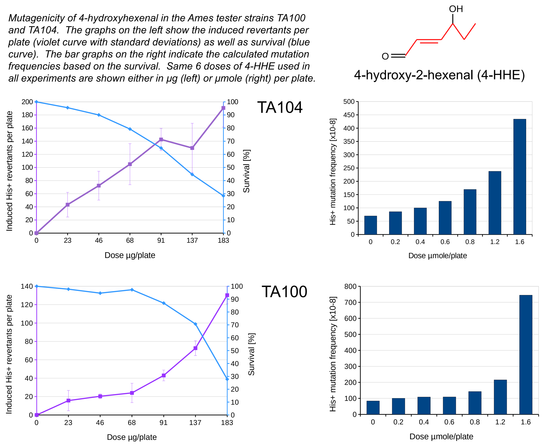
Materials & Methods

Chemicals: (±)-4-hydroxy-2*E*-hexenal (4-hydroxyhexenal, 4-HHE) and similar oxylipins were purchased from Cayman Chemical (USA), *trans*-2-hexenal (leaf aldehyde, 2-HE) and crotonaldehyde were from Wako (Japan). The AMT-S plates (Kyokuto, Japan) were used for plating in all experiments.

Mutagenicity assay: Ames test was carried out according to the standard protocol without the S9 mix but using 20 min preincubation with the test compound (dissolved in ethanol) in 0.02 M sodium phosphate buffer (pH 7.4) at 37°C. To decrease the toxicity of long chain enals, reduced glutathione chase was added following the preincubation period and before plating as described by Marnett et al.[1] The spontaneous revertant counts (about 90 and 250 colonies per plate for the TA100 and TA104 strains, respectively) have been subtracted from the data to obtain the induced revertants per plate values. To determine the survival, overnight culture of bacteria was used in the standard procedure after dilution to 10^{-5} and with extra histidine added to the top agar. At least 3 plates were used for each dose. Colonies were counted after 2 day incubation at 37°C with the DOT colony counter (Ieda Trading Corp. Japan).

Results & Discussion

Mutagenicity of 4-hydroxyhexenal in the Ames tester strains TA100 and TA104. The graphs on the left show the induced revertants per plate (violet curve with standard deviations) as well as survival (blue curve). The bar graphs on the right indicate the calculated mutation frequencies based on the survival. Same 6 doses of 4-HHE used in all experiments are shown either in μg (left) or μmole (right) per plate.



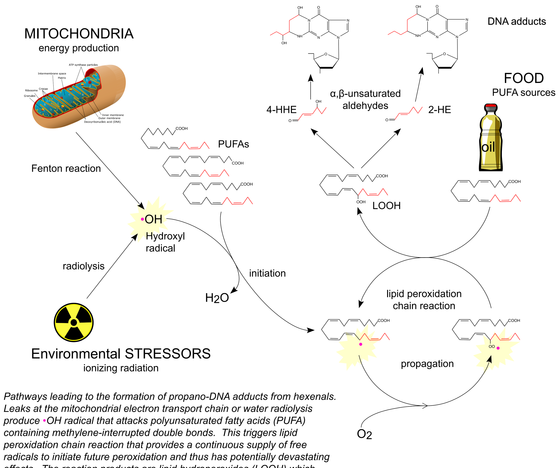
Lipid peroxide-derived endogenous DNA adducts are considered as mediators of cancer, cardiovascular disease and neurodegeneration, the three most prevalent diseases of aging. PUFAs are the direct source of lipid peroxides both *in vitro* and *in vivo*. The susceptibility to lipid peroxidation depends on the number of active methylene groups between two double bonds within a PUFA molecule. The ω-3 fatty acids contain the highest number of double bonds and therefore produce the largest amounts of mutagens by lipid peroxidation.

