Carcinogenesis vol.15 no.7 pp.1399–1404, 1994

# Peroxidation of linoleic, arachidonic and oleic acid in relation to the induction of oxidative DNA damage and cytogenetic effects

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**In the present study, the possible role of the polyunsaturated fatty acids linoleic and arachidonic acid in the chemical induction of carcinogenesis has been investigated. Analysis of 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxodG) levels in 2′-deoxyguanosine (dG) and isolated DNA has demonstrated that linoleic and arachidonic acid are capable of inducing this specific genotoxic damage. This effect appears to be related to the degree of fatty acid unsaturation, since it was not induced by monounsaturated oleic acid. Enzymatic peroxidation of linoleic and arachidonic acid resulted in a significant increase in oxidative DNA damage. Studies on the interference of radical scavengers with the induction of 8-oxodG in combination with electron spin resonance spectroscopy demonstrated that the superoxide anion was generated during peroxidation of these fatty acids and that singlet oxygen is most likely involved in the formation of oxidative DNA damage. The level of oxidative damage in dG and single-stranded DNA was higher as compared to that in native DNA after equimolar treatment. Exposure of human lymphocytes to linoleic or arachidonic acid did not result in a significant increase in levels of 8-oxodG. This may indicate that the rate of intracellular peroxidation is relatively low and/or that nuclear DNA in intact cells is effectively protected against genetic damage induced by reactive oxygen species. It is therefore concluded that relatively short periods of linoleic or arachidonic acid administration are not likely to impose a direct genotoxic risk. It can, however, not be excluded that chronic exposure to polyunsaturated fatty acids induces oxidative DNA damage or is related to cancer risk by epigenetic mechanisms, as is also indicated by the observed cytotoxic effects of linoleic and arachidonic acid.**

## Introduction

Dietary fat has been correlated with incidence of and mortality from cancer of the breast, colon and several other sites (1–5). Apart from the epidemiological indications for a fat–cancer correlation, there is supporting evidence for a causal relation between dietary fat and cancer from animal studies (6–8). Generally, tumour formation is more frequent and more rapid in rats or mice fed on diets containing high levels of polyunsaturated fatty acids as compared to animals on diets rich in saturated fat. It has been suggested that saturated fats may influence the initiation phase of carcinogenesis (9), whereas polyunsaturated fatty acids (PUFA*), such as linoleic acid (LA), act in the promotional phase (10,11). On the other hand, lipid oxidation products of linoleic and arachidonic acid (AA) have also been reported to exert genotoxic and cytogenetic effects in various short-term assays using mammalian cells (12–15). Furthermore, the mutagenic activity of repeatedly used deep-frying fats was found to correlate with levels of linoleic acid hydroperoxides (16,17), suggesting an initiating role of these compounds in carcinogenesis.

With regard to the mechanism of PUFA-induced genotoxic effects, the involvement of various reactive intermediates has been proposed. It is well known that OH radicals are generated during lipid peroxidation processes or arise directly from linoleic acid hydroperoxide decomposition (18). Furthermore, alkoxy and peroxyl radicals as well as reactive aldehydes can be formed from PUFA hydroperoxides (19–22). These fatty acid-derived free radicals are thought to be intermediates in the causation of damage of biomembranes, enzymes or other proteins and nucleic acids. Indeed, the induction of 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxodG), a marker of oxidative DNA damage, has previously been reported after exposure of calf thymus DNA to auto-oxidized methyl linoleate (23). An overview of possible interactions of lipid peroxidation products with DNA has been given by Vaca *et al.* (14). Although there is no experimental evidence for the involvement of lipid peroxidation products in the initiation or promotion of carcinogenesis, this hypothesis is supported by the fact that the most effective defence systems against mutagenic and carcinogenic effects also prevent oxygen radical-induced DNA damage and lipid peroxidation (24,25).

In the present study 8-oxodG formation in nucleosides, single (ss)- and double (ds)-stranded DNA has been examined after treatment with both PUFA linoleic and arachidonic acid and monounsaturated oleic acid (OA). The effect of peroxidative enzymes on the 8-oxodG levels induced by LA, AA and OA has also been determined. In order to elucidate the involvement of oxygen free radicals in this reaction, radical generation and the effect of radical scavengers on 8-oxodG induction have been established using electron spin resonance (ESR) spectroscopy and HPLC in combination with electrochemical detection. Since it is of crucial importance to establish whether possible effects in naked DNA are also observed in intact cell systems, 8-oxodG levels have also been analysed in cellular DNA after exposure of human lymphocytes to the test compounds. A possible correlation between the level of oxidative DNA damage and both cytogenetic effects and the inactivation of ss bacteriophage DNA has been evaluated.

