Oxidative DNA damage estimated by oxo^{8}dG in the liver of guinea-pigs supplemented with graded dietary doses of ascorbic acid and α-tocopherol

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Dietary antioxidants may influence cancer risk and aging by modifying oxidative damage. The effect of graded dietary doses of the antioxidant vitamins C and E on oxidative DNA damage was studied in the liver of guinea-pigs under normal conditions. Like human beings, guinea-pigs cannot synthesize ascorbate and α-tocopherol. In one experiment, three groups of 6–8 guinea-pigs were fed diets containing 15 mg of vitamin E/kg chow and three different amounts of vitamin C (33 660 or 13 200 mg/kg) for 5 weeks. In a second experiment, three groups of seven guinea-pigs were fed diets containing 660 mg of vitamin C/kg and three different amounts of vitamin E (15, 150 or 1500 mg/kg) for 5 weeks. The three graded levels of each vitamin respectively represent marginal deficiency, an optimum supplementation and a megadose. Oxidative damage to liver DNA was estimated by measuring 8-oxo-7,8-dihydro-2′-deoxyguanosine (oxo^{8}dG) referred to deoxyguanosine (dG) by means of high-performance liquid chromatography with simultaneous electrochemical-coulometric and ultraviolet detection. The level of ascorbate in the liver was 0.034 ± 0.051, 1.63 ± 1.06 and 1.99 ± 0.44 μmol/g in the low, medium and high dose ascorbate groups (59-fold variation). The liver concentration of α-tocopherol was 28 ± 11, 63 ± 18 and 187 ± 34 nmol/g in the low, medium and high dose α-tocopherol groups (7-fold variation). The level of oxo^{8}dG in the liver DNA was 1.89 ± 0.32, 1.94 ± 0.78 and 1.93 ± 0.65 per 10^5 dG in the low, medium and high dose ascorbate groups (no effect: $P > 0.05$). In the low, medium and high dose α-tocopherol groups oxo^{8}dG level in the liver DNA was 2.85 ± 0.70, 2.74 ± 0.66 and 2.61 ± 0.92 per 10^5 dG (no effect: $P > 0.05$). It is concluded that even very large variations in the content of the antioxidant vitamins C and E in the diet and liver have no influence on the steady-state level of oxidative damage to guanine in the liver DNA of normal unstressed guinea-pigs.

Introduction

Reactive oxygen species (ROS*) generated endogenously as by-products of aerobic or xenobiotic metabolism can cause

*Abbreviations: dG, deoxyguanosine; EC, electrochemical detection; HPLC, high performance liquid chromatography; oxo^{8}dG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; ROS, reactive oxygen species; UV, ultraviolet detection.

oxidative damage to cellular macromolecules. DNA lesions resulting from exposure to ROS include modified bases, abasic sites, single and double strand breaks, and DNA–protein crosslinks (1). Continuous damage to DNA by free radical mechanisms may contribute to aging, cancer and other age-related degenerative diseases (2,3).

A particularly useful, and one of the most widely used markers of oxidative damage to DNA, is 8-oxo-7,8-dihydro-2′-deoxyguanosine (oxo^{8}dG) (3). The oxo^{8}dG residue has been particularly implicated in carcinogenesis (4), since oxo^{8}dG in DNA induces misreading during DNA synthesis *in vitro* and leads to G→T transversion mutagenesis (5–7). Oxo^{8}dG is frequently measured by high-performance liquid chromatography with electrochemical detection (HPLC-EC) conveying a low risk of inducing artifactual increases in oxidative base products, which can occur during derivatization in some gas chromatography/mass spectrometry techniques (8). However, with the latter assay a range of oxidatively modified nucleobases can be determined (9).