Although the short chain aldehydic products of lipid peroxidation of ω-3 (e.g. acrolein, crotonaldehyde) and ω-6 (e.g. malondialdehyde) fatty acids are clearly mutagenic in the Ames test, their longer counterparts are rather toxic. We have confirmed the mutagenicity of crotonaldehyde but were unable to detect the mutagenicity of 4-hydroxynonenal (4-HNE) and 4-oxononenal (4-ONE) in the Ames test. Despite the lack of mutagenicity in bacteria, 4-HNE is an important genotoxin derived from the ω-6 dietary fatty acids and a potent inducer of the bacterial DNA damage SOS response[4]. It can be further activated by a reaction with peroxides to its epoxide which is a potent mutagen in both TA100 and TA104 Ames tester strains[5]. As demonstrated in the 4-HNE case, epoxy aldehydes are considerably more reactive with DNA than their parent aldehydes which conjugate more rapidly with the sulfhydryl groups on proteins leading to higher toxicity than mutagenicity[5]. The epoxy aldehydes form a variety of etheno DNA adducts while the parental α,β-unsaturated aldehydes form the propano adducts[6]. The major propano N^2-dG DNA adducts are bypassed in an *error-free* manner with the assistance of the DinB-type DNA polymerases what may effectively lower their mutagenicity[7].

The ω-3 counterparts of the ω-6 nonenals 4-HNE and 4-ONE are the hexenals 4-HHE and 4-OHE which are 3 carbons in length shorter. The direct mutagenicity of 4-oxo-2-hexenal (4-OHE) in the Ames test has been demonstrated previously by Kasai et al. and its DNA footprint was found to constitute a significant part of the human adductome[2,3]. Here we present the positive mutagenicity of related aldehydes 4-HHE and 2-HE in the standard Ames assay. Because strong toxicity was observed at higher doses, we have attempted to determine the survival and calculate the mutation frequencies in addition to the standard revertant per plate values similarly as it was done in the 4-ONE case[3]. The survival data can be informative since bacteria were exposed to the agents during preincubation phase in buffer and thus residual growth on the plates should not substantially skew the calculated values.

Strain	Genotype	Mutation target	DNA Pol RI	rev./μmol 4-HHE	rev./μmol 2-HE
YG5144	*his*G428	TAA (base change)			
TA104			+	181	31
YG5151	*his*G46	GGG (base change)			
TA100			+	51	108
TA1537	*his*C3076	CCC (+1 frameshift)			
TA2637			+		
TA97	*his*D6610	CCCCCC GCGC (+1 frameshift)	+		
YG5147	*his*D3052	CGCGCGCG (-2 frameshifts)			
TA1538					
TA98			+		

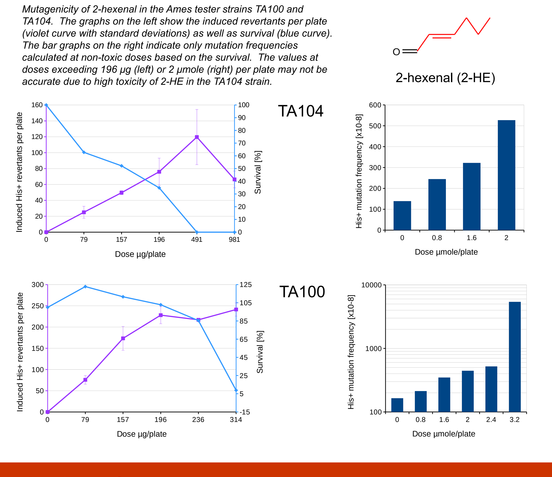
Ames tester strains used to screen for the mutagenicity of hexenals. Relevant genotypes and target sequences for the reversions to histidine prototrophy are indicated. Strains harboring the R-factor plasmid pKM101 expressing DNA polymerase RI (MucAB) are labelled with "+". For the two strains which tested positive, specific mutagenicities were calculated within the linear non-toxic range at the doses of 0.4 and 1.6 μmol/plate for 4-HHE and 2-HE, respectively.