## Materials and methods

### *Chemicals*

Sodium salts of oleic acid (18:1, n-9), linoleic acid (18:2, n-6) and arachidonic acid (20:4, n-6), as well as nuclease P1, alkaline phosphatase, RNase T1, RNase

*Abbreviations: PUFA, polyunsaturated fatty acids; FCS, fetal calf serum (heat-inactivated); LA, linoleic acid (18:2); AA, arachidonic acid (20:4); 8-oxodG, 7,8-dihydro-8-oxo-2′-deoxyguanosine; OA, oleic acid (18:1); ss, single-stranded; ds, double-stranded, PHS, prostaglandin H synthase; LO, lipoxidase; SOD, superoxide dismutase; tBuOH, t-butylalcohol; TMP, 2,2,6,6-tetramethylpiperidine; dG, 2′-deoxyguanosine; CytB, cytochalasin B; DMPO, 5,5 dimethyl-1-pyrroline *N*-oxide; ESR, *electron spin resonance*; *pen/strep*, *penicillin–streptomycin* solution; PHA, phytohaemagglutinin (M-form); HBSS, Hank's balanced salt solution; DAM, desferroxamine; MN, micronucleus; SCE, sister chromatid exchange.

A, prostaglandin H synthase (PHS) and lipoxidase (LO), superoxide dismutase (SOD), sodium azide, 2'-deoxyguanosine (dG), bisbenzimide (Hoechst 33258) and cytochalasin B (CytB) were from Sigma Chemical Co. (St Louis, MO). t-Butylalcohol (tBuOH) and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) were purchased from Aldrich (Steinheim, Germany). Proteinase K was from Boehringer (Mannheim, Germany). DMEM, RPMI 1640, fetal calf serum (FCS), L-glutamine, penicillin–streptomycin (pen/strep), trypsin (1:250), phytohaemagglutinin (PHA) (M-form), colcemid (10 μg/ml) and Hank's balanced salt solution (HBSS) (10×) were obtained from Gibco. Sodium heparin (Thromboliquine, 25000 IE) was from Organon Technika (Oss, The Netherlands).

The sodium salts of oleic, linoleic and arachidonic acids were dissolved in water. At the applied concentrations (mM range), stearic acid could only be dissolved in methanol or dimethyl sulphoxide. Since both compounds are known to possess radical scavenging effects, this unsaturated fatty acid appeared unsuitable as a negative control. The total amount of test solution in the culture medium was 5–10%.

The culture media DMEM and RPMI 1640 were supplemented with 10% FCS, L-glutamine (final concentration 30 g/l), heparin and antibiotics (penicilllin and streptomycin, both 100 U/ml final concentration).

*Induction of 8-oxodG in dG*

In order to establish the quantitative relationship between exposure of dG to linoleic (18:2, n-6), arachidonic (20:4; n-6) or oleic acid (18:1; n-9) and the induction of 8-oxodG as a marker of oxidative damage, various time–response and dose–response curves have been determined. Solutions of 2.6 mM dG in 10 mM Tris–HCl (pH 7.5) were incubated at 37°C for periods of 15 min up to 28 h in the presence of the sodium salts of the various fatty acids in concentrations ranging from 0.2 to 10 mM in a total volume of 500 μl. Effects of enzymatic peroxidation of LA and OA by 400–1000 units of LO have been investigated, whereas the effect of enzymatic peroxidation of AA on the induction of 8-oxodG was also studied by addition of 50–200 units of PHS. All incubations using LO contained 1 nM $H_2O_2$ as an additional cofactor. Incubations with PHS were supplemented with 1 μM haematine.

In order to study the involvement of superoxide in the formation of 8-oxodG, SOD (120–360 units) and DMPO (10–30 mM) were added to the incubation mixtures. Since DMPO may react with both superoxide and OH radicals, an effect of DMPO observed in these incubations does not discriminate between either intermediate. Therefore, effects of the more selective OH radical scavenger tBuOH (105, 210, 315 or 630 mM) were also studied. The possible involvement of singlet oxygen in the formation of 8-oxodG was investigated by quenching singlet oxygen with sodium azide (10–50 mM). Furthermore, desferroxamine mesylate (DAM; 1 and 3 mM) was added as an iron chelator.