The possible role of naturally occurring antioxidants in protection against tumorigenesis has been a recent focus of attention. It is widely accepted that diets rich in fruits and vegetables are protective against the development of cancer and other degenerative diseases in humans (10–12). Although controversial, many epidemiological studies show inverse correlations in humans between plasma concentrations of certain antioxidants and the development of cancer (13–15) and other degenerative diseases. Some large-scale long-term controlled intervention trials in humans have also shown protective effects of vitamin E and β-carotene against cancer development (16), and others, although performed frequently only in smokers, have not found those effects (17,18). While the nature of the chemical substances responsible for the protective effects of fruits and vegetables are still not known, there is much interest in antioxidant vitamins C and E since they can be incorporated easily and safely in the human diet for *in vivo* protection against oxidative stress.

Despite the broad epidemiological evidence available concerning antioxidant protection against diseases, little is known whether ascorbic acid or α-tocopherol content itself actually influences the level of oxidative DNA damage in normal unstressed tissues. In this work the effect of dietary supplementation with vitamins C and E on hepatic oxo^{8}dG of normal unstressed guinea-pigs was studied at dietary doses that are known to effectively modify hepatic ascorbate and α-tocopherol levels (19,20). The guinea-pig is an ideal laboratory animal to perform controlled dietary experiments with anti-oxidant vitamins because it lacks, like human beings, the capacity to endogenously synthesize vitamins C and E (21), whereas common laboratory rodents are not appropriate for this purpose due to their capacity for tissue ascorbate synthesis and homeostasis.

The three dietary levels of each vitamin used in this study were selected to cover a broad spectrum from marginal

deficiency to an optimum supplementation and from this last level to a megadose. Previous studies from our laboratory have shown protective effects against increases in tissue oxidative damage to proteins *in vivo* or to lipid peroxidation *in vitro* with exactly the same time-dose protocol used here in normal (19,20,22,23) or endotoxin-treated (24) guinea-pigs.

Materials and methods

Animals and diets

Dunkin–Hartley male guinea-pigs, weighing ~250 g, were obtained from Iffa-Creddo, Lyon, France.

Ascorbic acid supplementation

A basal vitamin C-deficient laboratory diet (UAR, Usine Alimentation Rationelle, Billemoisson-Sur-Orge, Epinay, France) containing 19.5% protein, 3.1% fat, 68% carbohydrates, and 4.4% non-nutritive bulk was used. The content of minerals and vitamins per kg of diet was: phosphorus, 6791 mg; calcium, 9450 mg; potassium, 6700 mg; sodium, 1900 mg; magnesium, 2003 mg; manganese, 90.7 mg; iron, 252 mg; copper, 30.6 mg; zinc, 1134 mg; cobalt, 1.61 mg; iodine, 0.9 mg; vitamin A, 19 000 IU; vitamin D3, 1500 IU; vitamin D2, 1150 IU; thiamine, 18.5 mg; riboflavin, 18 mg; pantothenic acid, 16.5 mg; pyridoxine, 3.15 mg; α-tocopherol, 15 mg; menadione, 2.5 mg; niacin, 190 mg; folic acid, 0.5 mg; biotin, 0.04 mg; choline, 1600 mg; and vitamin B12, 0.02 mg. The diets with different vitamin C levels were prepared by adding 33 (Low C), 660 (Medium C) or 13 200 (High C) mg of L-ascorbic acid/kg of basal diet. These concentrations were checked on arrival at the laboratory by HPLC analyses of diet samples. Mean vitamin C intakes were 4.2 mg/kg body wt per day (Low C), 95 mg/kg body wt per day (Medium C) and 2125 mg/kg body wt per day (High G).

