Lipid peroxidation and DNA adduct formation in lymphocytes of premenopausal women: role of estrogen metabolites and fatty acid intake

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A diet high in linoleic acid (an ω-6 PUFA) increased the formation of miscoding etheno (ε) - DNA adducts in WBC-DNA of women, but not in men (Nair *et al.*, *Cancer Epidemiol Biomark Prev* 1997;6:597-601). This gender specificity could result from an interaction between ω-6 PUFA intake and estrogen catabolism, *via* redox-cycling of 4-hydroxyestradiol (4-OH-E_2) and subsequent lipid peroxidation (LPO). In this study, we investigated whether in premenopausal women LPO-derived adducts in WBC-DNA are affected by serum concentration of 2- and 4-hydroxyestradiol metabolites and by fatty acid intake. DNA extracted from buffy coat and plasma samples, both blindly coded from healthy women (N = 124, median age 40 year) participating in the EPIC-Heidelberg cohort study were analyzed. Three LPO-derived exocyclic DNA adducts, εdA, εdC and M_1dG were quantified by immuno-enriched ^{32}P-postlabelling and estradiol metabolites by ultra-sensitive GC-mass spectrometry. Mean M_1dG levels in WBC-DNA were distinctly higher than those of εdA and εdC, and all were positively and significantly interrelated. Serum levels of 4-OH-E_2, but not of 2-OH-E_2, metabolites were positively related to etheno DNA adduct formation. Positive correlations existed between M_1dG levels and linoleic acid intake or the ratios of dietary linoleic acid/oleic acid and PUFA/MUFA. Aerobic incubation of 4-OH-E_2, arachidonic acid and calf thymus DNA yielded two to threefold higher etheno DNA adduct levels when compared with assays containing 2-OH-E_2 instead. In conclusion, this study is the first to compare M_1dG and etheno-DNA adducts and serum estradiol metabolites in human samples in the same subjects. Our results support a novel mechanistic link between estradiol catabolism, dietary ω-6 fatty acid intake and LPO-induced DNA damage supported by an *in vitro* model. Similar studies in human breast epithelial tissue and on amplification of DNA-damage in breast cancer patients are in progress.

Persistent cellular oxidative stress and enhanced lipid peroxidation (LPO), leading to macromolecular damage and disruption of signaling pathways are implicated in the development of human malignancies.[1–3] LPO of ω-6 polyunsaturated fatty acids (ω-6 PUFA) generates reactive α, β-unsaturated aldehydes, such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), which can form promutagenic exocyclic DNA adducts in human cells and thus may contribute to human cancers.[4–11] Earlier we have used an ultra-sensitive detection method to analyze etheno-DNA adducts in WBC collected from healthy volunteers in a carefully controlled dietary study. We showed that a very high intake of ω-6

Key words: lipid peroxidation, LPO, DNA adducts, fatty acids, estrogen, metabolism, premenopausal women

Abbreviations: E_2: 17β-estradiol; εdA: 1,N^6-ethenodeoxyadenosine; εdC: 3,N^4-ethenodeoxycytidine; HNE: 4-hydroxy-2-nonenal; I.S.: internal standard; LA: linoleic acid; LPO: lipid peroxidation; M_1dG: 3-(2-deoxy-β-D-erythro-pentofuranosyl)pyrimido[1,2-α]purin-10[3H]one; 2-/4-OH-E_2: 2-/4-hydroxyestradiol; OA: oleic acid; ω-6 PUFA: omega (ω)-6 polyunsaturated fatty acids; WBC: white blood cells

Additional Supporting Information may be found in the online version of this article.

This article is dedicated to Jagadeesan Nair who died prematurely in 2007.