*Induction of 8-oxodG in isolated DNA*

Rat liver DNA was isolated applying methods described by Gupta with minor modifications (26). Four grams of rat liver were homogenized in 40 ml PBS containing 1% SDS/1 mM EDTA. The homogenate was incubated with proteinase K (500 μg/ml) for 30 min at 37°C. After addition of 2 ml M Tris–HCl (pH 7.4), the homogenate was successively extracted for 5 min with equal volumes of phenol, phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1). After addition of 4 ml 5 M NaCl, DNA was precipitated with 2 volumes of 96% ethanol (4°C). High molecular weight DNA was isolated and rinsed twice with 70% ethanol. After drying, DNA was dissolved in 8 ml 1.5 mM NaCl/0.15 mM Na citrate/1 mM EDTA and treated during 30 min at 37°C with 50 units/ml RNAse $T_1$ and 100 μg/ml RNAse A. Excess of glycogen was removed by treatment with 500 μl amylase (1 mg/ml, 30 min at 37°C). This DNA solution was again extracted with chloroform/isoamyl alcohol (24:1) and precipitated, washed and dissolved as described above. Final DNA concentration was determined spectrophotometrically at 260 nm (1 unit $A_{260}$ = 50 μg ds DNA/ml). ss DNA was obtained by denaturation of native DNA at 100°C for 5 min and subsequent cooling in ice–water. Denaturation was checked by spectrophotometric measurement of the hyperchromic effect at 260 nm (1 unit $A_{260}$ = 40 μg ss DNA/ml).

Both ss and ds DNA (500 μl, 567 μg/ml) were incubated in duplex during 21 h at 37°C with LA, AA or OA (OA was only incubated with ds DNA), with or without LO or PHS. After incubation, peroxidative enzymes were removed after denaturation at 100°C (3 min) by centrifugation. The supernatant was removed from the pellet and DNA was precipitated with 2 volumes of 96% ethanol (4°C), washed twice with 70% ethanol and dissolved in 5 mM Tris–HCl (pH 7.4).

*Induction of 8-oxodG in cellular DNA*

Lymphocytes from healthy donors were isolated using lymphoprep density centrifugation. For each incubation, $5 \times 10^5$ lymphocytes at a density of $5 \times 10^6$/ml were stimulated for 12 h by 1.5 ml PHA. At $t$ = 12 h, the test substance was added for 24 h. The cells were washed in HBSS and were frozen in RPMI 1640.

Isolated human fibroblasts were cultured in DMEM with supplementation. Before confluency fresh culture medium with test solution was added to the cultures for 24 h, whereupon the cells were washed in HBSS and trypsinized by 0.25% trypsin/0.04% EDTA.

Since it could be expected that stimulation of extracellular radical generation results in cytotoxic effects rather than increased cellular DNA damage, no peroxidative enzymes were added during the incubations. Cytotoxicity of LA, AA and OA was established by determination of trypan blue exclusion. Only non-cytotoxic concentrations were applied for the cellular induction of 8-oxodG. DNA was isolated from lymphocytes according to the methods described above.

*HPLC analysis of 8-oxodG*

DNA solutions containing approximately 1 mg DNA/ml in 33 mM sodium acetate (pH 5.1) and 1 mM $ZnCl_2$ were digested to 2'-deoxyribonucleoside 3'-monophosphates by 2.5 units nuclease P1/100 μg DNA. This solution was adjusted to pH 7.5 by adding 0.33 volumes of 0.4 M Tris–HCl (pH 7.5) and incubated for 30 min at 37°C with alkaline phosphatase (2.5 units/100 μg DNA). Analysis of 8-oxodG was performed on HPLC, applying a Gyncotech 480 high precision pump coupled with a Spectroflow 783 programmable absorbance detector and an Antec electrochemical detector (850 mV). As stationary phase a Supelcosil™ column (Supelco; 250 × 4.6 mm) was used in combination with a guard column (ODS pellicular; 75 × 2.1 mm). The mobile phase consisted of 15% aqueous methanol containing 12.5 mM citric acid, 25 mM sodium acetate, 10 mM acetic acid and 30 mM NaOH and elution was performed at a flow rate of 1.0 ml/min. For the analysis of 8-oxodG in incubations containing PHS, eluents with 10% methanol and a flow rate of 1.1 ml/min were used to provide optimal separation from an unknown contaminant present in the commercial enzyme preparation.

*Electron spin resonance spectroscopy*

ESR measurements were performed at room temperature on a Bruker ESP-300 with an ESP 1600 data processor, equipped with an ER 4102 ST standard rectangular cavity. Instrumental conditions were as described in the legends of the figures. DMPO was used for trapping both hydroxyl and superoxide radicals. Solutions of DMPO in nitrogen-flushed milli Q water were purified by gentle mixing with 30 mg/ml charcoal during 20 min at 35°C. This procedure was repeated three times to reduce the background ESR signal. Incubations with LA, AA or OA were performed during 15 min at 37°C in a total volume of 0.5 ml PBS (pH 7.0). The reference spectrum of the DMPO–OH adduct was obtained from incubations with $H_2O_2$.