α-Tocopherol supplementation

Three diets differing in vitamin E (dl-α-tocopherol acetate) content were prepared by UAR (France) by adding different quantities of vitamin E to a vitamin E-free standard guinea-pig diet (diet 114, UAR.): 15 (Low E), 150 (Medium E) or 1500 (High E) mg of vitamin E/kg diet. These amounts were confirmed by HPLC analysis (for method see below) in two samples of each diet after arrival at the laboratory. Mean vitamin E intakes were 2.3 mg/kg body wt per day (Low E), 18.3 mg/kg body wt per day (Medium E) and 200 mg/kg body wt per day (High E). The basal diet contained 18.5% protein, 2.9% fat, 46.9% carbohydrates, 8.4% mineral mix, 1.4% vitamin mix, 11% humidity and 10.9% non-nutritive bulk. The content of minerals and vitamins per kg of diet was: phosphorus, 8600 mg; calcium, 10 600 mg; potassium, 12 000 mg; sodium, 3450 mg; magnesium, 3130 mg; manganese, 100 mg; iron, 320 mg; copper, 26 mg; zinc, 85 mg; cobalt, 1.61 mg; vitamin A, 19 000 IU; vitamin D3, 2031 IU.; thiamine, 22.5 mg; riboflavin, 21 mg; pantothenic acid, 123 mg; pyridoxine, 10.7 mg; menadione, 55 mg; niacin, 1930; folic acid, 7.3 mg; biotin, 0.275 mg; choline, 1740 mg; M-inositol, 250 mg; selenium, 114 μg; and vitamin C, 660 mg.

Guinea-pigs were maintained during 5 weeks receiving the three experimental diets and were caged inside aseptic air positive-pressure animal cabinets (A 130 SP, Flufrance, Cachan, France) equipped with an HEPA air filter (99.999% for particles >0.3 μm) at the inlet. At the end of the dietary regimen, the animals were killed by decapitation and liver samples were immediately dissected and stored at –75°C.

Ascorbic acid and α-tocopherol

Samples of liver tissue were homogenized in 50 mM perchloric acid for the analysis of ascorbic acid by ion pair HPLC with UV detection at 280 nm (25). Liver content of α-tocopherol was measured by HPLC with UV detection at 292 nm (26).

Isolation of DNA

Liver samples (500 mg) were homogenized in 500 μl of 150 mM NaCl. After homogenization, 1.8 ml of buffer (150 mM NaCl, 10 mM Tris, 10 mM Na2EDTA, pH 8.0) and 200 μl 10% SDS were added to 200 μl of liver homogenate. Samples were rotated in an extraction bench for 15 min and then incubated at 37°C for 10 min. Chloroform extraction (27) was performed as follows: 200 μl 3 M sodium acetate (pH 5.2), 550 μl of 5 M sodium perchlorate and 2 ml chloroform:isoamyl alcohol (24:1) were added. After rotation in an extraction bench for 10 min the samples were centrifuged at 2100 *g* for 10 min at 4°C. The chloroform extraction was performed twice to each sample. Subsequently, the DNA was precipitated by addition of 5 ml of ice-cold 96% ethanol. The DNA was allowed to precipitate at –20°C overnight. The DNA precipitate was washed twice with 70% ethanol, dried under a stream of nitrogen, and redissolved in 200 μl of 20 mM sodium acetate (pH 4.8). The isolated DNA was digested to deoxynucleoside level by incubation
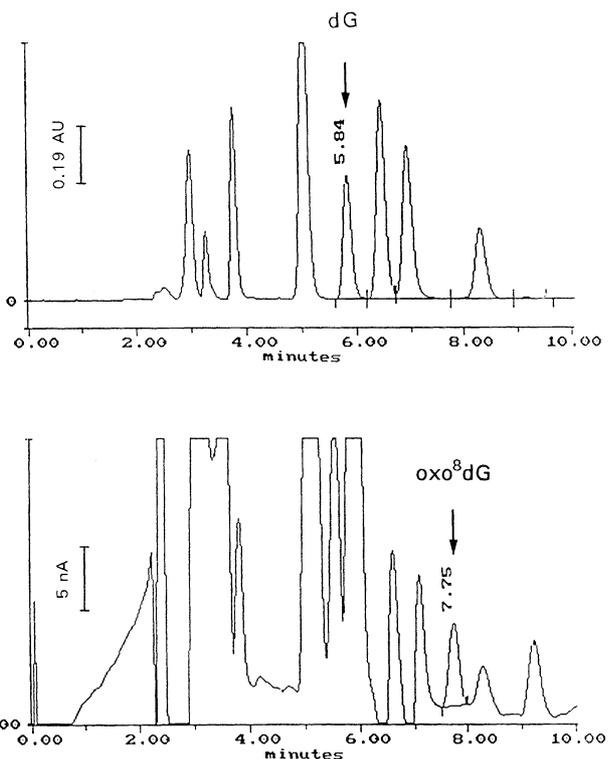
Fig. 1. Representative simultaneous ultraviolet and electrochemical chromatograms of a liver guinea-pig sample from the group Medium C, respectively showing the separation of the dG and the oxo^{8}dG peaks.