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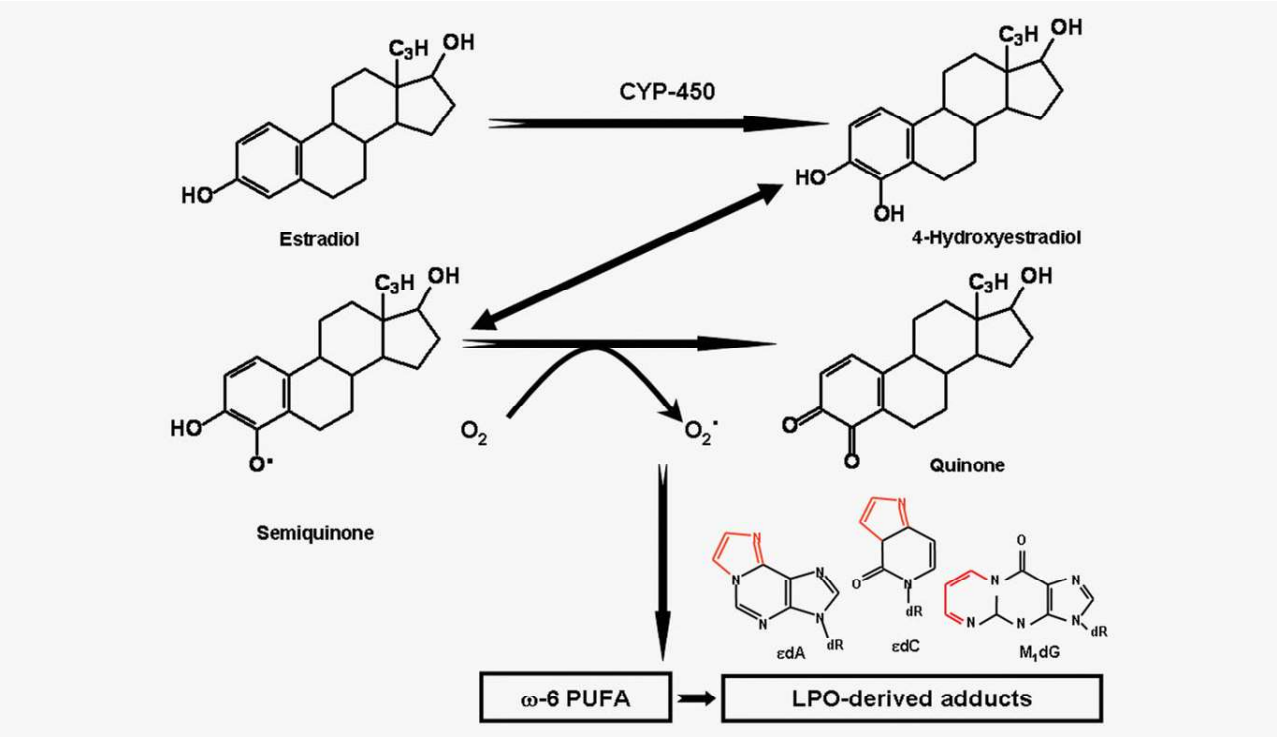

Figure 1. Proposed scheme for metabolic redox-cycling of 4-hydroxyestradiol leading to reactive oxygen species and lipid peroxidation (LPO) of ω-6 PUFA; the resulting LPO byproducts such as 4-hydroxy-2-nonenal and malondialdehyde generate miscoding etheno DNA adducts (εdA and εdC) and M_1dG, respectively, that were analyzed in WBC of healthy female volunteers. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

PUFA, linoleic acid (LA), in sunflower oil but not of oleic acid (OA) in rape seed oil increased the level of etheno-DNA adducts in WBC some 40-times in women but not in men.[12] These results indicated that the DNA adduction was attributable to consumption of an LA-rich diet, which resulted in increased LPO of membrane lipids. The marked inter-individual differences in etheno-DNA adduct levels in women on the same high ω-6 PUFA diet and the gender-specificity, however, remained unexplained. We hypothesized that these could be related to an interaction between ω-6 PUFA intake and estrogen catabolism.[13–17] A feeding experiment in rats supported our assumption and confirmed our previous findings in human volunteers.[12] Male and female rats were gavaged with LA (ω-6 PUFA), OA (MUFA), or coconut oil (saturated fatty acids).[18] Etheno-DNA adducts in LA-treated female rats were found to be 8–13 times higher in WBC when compared with males.[18,19] Females seemed to be more susceptible to LPO-induced DNA damage, possibly as a consequence of interaction of ω-6 PUFA with 4-hydroxylated estradiol metabolites which may undergo redox-cycling (Fig. 1), thereby producing reactive oxygen species and increasing LPO of membrane fatty acids as proposed earlier.[13,14,16] Indeed, 4-hydroxylation of estradiol with subsequent estradiol-3,4-quinone formation (Fig. 1), but less so 2-hydroxylation, has been implicated as a pathway for genotoxicity and tumor-initiating activity.[20,21] Therefore, high etheno-DNA adduct levels with concomitantly huge inter-individual variation in women may be, at least in part, due to variations in individual levels and patterns of 2- *versus* 4-hydroxylated estradiol metabolites.