*Cytogenicity testing*

Blood from a healthy donor was used (0.4 ml) and cultured in 4.4 ml RPMI 1640 containing L-glutamine, heparin, pen/strep and 10% FCS in a sterile 10 ml tube. The blood cultures were incubated at 37°C with 5% $CO_2$ in humidified air. At $t$ = 0 h, each incubation was stimulated with 0.2 ml PHA. For micronucleus (MN) analysis, the blood cultures were exposed in duplex to LA, AA and OA at $t$ = 24 h. At $t$ = 44 h, CytB was added to a final concentration of 6 μg/ml. At $t$ = 72 h, cells were washed in HBSS and treated with hypotonic 75 mM KCl for 20 min at 37°C. After centrifugation at 1500 r.p.m. for 8 min, the exposed lymphocytes were fixed three times in methanol:acetic acid (v/v 3:1) and dropped on cold slides. Slides were stained with 5% Giemsa in phosphate buffer for 20 min. Slides from each treatment were randomized and coded prior to scoring. The number of micronuclei (MN) for each dose level was determined by scoring 2000 binucleated cells.

For the sister chromatid exchange (SCE) assay, stimulated blood cultures were incubated in the presence of bromodeoxyuridine (20 μg/ml final concentration) at $t$ = 24 h. At $t$ = 48 h, whole blood cultures were exposed to LA, AA or OA until cell harvesting at $t$ = 72 h. One hour prior to cell harvesting, colcemid (final concentration 2 μg/ml) was added to the cultures. Fixed cells were dropped on cold slides and stained according to standardized procedures (27). Slides from each treatment were randomized and coded prior to scoring. The number of SCEs for each dose was determined by scoring 40 metaphases containing at least 40 chromosomes.

*Inactivation of ss φX-174 DNA*

Studying the induction of DNA damage in relation to its effect on the biological activity of DNA might contribute to a better understanding of the processes involved in the genotoxic potential of polyunsaturated fatty acids. Therefore, biological inactivation of ss bacteriophage (φX-174) DNA by LA and AA has been quantified. This assay provides a relatively simple system as compared to the *Salmonella* mutagenicity test, because the biological consequences of interactions between naked ss phage DNA and oxygen radicals generated during (enzymatic) peroxidation can be studied without the interference of cellular components.

φX-174 DNA was isolated from wild-type φX-174 bacteriophage according to Blok *et al.* (28). LA and AA (both 0.2 mM) were incubated with 1 μg/ml ss φX-174 DNA in the presence of 400 units of LO or 87 units PHS, respectively. Incubations were performed at 37°C during 48 h in a volume of 500 μl. Every hour, during the first 6 h, reaction mixtures were oxygenated for 1 min. At various time intervals, 20 μl samples were taken and diluted 50-fold in ice-cold 0.05 M Tris–HCl (pH 8.0) to stop the reaction. Biological inactivation was determined after transfection of the phage DNA to freshly prepared *Escherichia coli* (AB1157)

spheroplasts (29). After incubation of mixtures of 100 μl DNA solution (1 μg/ml) and 100 μl spheroplast suspension at 37°C for 10 min, 0.8 ml LBM (Luria Broth Medium with 10% (w/v) sucrose, 0.1% glucose, 0.2% $MgCl_2$) was added and the incubation was continued for 90 min. Bacteriophage yield was determined after addition of 4 ml cold distilled water by plating with *E.coli* (28). The number of plaques provides a measure for the number of non-damaged DNA strands.

*Statistics*

Student's *t*-test was used for the comparison of averaged hydroxylation ratios, calculated from duplex incubations and analyses. This test was furthermore used to compare the averaged number of MN and SCEs, calculated from duplex incubations.