Pathways leading to the formation of propano-DNA adducts from hexenals. Leaks at the mitochondrial electron transport chain or water radiolysis produce •OH radical that attacks polyunsaturated fatty acids (PUFA) containing methylene-interrupted double bonds. This triggers lipid peroxidation chain reaction that provides a continuous supply of free radicals to initiate future peroxidation and thus has potentially devastating effects. The reaction products are lipid hydroperoxides (LOOH) which homolytically decompose to genotoxic α,β-unsaturated aldehydes.

Considering the mutation frequencies, the standard revertants per plate data would rather underestimate the mutagenicities of 4-HHE and 2-HE. Actually in the case of 4-HNE (data not shown) we also observed induction of mutagenesis if only mutation frequencies were taken into account. Only base substitutions were induced in a Y-family DNA polymerase RI dependent manner. This *error-prone* polymerase is probably necessary to overcome an *error-free* bypass of the putative major N^2-dG adducts by DNA polymerase IV. All tested strains carried the *uvr*B deletion to prevent DNA adduct removal by the nucleotide excision repair. In contrast to some dicarbonyl compounds such as malondialdehyde, both tested hexenals did not promote frameshift mutagenesis in the *his*D3052, *his*D6610 or *his*C3076 Ames strains. When comparing the specific mutagenicities on molar basis, 4-HHE was more mutagenic in TA104 (*his*G428) than TA100 (*his*G46) strain while 2-HE was more mutagenic in TA100 than TA104 strain (see the Table).

Mutagenicity of 2-hexenal in the Ames tester strains TA100 and TA104. The graphs on the left show the induced revertants per plate (violet curve with standard deviations) as well as survival (blue curve). The bar graphs on the right indicate only mutation frequencies calculated at non-toxic doses based on the survival. The values at doses exceeding 196 μg (left) or 2 μmole (right) per plate may not be accurate due to high toxicity of 2-HE in the TA104 strain.



Implications for carcinogenesis

As has been discussed previously[8], endogenous lipid peroxide mediated DNA damage plays a key role in the aging process and can initiate cancer. Despite their high toxicity in bacteria, we have demonstrated the mutagenicity of two major ω-3 fatty acid peroxidation products in the classical Ames test. 4-HHE is a neurotoxin derived from the docosahexaenoic acid (DHA), the most abundant ω-3 fatty acid in human brain and retina[9]. It is clearly mutagenic in the mouse lymphoma assay[10] and has been shown to form a cyclic N^2-dG DNA adduct *in vitro*[11]. 2-HE is another common peroxidation product of the linolenic acid present e.g. in grass, fruits and vegetables also forming similar N^2-dG DNA adduct[13]. It is a potent aneuploidy inducer[25] and has been shown to induce chromosomal aberrations in somatic cells as well as abnormal sperm head morphology in germ cells[12].

Although the genotoxic risks from 4-HHE and 2-HE exposure could be questioned by recent work suggesting a hormetic response to the former and an efficient detoxification of the latter[14,15], some worrying data about the carcinogenic affects of long term exposure to the ω-3 fatty acids should not be ignored. This particularly includes the involvement of ω-3 PUFAs in prostate and breast tumorigenesis[16-19]. A recent trend of balancing high ω-6 intake in modern diet with increased ω-3 consumption can prove futile if one considers the ω-3 PUFAs as initiators and ω-6 PUFAs as promoters of cancer. The role of ω-6 PUFAs in cancer promotion through the eicosanoid metabolism is widely acknowledged and demonstrated experimentally e.g. on the mouse skin cancer model[20].

Compared to 4-HNE, 4-HHE has been rather underinvestigated despite of being more prominent in human plasma and forming adducts at higher levels in some disease states and aging[21,22]. 4-HHE is also present at 20x higher levels in commercial infant formulas and baby foods than in human milk[23]. This should be of concern since babies maintained exclusively on the commercially available foods could be exposed to two orders of magnitude higher levels of genotoxic 4-hydroxy-2-alkenals at their early stage of development than the adults[24].

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