To further support this postulated pathway of LPO-derived DNA damage (Fig. 1), *e.g.*, a synergistic interaction of ω-6 PUFA and 17β-estradiol catabolism, we have investigated whether the levels of three exocyclic DNA adducts in WBC are affected by concentrations of 2- and 4-hydroxyestradiol metabolites in plasma and by dietary fatty acid intake. WBC and plasma samples from healthy premenopausal women participating in the Heidelberg-EPIC cohort study were used to analyze the εdA, εdC and M_1dG content by immuno-enriched ^{32}P-postlabelling; 17β-estradiol metabolites were analyzed by an ultra-sensitive GC-mass spectrometry method.

Material and Methods

Materials

17β-estradiol (E_2), 2-hydroxy-estradiol (2-OHE_2), 4-hydroxy-estradiol (4-OHE_2), 2-methoxy-estradiol (2-$MeOE_2$), 4-methoxy-estradiol (4-$MeOE_2$) and 4-methoxy-estradiol 3-methyl ether (4-$MeOE_2$ Me) were obtained from Steraloids Inc. (Newport, Rhode Island, USA), the stock solutions of estradiol metabolites were prepared by dissolving 5 mg in 1 ml of ethanol. Arachidonic acid, hexamethyldisilazane, trimethylchlorosilane and pyridine were obtained from Sigma Chemical Co. (Steinheim, Germany),

(arachidonic acid is air and moisture sensitive compound and should be handled under Argon gas). Micrococcal nuclease (MN) was purchased from Sigma-Aldrich (Taufkirchen, Germany). Spleen phosphodiesterase (SPD) was obtained from Worthington Biochemical Corp. (Lakewood, NJ), cloned T4 polynucleotide kinase (PNK) from MBI/Fermentas (St. Leon-Rot, Germany). [γ-^{32}P]ATP with a specific activity of ~220 TBq/mmol (~6,000 Ci/mmol) was purchased from Hartman Analytic (Braunschweig, Germany), (caution: [γ-^{32}P]ATP is a hazardous and radioactive compound and should be handled with sufficient protection and shielding). Polyethyleneimine (PEI) was obtained from J.T. Backer, Inc. (Phillipsburg, NJ).

For analyses of 17β-estradiol metabolites, all glassware including Pasteur pipettes was silanized with 1% dimethyldichlorosilane in toluene because of the known creepage of estrogens and subsequent decomposition.[22] All solvents were of HPLC grade.

Study subjects and biological sample collection

At recruitment, detailed information from all EPIC-Heidelberg participants was obtained by questionnaire, face-to-face interview and anthropometric measurements. Recruitment and blood sampling procedures followed standard protocols as described elsewhere.[23,24] For the present study, premenopausal women not taking hormone preparations (oral contraceptives, hormone replacement therapy) were identified from a sub-group of EPIC-Heidelberg participants for which buffy coat and plasma samples were available. Among these, 200 apparently healthy women in the age range of 35–49 years (median 40 years) were randomly selected.

DNA isolation from WBCs

Blood samples for plasma preparation were collected in citrate buffer (0.05 M sodium citrate, pH 7.0), cooled to 4–8°C and subsequent centrifugation (2900 rpm, 20 min). Buffy coat (PBMC, mainly leukocytes) and plasma fractions were separated and stored at −80°C for further analyses. The isolation of genomic DNA from buffy coats was achieved using the blood and cell culture Midi kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol except for the modification of the supplied pH to 7.4 and adjusting the NaCl concentration in the elution buffer to 1.4 M. Some DNA samples were isolated using the hydroxylapatite method modified as described, yielding the same results as the Qiagen extraction procedure.

In vitro assay for the formation of DNA adducts

The reaction was performed in 1.5 ml glass vials with screw caps under argon gas.[19] The incubation mixture typically contained 40 μg purified calf thymus DNA (CT-DNA), 20 μM substrate (2- / 4-OH-E_2) and 50 μM of arachidonic acid in a 100 μl PBS buffer pH 7.0. After shaking at 37°C for 6 hr, the DNA was precipitated by adding 0.1 vol. of 5 N NaCl and 1.5 vol. of cold ethanol. The resulting DNA pellet was washed three times with 70% ethanol, dried and dissolved in distilled water to measure DNA concentration and stored at −20°C for further DNA adducts analyses. A control experiment (CT-DNA alone) was similarly performed at 37°C for 6 hr. Reaction assays were done in triplicate.