## Results

### *Analysis of DNA damage and radical generation*

*Induction of 8-oxodG in dG.* The induction of 8-oxodG by LA, AA and OA during incubations with 2.6 mM dG is presented in Figure 1. The results show that concentrations of both LA and AA of 1 mM and higher induce a significant increase in the hydroxylation ratio. On the other hand, monounsaturated OA is not found to induce 8-oxodG levels above the background of $2.0 \times 10^{-5}$ 8-oxodG/dG. The effects on the induction of 8-oxodG by 2 mM LA caused by various radical scavengers and compounds which are known to interfere with radical generation by other mechanisms are summarized in Table I. Since the reference values are found to vary between experiments (26.9 8-oxodG/dG ± 6.3, $n = 5$), the results are expressed as a percentage of the hydroxylation ratio in the 2 mM LA reference incubation in each separate experiment. The LA-induced 8-oxodG is found to be reduced to approximately background levels (averaged background 5.9 ± 1.3%, $n = 5$) by addition of DMPO, indicating the involvement of OH radicals and/or superoxide. In the presence of 25 units SOD this hydroxylation ratio is also found to be significantly lower as compared to the reference value, although the reduction is not total and cannot be increased by raising the concentration of this enzyme. No significant effect is found for tBuOH. In the presence of sodium azide, the hydroxylation ratio is reduced to about 50% of the 2 mM LA reference, whereas addition of the iron chelator DAM is found to interfere most effectively with the induction of 8-oxodG by LA.

The effect of enzymatic peroxidation during the first 4 h of LA treatment by 400 or 1000 U LO is shown in Figure 2. After 21 h of incubation, hydroxylation ratios with and without 1000 units LO are 43.8 ± 2.4 and 24.2 ± 1.1 8-oxodG/dG × $10^5$ ($n = 5$), respectively. PHS (87 units) was found to stimulate the induction of 8-oxodG by 2 mM AA significantly, whereas peroxidation of OA by 400 or 1000 units LO is not found to result in increased hydroxylation levels (data not shown).

*Induction of 8-oxodG in isolated and cellular DNA.* The induced levels of oxidative damage in isolated ss and ds DNA are illustrated in Figure 3a and b. The level of LA- or AA-induced 8-oxodG is found to be considerably higher in ss as compared to that in native DNA. Enzymatic peroxidation of LA and AA

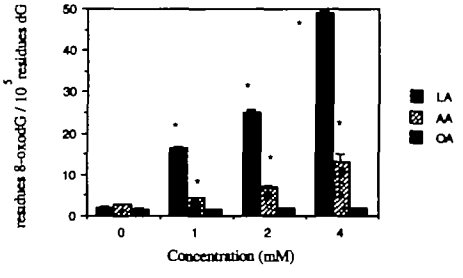


**Fig. 1.** Effects of various concentrations of LA, AA and OA on the hydroxylation of dG (2.6 mM) induced during 21 h of incubation at 37°C. *All ratios determined at various exposure levels of both LA and AA are significantly different from the control ($P < 0.05$).

**Table I.** Effect of various compounds on the induction of 8-oxodG after incubation of 2.6 mM dG with 2 mM sodium linoleate for 21 h at 37°C

| Compound | Concentration | Response (%)[a] | SD (%) | *P* value[b] |
|---|---|---|---|---|
| DMPO | 25 mM | 13.8 | 1.3 | <0.01 |
| | 50 mM | 15.2 | 1.2 | <0.01 |
| | 75 mM | 13.3 | 1.4 | <0.01 |
| SOD | 25 units | 60.3 | 4.9 | <0.01 |
| | 125 units | 65.8 | 5.8 | <0.01 |
| | 250 units | 72.9 | 6.0 | <0.01 |
| tBuOH | 53 mM | 109.4 | 3.4 | NS |
| | 106 mM | 108.7 | 0.9 | NS |
| | 212 mM | 103.6 | 8.1 | NS |
| Sodium azide | 10 mM | 48.1 | 4.1 | <0.01 |
| | 25 mM | 50.1 | 11.2 | <0.01 |
| DAM | 1 mM | 5.0 | 0.3 | <0.01 |
| | 3 mM | 3.4 | 0.1 | <0.01 |

[a]Response is expressed as a percentage of the hydroxylation ratio established in the 2 mM linoleate reference in each separate experiment.
[b]Unpaired Student's *t*-test (compared to the 2 mM linoleate reference incubation = 100%).
NS, non-significant.