DNA hydrolysis and enrichment

Briefly, ~10 μg of DNA was hydrolysed by adding micrococcal endonuclease and spleen phosphodiesterase to the corresponding 2′- deoxyribonucleoside 3′-monophosphates, εdA, εdC and M_1dG were enriched by using immuno-affinity columns prepared by monoclonal antibodies EM-A-1 for εdA, EM-C-1 for εdC and D 10A1 (the latter generously provided by L. Marnett) for M_1dG.[25–27]

Etheno-DNA adducts analyses by ^{32}P-postlabeling/TLC

The εdA, εdC were analyzed according to a procedure described in detail elsewhere.[25,26] The DNA sample was labelled with [γ-^{32}P]ATP and T4-polynucleotide kinase to 5′-deoxynucleoside monophosphate. 500 amol of deoxyuridine 3′-monophosphate (dU 3′-MP) as an internal standard (I.S.) was added to each DNA samples. The mixture was incubated at 37°C for 1 hr and spotted onto the prewashed PEI-cellulose plates. The plates were developed in two-dimensional TLC on polyethyleneimine (PEI) cellulose (D1: 1.0 M acetic acid, pH 3.5; D2: saturated ammonium sulphate, pH 3.5). The plates were exposed to X-ray films (Fuji medical X-ray film, 100 NIF, 18 × 20 cm, Düsseldorf, Germany) in cassettes supported with intensifying screens at −80°C for 2 hr.

The spots corresponding to εdA, εdC and deoxyuridine 5′-monophosphate (I.S.) were cut from the TLC plates, and the radioactivity was determined by liquid scintillation counting. Background counts were determined from appropriate blank areas on the TLC plates and subtracted. The absolute adduct levels were quantitated using standards and the relative adduct level per parent nucleotides were determined with amount of deoxycytidine (dC) and deoxyadenosine (dA) obtained from high performance liquid chromatography analyses as described.[25,26]

M_1dG analyses by ^{32}P-postlabeling-HPLC

M_1dG adduct was analysed following an established procedure described in previously.[27,28] 1.0 fmol of O^4-ethylthymidine 3′-monophosphate (O^4-etT 3′-MP) as an internal standard was added to the dried DNA samples. After addition [γ-^{32}P]ATP (10 μCi, spec. act. 6,000 Ci/mmol) and T4 polynucleotide kinase, the labeled samples were pipetted onto a PEI mini-column, whereas excess ATP remains bound to PEI. Elute was injected into a reverse HPLC with a Prodigy ODS column (Phenomenex, 4.6 × 250 mm, 5 μ) connected to radioactivity detector. The non-radioactive MDA-5′-MP standard was added to each sample as a UV marker to verify that radioactive chromatographic peak (retention time) contained the [^{32}P]M_1dG -5′-MP adduct.

The amount of M_1dG adduct was calculated as (F_2/F_1), whereby F_1 = [c.p.m. of the peak of M_1dG adduct in standard]/[c.p.m. of the peak of O^4-etT (I.S.) in standard] and

F_2 = [c.p.m. of the peak of M_1dG adduct in sample]/[c.p.m. of the peak of O^4-etT in sample].

17β-estradiol metabolites analyses by GC-MS

Plasma samples were prepared following the protocol reported with minor modifications.[29] Briefly, 100 μl of aliquots of plasma from female participants were extracted twice with 1 ml of methylene chloride, 0.25 ng of 4-methoxy-estradiol 3-methyl ether (4-MeOE_2-Me) as an internal standard (I.S.) prepared by dissolving in ethanol before the extraction was spiked into each plasma samples. The supernatant was collected and concentrated to complete dryness under a gentle stream of nitrogen.

To the dried samples, 50 μl of a mixture of hexamethyldisilazane:trimethylchlorosilane:pyridine (3:1:9, by vol.) was added to convert 17β-estradiol metabolites to their trimethylsilyl (TMS)- ether derivatives by incubating at 60°C for 30 min.[30]

Finally, following TMS derivatives, the estrogens were analyzed by GC/MS in the selected ion monitoring mode. GC-MS analyses was carried out by gas chromatography-mass spectrometry (model: HP 5890-5970) equipped with a 5% phenyl-methyl silicone capillary column (30 × 0.25 cm i.d., 0.25 μm film, HP 19091S-433). 1.0 μl of aliquot from 50 μl of reaction volumes were injected into the gas chromatography/mass spectrometry. Helium was used as carrier gas with a linear velocity of 0.9 ml/sec. The oven temperature program was: initial temperature 160°C, and increased to 270°C in 4 min, 270°C for 20 min, running time was 47.5 min. The GC injector temperature was 250°C; the transfer line temperature was held at 280°C. The mass spectrometer parameters for EI mode were: ion source temperature, 230°C; electron energy, 70 eV; filament current, 34.6 μA; electron multiplier voltage, 1,200 V.

Statistical analyses

WBC and plasma samples from 200 randomly selected female participants of the Heidelberg-EPIC study were originally coded. After exclusion of women taking hormones (oral contraceptives, hormone replacement therapy) or without regular menstruation, 124 samples successfully analyzed for DNA adducts in WBC were included in the current analysis. Among those, data on plasma estrogen metabolites were available for 118 samples (116 for 4-MeO-E_2, and 117 for 2- and 4-OH-E_2). Neither adducts nor estrogen metabolite data followed a normal distribution, and were thus log-transformed. The correlation between parameters was determined by means of the Pearson correlation, and multivariable linear regression models (ANOVA) were run to explore the contribution of the estrogen metabolites to explain the variation in DNA adduct levels.

Results

Some characteristics of the premenopausal women included in this study are given in Table 1.

In Figure 2, the typical auto-radiograms of etheno-DNA adduct and HPLC profiles of M_1dG detected in WBC-DNA of the female volunteers are presented. Levels of etheno-DNA and M_1dG adducts detected in WBC-DNA are shown as Box-Whisker plots (Supporting Information Fig. 1S). The levels expressed per 10^8 parent nucleotides (means ± SE) [median] were: 1.56 ± 0.24 [0.77] for εdA/dA; 6.45 ± 0.48 [4.71] for εdC/dC and 25.5 ± 4.04 [8.30] for M_1dG/dG. M_1dG levels were distinctly higher than those of etheno-DNA adducts. Comparison between adduct levels yielded statistically significant correlation coefficients (Pearson correlation) of $r = 0.447$ ($p < 0.001$; εdA and εdC), $r = 0.270$ ($p = 0.002$; εdA and M_1dG) and $r = 0.243$ ($p = 0.007$; εdC and M_1dG).

2- and 4-hydoxylated estradiol metabolites were analyzed by an ultra-sensitive GC-mass spectrometry method based on single ion monitoring (SIM) that we have developed. This

Table 1. Description (means ± SD, median, range) of the random sample of healthy premenopausal women, not taking hormones at the time of blood collection

	%	Mean ± SD
Age (years)		40.5 ± 4.0
Height (cm)		166.0 ± 6.1
Weight (kg)		71.6 ± 17.1
BMI (kg/m^2)		26.1 ± 6.7
Waist (cm)		82.1 ± 14.5
Hip (cm)		103.2 ± 12.7
WHR		0.79 ± 0.07
BMI categories		
<25 kg/m^2	60.7	
25 to 30 kg/m^2	15.5	
≥30 kg/m^2	23.8	
Physical activity (quartiles)		
1 (lowest)	17.5	
2	32.5	
3	35.5	
4 (highest)	14.5	
Educational attainment		
None or primary	15.7	
Secondary/technical	57.3	
Longer education	26.8	
Smoking status		
Never	37.1	
Former	41.3	
Current	21.6	
Number of children		
0	27.4	
1	19.6	
2	39.3	
>2	13.7	