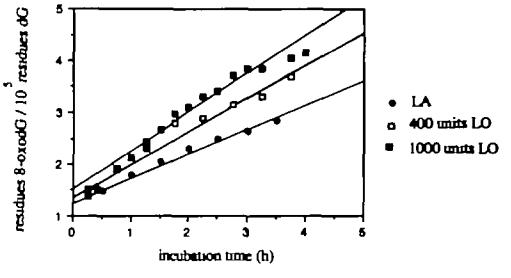


**Fig. 2.** Effect of 400 and 1000 units of LO on the hydroxylation ratio induced by 2 mM LA.

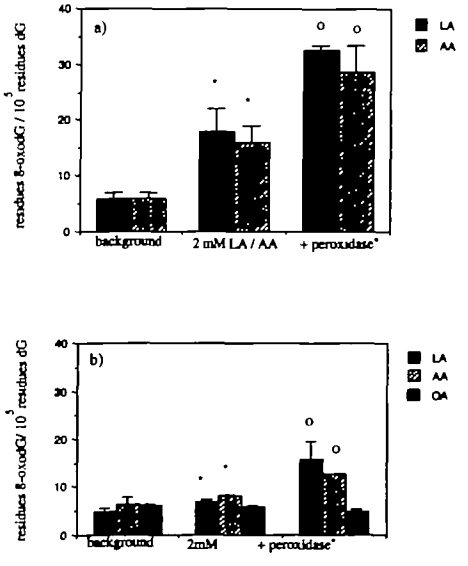


**Fig. 3.** Effect of LA, AA and OA on the hydroxylation of the guanine residue induced during 21 h of incubation at 37°C in isolated ss (a) and ds (b) rat liver DNA. *400 units LO (incubations with LA); 1000 units LO (incubations with OA); 200 units PHS (incubations with AA). *Significantly higher as compared to the background ($P < 0.05$). °Significantly higher as compared to the non-enzymatic incubations ($P < 0.05$).

Table II. Induction of 8-oxodG in DNA from human lymphocytes

| Compound | Concentration (mM) | Ratio 8-oxodG/dG ($\times 10^{-5}$) | SD ($\times 10^{-5}$) | $P^a$ |
|---|---|---|---|---|
| Reference | 0.0 | 7.6 | 1.5 | >0.05 |
| Linoleic acid | 0.5 | NQ | | |
| | 1.0 | 8.9 | 2.0 | >0.05 |
| | 1.5 | 8.7 | 1.6 | >0.05 |
| Arachidonic acid | 0.15 | NQ | | |
| | 0.3 | 6.6 | 3.2 | >0.05 |
| | 0.5 | 10.5 | 2.5 | >0.05 |
| Oleic acid | 0.15 | 2.2 | 0.4 | 0.02 |
| | 0.3 | 12.2 | 1.6 | 0.04 |
| | 0.5 | 6.5 | 0.7 | >0.05 |

[a]Unpaired Student's *t*-test (compared to the 0 mM reference incubation).

is found to increase the hydroxylation ratio significantly in both types of DNA. OA as well as the combination of OA and 1000 units LO again did not induce 8-oxodG in ds DNA. Levels of 8-oxodG analysed in DNA isolated from human lymphocytes are shown in Table II. These results indicate that only the incubation with 0.3 mM OA results in a significantly increased hydroxylation ratio. Since the hydroxylation ratio was not increased using 0.5 mM OA and was even significantly lower using 0.15 mM OA, no distinct dose–response relationship could be assessed.

*Electron spin resonance spectroscopy.* ESR spectra recorded after incubation of 100 mM DMPO with 2 mM LA with and without 1000 units LO are compared with the typical DMPO–OH spin trap adduct induced by 100 mM $H_2O_2$ in a solution containing 10 mM DMPO (Figure 4). These results show that the ESR signal induced by LA is identical to the DMPO–OH signal ($A_N = A_H^\beta = 15.0$ G). Furthermore, the signal intensity is found to be increased by a factor of two after addition of LO, as calculated from the signal-to-noise ratios (11.1 and 20.6, respectively). In Figure 5, signal intensities recorded during incubations with different fatty acids are compared. A dose–response relationship was observed between the concentrations of LA and AA as well as OA and the signal intensity of the typical DMPO–OH spin trap adducts. Furthermore, addition of 1000 units LO stimulated the induction of this spin trap adduct in incubations with both LA and OA. The effect of LO was also determined in combination with 2 mM AA, and resulted in an increase in the signal-to-noise ratio from 14.0 to 19.7.

*Analysis of biological effects*

*Inactivation of ss φX-174 DNA.* The results in Figure 6 demonstrate that after 24 h of incubation with LA/LO and AA/PHS, φX-174 DNA is inactivated by a factor of $10^3$ and $10^5$, respectively. After incubation during 48 h with LA/LO, no surviving DNA was found. These results confirm our previous observation that peroxidation of these polyunsaturated fatty acids may result in considerable genetic damage.

*Cytogenicity testing*

The induction of cytogenetic effects after exposure of human lymphocytes to LA, AA or OA is presented in Table III. From these results it has to be concluded that under the applied experimental conditions no micronuclei are induced. The same holds true for the number of SCEs after incubation with LA. Quantification of the number of SCEs after incubation with AA and OA was impossible, since each slide showed only two to three properly stained metaphases. This effect was repeatedly found in different experiments and could unfortunately not be eliminated.