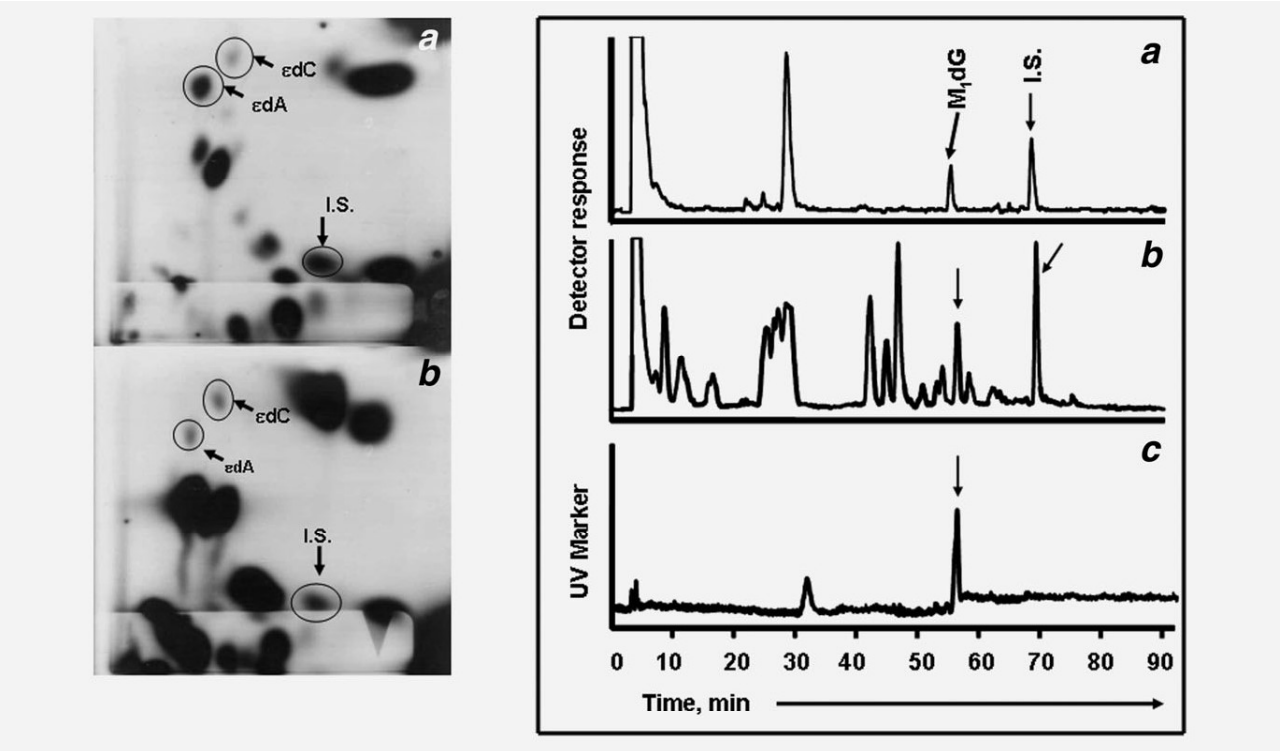

Figure 2. Typical autoradiograms of etheno and M_1dG adducts detected by immuno-^{32}P-postlabeling/TLC (εdA, εdC) and immuno-^{32}P-postlabeling/HPLC (M_1dG) in WBC-DNA from healthy female volunteers. Left panel *a*: TLC of εdA and εdC standards; left panel *b*: TLC of a human WBC sample. Right hand HPLC profile *a*: M_1dG 3′-monophosphate (MP) and O^4-ethylT 3′- MP standards; profile *b*: a human WBC sample; profile *c*: unlabeled M_1dG 5′-MP as a UV- marker.

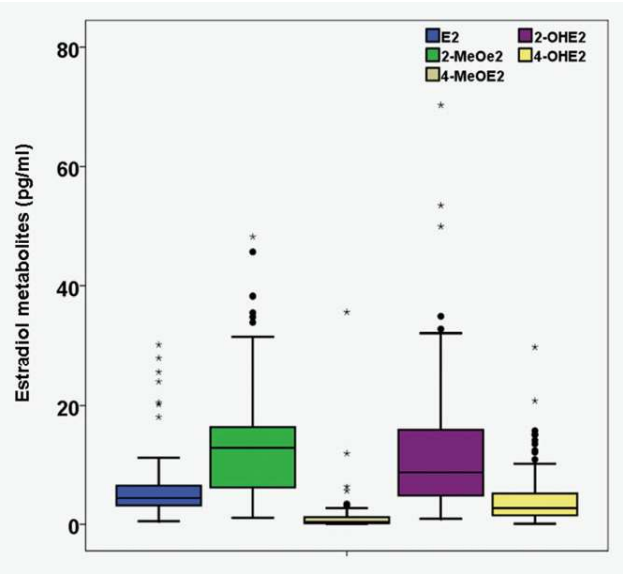

Figure 3. Concentrations of estradiol metabolites measured in plasma samples of premenopausal women (pg/ml). E_2, 17β-estradiol; 2-MeO-E_2, 2-methoxy-estradiol; 4-MeO-E_2, 4-methoxy-estradiol 3-methyl ether; 2-OH-E_2, 2-hydroxy-estradiol; 4-OH-E_2, 4-hydroxy-estradiol. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

method offered a detection limit of 0.1 picogram per injection.

Supporting Information Figure 2S depicts typical GC-MS chromatograms of estradiol metabolites found in plasma in comparison with authentic standards. Concentrations of estradiol metabolites in the plasma samples of premenopausal women are plotted in Figure 3. Mean ± SE [median] concentrations were: 13.4 ± 0.70 [13.5] for 2-$MeOE_2$; 12.9 ± 1.39 [8.8] for 2-OHE_2; 1.12 ± 0.16 [0.4] for 4-$MeOE_2$; and 4.59 ± 0.40 [2.7] for 4-OHE_2.

Applying multivariate linear regression models (ANOVA), significant effects of 4-$MeOE_2$ ($p = 0.001$) and 4-OHE_2 ($p = 0.01$) on εdA adduct levels were observed. Similarly, concentrations of εdC and 4-$MeOE_2$ ($p = 0.007$) and of M_1dG and 4-$MeOE_2$ ($p = 0.018$) were significantly associated. In contrast, 2-$MeOE_2$ and 2-OHE_2 plasma concentrations were associated with lower adduct levels (negative ß-value), significant so for εdA *vs.* 2-OHE_2 ($p = 0.004$) (Table 2).

Searching for possible associations between DNA adduct levels in WBC and food frequency questionnaire data on individual fatty acid intake (Table 3). A significant positive association was found for M_1dG levels and linoleic acid (ω-6 PUFA) intake ($r = 0.198$, $p = 0.027$); M_1dG and dietary LA/OA ratio ($r = 0.218$, $p = 0.015$); and M_1dG and dietary PUFA/MUFA ratio ($r = 0.227$, $p = 0.001$).

Binding to calf thymus (CT-DNA) of LPO by-products produced after aerobic incubation of the 2-OHE_2 or 4-OHE_2 in the presence of arachidonic acid (ω-6 PUFA) was then studied (Supporting Information Table 1S). As analyzed by immunoaffinity-^{32}P-postlabeling and comparison with authentic standards, εdA and εdC were identified as the only detectable adducts. A two to threefold increase in their adduct levels was only observed after incubation at 37°C for 6 hr in the presence 4-OHE_2 but not of 2-OHE_2. Results of a time course study (data not shown), incubating DNA with 4-OHE_2 in the presence of arachidonic acid aerobically for 0.25, 0.5, 1, 2, 6 and 24 hr revealed that etheno-DNA adduct levels increased up to 6 hr. Thereafter, only a marginal further increase was seen. A concentration-dependent increase of adducts was observed by incubating 2 to 200 μM 4-OHE_2 with fixed amounts of DNA and arachidonic acid in100 μl reaction mixture (data not shown).

Table 2. Linear association of WBC DNA adducts and estrogen metabolites measured in plasma samples of premenopausal women (all data were log-transformed; multivariate ANOVA, additionally adjusted for plasma estrogen and intake of saturated, monounsaturated and polyunsaturated fatty acids)

DNA adduct		β	*P* value
εdA			
	2-$MeOE_2$	−0.299	0.123
	4-$MeOE_2$	0.583	0.001
	2-OHE_2	−0.679	0.004
	4-OHE_2	1.208	0.010
εdC			
	2-$MeOE_2$	−0.239	0.228
	4-$MeOE_2$	0.467	0.007
	2-OHE_2	−0.253	0.293
	4-OHE_2	0.534	0.262
M_1dG			
	2-$MeOE_2$	−0.367	0.068
	4-$MeOE_2$	0.415	0.018
	2-OHE_2	−0.313	0.195
	4-OHE_2	0.794	0.100

Discussion

Exocyclic etheno-DNA and M_1dG DNA adducts are formed by reactive aldehydes, such as HNE and MDA, generated by oxidative stress and LPO. The resulting damage to the genome may cause genetic instability and malignant growth.

A major finding in our study was that 4-hydroxy-estradiol metabolite formation and high ω-6 PUFA intake were both linked to increased formation of LPO-derived DNA adducts in WBC of healthy women, participating in the EPIC-Heidelberg cohort study. Also our results of *in vitro* studies (Supporting Information Table 1S) support the postulated pathway (Fig. 1). Elevated etheno-DNA adducts were only observed when DNA was incubated under aerobic conditions together with 4-hydroxy-estradiol and arachidonic acid (ω-6 PUFA). Adduct levels in identical assays with 2-hydroxyestradiol instead were not different from background controls. The plausible mechanism as discussed earlier[14,16] is depicted in Figure 1. Hereby, 4-hydroxy-estradiol could generate ROS *via* redox-cycling and triggers peroxidation ω-6 PUFA to MDA and HNE, which are precursors of exocyclic-DNA adducts. Based on this mechanism, it is plausible to assume that the large inter-individual variations of etheno adducts in WBCs found in women on a controlled[12] or free diet,[31] may be caused by inter-individual variations in the levels and patterns of 2- and 4-hydroxy-estradiol metabolites in addition to modifying effects by dietary vitamin E- and C intake.[31] Such a mechanism (Figure 1) would also explain the gender specificity of DNA adduction in women and rats after a high w-6 PUFA intake.[12,18]