Fig. 4. ESR, signals recorded after 15 min of incubation in the presence of 10 mM DMPO of (a) 100 mM $H_2O_2$ 10 scans ($A_N$ = 15.0 G) and in presence of 100 mM DMPO of (b) 2 mM LA, 20 scans and (c) 2 mM LA with 1000 units LO, 20 scans. Instrumental conditions: modulation amplitude, 1 G; receiver gain 2 × $10^5$ ms; time constant, 40.96 ms; scan time, 20.97 s; power, 100 mW; magnetic field, 3480 ± 40 G.

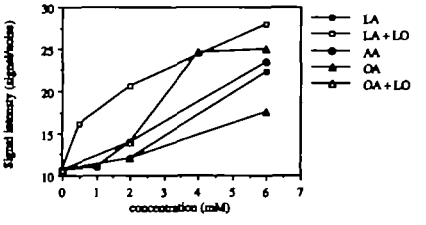


Fig. 5. Dose–response relationship between concentration of the fatty acids LA, AA or OA and the ESR signal intensity of the DMPO–OH spin trap adduct, expressed as signal-to-noise ratio.

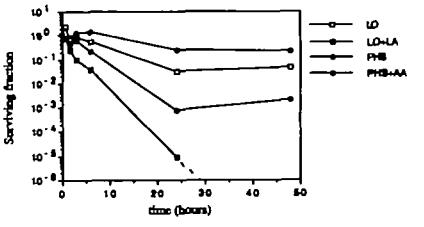


Fig. 6. Surviving fraction of φX-174 DNA after incubation with 0.2 mM LA or 0.2 mM AA in combination with the peroxidative enzymes LO (400 units) or PHS (87 units) at 37°C during 48 h. Inactivation of bacteriophage DNA by PHS and LO alone is presented as a reference.

Table III. Cytogenetic effects of various fatty acids in human lymphocytes

| Compound | Concentration (mM) | Micronuclei/2000 cells | SCEs | SD |
|---|---|---|---|---|
| Linoleic acid | 0.0 | 15 | 7.26 | 2.59 |
| | 0.5 | 15 | 9.69 | 2.93 |
| | 0.75 | 7 | 6.33 | 2.65 |
| | 1.0 | 9 | ND | |
| | 1.25 | 9 | 8.37 | 2.57 |
| | 1.5 | 10 | ND | |
| Arachidonic acid | 0.0 | 7 | – | |
| | 0.1 | 7 | – | |
| | 0.2 | 6 | – | |
| | 0.3 | 4 | – | |
| | 0.4 | 8 | – | |
| | 0.5 | 10 | – | |
| Oleic acid | 0.0 | 6 | – | |
| | 0.1 | 8 | – | |
| | 0.2 | 7 | – | |
| | 0.3 | 7 | – | |
| | 0.4 | 6 | – | |
| | 0.5 | 8 | – | |

–, not quantifiable.
ND, not determined.

## Discussion

In the present study, the hypothesized role of the polyunsaturated fatty acids LA and AA in the chemical induction of carcinogenesis has been investigated at various levels. At the molecular level, the induction of 8-oxodG has been determined as a marker of oxidative DNA damage, whereas ESR spectroscopy has been applied for the detection of (oxygen) radical formation. At the level of biological activity, inactivation of phage DNA in a non-cellular environment has been determined. Additionally, cytogenetic effects have been analysed in human lymphocytes.

The results of the chemical analysis of oxidative DNA damage as measured by the induction of 8-oxodG clearly confirm previous observations that both polyunsaturated fatty acids induce this type of DNA damage in a dose-dependent manner (23). It was furthermore shown that enzymatic peroxidation of the compounds results in a significant increase in this genotoxic effect. Neither the induction of 8-oxodG nor the stimulating effect of enzymatic peroxidation have been established after incubation with monounsaturated OA. This indicates that this particular genetic risk only relates to exposure of the polyunsaturated fatty acids. The fact that the hydroxylation ratio is found to be higher in incubations with purified nucleotides and lower in ds DNA suggests protection by steric hindrance of the critical target sites. In contrast to these results, the hydroxylation ratio in cellular DNA was not found to be elevated after incubation with LA or AA. It may therefore be suggested that in intact cell systems either the reactive intermediates do not reach the DNA in the nucleus as a consequence of interaction with intracellular anti-oxidative systems or that the induced oxidative DNA damage is effectively removed by cellular DNA repair activity. This observation could furthermore be explained by a relatively low level of cellular peroxidase activity as compared to the experimental conditions we have applied *in vitro*. Based on the results from the present study, no discrimination can be made between these three alternatives.