Another supportive mechanistic findings in our study were the positive correlations found between M_1dG levels in WBC and LA (ω-6 PUFA) intake, between M_1dG and the ratio of LA/OA intake and between M_1dG and the ratio of

Table 3. Pearson correlation coefficients for the association of levels of DNA adducts in WBC and individual fatty acid intake in premenopausal women

		Oleic acid (OA)	Linoleic acid (LA)	α-Lino-lenic Acid	Arachidonic acid	Eicosapen-taenoic acid	LA/OA	PUFA/MUFA
εdA	*r*	0.105	0.002	0.058	0.163	0.231	−0.008	−0.071
	p	(0.245)	(0.985)	(0.520)	(0.070)	(0.010)	(0.379)	(0.433)
	n	124	124	124	124	124	124	124
εdC	*r*	0.058	−0.022	−0.008	0.134	0.141	−0.081	−0.077
	p	(0.524)	(0.808)	(0.927)	(0.139)	(0.119)	(0.372)	(0.398)
	n	124	124	124	124	124	124	124
M_1dG	*r*	−0.032	0.198	0.169	0.048	0.051	0.218	0.227
	p	(0.727)	(0.027)	(0.061)	(0.597)	(0.575)	(0.015)	(0.011)
	n	124	124	124	124	124	124	124

Adduct data were log-transformed; fatty acid intake data are expressed as percentage of total energy intake. (Supporting Information).

PUFA/MUFA intake. No such apparent correlations were found for etheno-DNA adducts. These results seem to confirm our earlier report where no difference in mean etheno-DNA adducts in WBC-DNA was observed in two groups of female subjects on a free diet differing significantly in their daily LA intake and LA serum concentration.[31] We assume that etheno adducts in WBC are not determined by individual fatty acid alone but depend on the ratio of ω-6 PUFA to other fatty acids in the diet. The positive correlations found between M_1dG and ω-6 PUFA or LA/OA intake might also be due to faster formation and/ or higher yield of MDA-derived DNA adducts in WBC. To form etheno adducts an additional metabolic step, *e.g.*, oxidation of HNE to an epoxide is required, possibly explaining why etheno adducts in WBC were found to be 5–15 times lower than M_1dG levels.

Earlier findings reported in general a poor correlation between M_1dG and etheno adducts in human DNA samples. In the present study, however, the two adduct types were moderately correlated, suggesting alternative routes to its formation, *e.g.*, base propenals generated *in vivo via* hydroxyl radical attack on DNA, might be more important in estrogen-related etiopathogenesis.[32]

In conclusion miscoding exocyclic DNA adducts such as M_1dG, etheno-dA and etheno-dC were found in WBC of healthy premenopausal women, likely to be formed through LPO after high dietary intake of ω-6 PUFA, a DNA damaging process that was found to be enhanced by 4-hydroxy-estradiol in vitro. Studies on the existence of such an inter-relationship in human breast epithelial tissue, and on amplification of this type of DNA-damage in breast cancer patients are in progress.[33] Previous adduct analyses in a variety of cancer prone organs from humans and rodents[34–36] suggested that LPO-derived DNA lesions could be a cause of gene mutations, acting as a driving force to malignancy. Moreover, epigenetic processes could also be affected, since etheno adducts could be formed in dCpdG islands, *e.g.*, on dC and 5-methyl-dC residues. Our recent study first detected the presence of such etheno modified 5mdC residues (ε5mdC) in human tissue DNA.[37]

The frequency of ε5mdC residues was relatively high (*ca.* 7 per 10^6 5mdC) in WBC samples from women on a high ω-6 PUFA diet.[12] In 10 non-tumorous lung samples from lung cancer patients the ε5mdC/dC ratio was about 10:1. Our results provide support for a possible role of etheno adducts in the alteration of the global methylation status of DNA, currently implicated in the onset of chronic degenerative diseases.

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