Regarding the mechanism of *in vitro* induction of 8-oxodG, the involvement of superoxide has been demonstrated by the reduction of the hydroxylation ratio after addition of DMPO, as well as SOD. This is in accordance with the hypothesis of Chamulitrat *et al.* (30) that hydroperoxides of PUFA are reduced to form alkoxyl radicals which react after intramolecular rearrangement with oxygen and eventually release the superoxide radical anion. Since superoxide itself is not likely to induce biologically relevant DNA damage (28,31), the induction of oxidative DNA damage may be more directly related to the presence of either or both OH radicals and singlet oxygen. These reactive oxygen species may be generated from superoxide by a combination of dismutation of superoxide and the iron catalysed Haber–Weiss reaction, as described previously (32). Singlet oxygen may furthermore be generated directly during lipid peroxidation processes (33). In our experiments, the formation of singlet oxygen was demonstrated by the observed reduction of the hydroxylation ratio during incubations in the presence of singlet oxygen quenching sodium azide. On the other hand, no reduction of the hydroxylation ratio was found after scavenging OH radicals with tBuOH. Since ESR measurements using $H_2O_2$ demonstrated that OH radicals generated by the Fenton reaction can be effectively scavenged, we conclude that OH radicals are not likely to be involved in the induction of 8-oxodG. In addition, ESR measurements have been performed in order to detect reactive oxygen species and/or lipid radicals generated during peroxidation of fatty acids. The only ESR signal detected after incubation with LA and AA using DMPO as spin trap was found to be identical to the DMPO–OH spectrum. Theoretically, this spin trap adduct may be formed by direct trapping of OH radicals by DMPO or result from the chemical decay of the DMPO–OOH adduct which in turn results from trapping of superoxide (34). During incubations of OA and DMPO, the same spectrum has been recorded, showing a dose-related increase in signal intensity comparable to that of incubations with LA and AA. This signal intensity is furthermore increased by enzymatic peroxidation with LO. Since OA, in contrast to LA and AA, was not found to induce 8-oxodG, whereas ESR measurement demonstrated the DMPO–OH spin adduct using all three fatty acids, the formation of this spin adduct might not be related to the induction of 8-oxodG. This can only be explained when the DMPO–OH adduct is a result of trapping OH radicals, which, as we suggested above, are not likely to contribute substantially to the induction of 8-oxodG. We therefore suggest that the

induced 8-oxodG is mainly the result of the interaction of DNA or nucleotides with singlet oxygen.

It should furthermore be mentioned that we were unable to detect the composite ESR signal reported previously (30) and which was suggested to be the resultant of five simultaneously formed DMPO−adducts, including DMPO−OH, DMPO−OOH, a peroxyl radical adduct (DMPO−OOL) and two species of carbon-centred radical adducts (DMPO−COR and DMPO−R). Since all experimental and measurement conditions were identical to those described in this report (30), except that enzyme preparations and fatty acid suspensions were not chelex treated, it should be concluded that in the presence of trace amounts of metal ions the induced DMPO−OH signal surpasses all other spectra. This raises the question whether at physiological conditions these peroxyl and carbon-centred radicals may contribute to the induction of genetic damage, for instance by one-electron oxidation of guanine residues.

The absence of significant cytogenetic effects after incubations of human lymphocytes with LA or AA is in accordance with the observation that cellular levels of 8-oxodG were also not increased. Most of the mutagenic and other cytogenetic effects which have previously been established in mammalian cells were studied using aldehydic or other lipid peroxidation products of PUFA. These compounds, including malondialdehyde and 4-hydroxynonenal, were found to induce SCE, DNA fragmentation and mutations at the hypoxanthine−guanine phosphoribosyltransferase locus (12,13,35). Although intracellular peroxidation of PUFA may generate reactive oxygen species, this probably does not impose a genotoxic risk, due to effective defence and steric hindrance of DNA target sites. However, chronic exposure to PUFA-derived radicals may result in depletion of various cell defence systems or compounds such as glutathione, vitamins C and E, β-carotene, uric acid and possibly DNA repair capacity. Due to these long term effects of oxidative stress, susceptibility to DNA damage induced by either reactive oxygen species or other initiating factors may increase substantially.

Furthermore, exposure to PUFA may relate to carcinogenic events as a result of epigenetic mechanisms. Cytotoxic effects, which have been demonstrated by trypan blue exclusion tests in this study (data not shown), may stimulate cell proliferation *in vivo*, for instance in the gastrointestinal tract, and thereby also affect tumour promotion processes.

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*Received on January 21, 1994; revised on April 7, 1994; accepted on April 12, 1994*