at 37°C with 5 U of nuclease P1 (in 20 μl of 20 mM sodium acetate, 10 mM ZnCl2, 15% glycerol, pH 4.8) during 30 min and 1 U alkaline phosphatase (in 20 μl of 1 M Tris–HCl, pH 8.0) for 1 h.

The ratio of oxo^{8}dG to dG in the DNA was measured by HPLC-EC. For analysis, 15 μl of the nucleoside mixture was injected into a reverse-phase Beckman Ultrasphere ODS column (5 μm, 4.6 mm×25 cm), eluted with 4% acetonitrile in 50 mM phosphate buffer (pH 3.2). Oxo^{8}dG and dG were detected with an ESA Coulochem II electrochemical coulometric detector (ESA, Inc., Bedford, MA) with a 5011 analytical cell run in the oxidative mode (E1 = 100 mV, E2 = 180 mV) and a Waters model 440 UV detector at 254 nm in series, respectively. For quantization peak areas and a three level calibration of pure oxo^{8}dG standard were used. Figure 1 shows a representative simultaneous electrochemical and UV chromatogram of a liver from the guinea-pig group Medium C.

Statistical methods

Data were analyzed by one-way analysis of variance. The $P < 0.05$ level was selected as the point of minimal statistical significance in every comparison. The 95% confidence intervals of the oxo^{8}dG levels in the high and low vitamin groups were calculated relative to the medium vitamin groups.

Results

All animals increased their body wt during the treatment, but it was significantly higher (25–31%) in the group Medium C than in the other two groups after 5 weeks of dietary supplementation with vitamin C (Table I). The group Low C showed a significant decrease in daily food intake and growth rate (Table I), typical in marginal vitamin C deficiency. Growth rate was also lower in High C than in Medium C animals. On the other hand, the three groups of animals supplemented with different vitamin E levels significantly increased their body wt during the experimentation, and neither the body growth rate nor the daily food intake were affected by the dietary treatment with vitamin E (Table II). No apparent signs of vitamin C and E deficiency were observed in groups Low C and Low E either at the behavioral or nutritional level, or

Table I. Body wt, growth rate, food intake, and hepatic vitamin C and oxo^{8}dG concentration in guinea-pigs fed three diets containing different levels of vitamin C for 5 weeks

	Dietary group		
	Low C 33 mg/kg diet	Medium C 660 mg/kg diet	High C 13 200 mg/kg diet
Body wt (g)	282.3 ± 64.5	369.0 ± 60.9^a	294.2 ± 55.4
Growth rate (g/day)^e	0.95 ± 0.70	4.84 ± 0.97^b	1.72 ± 0.46
Food intake (g/day)^e	33.6 ± 2.6	44.8 ± 5.2^c	43.8 ± 11.5^c
Vitamin C (μmol/g)	0.034 ± 0.051	1.63 ± 1.06^d	1.99 ± 0.44^d
Oxo^{8}dG/10^5 dG	1.89 ± 0.32	1.94 ± 0.78	1.93 ± 0.65

Values are mean ± SD from 6–8 guinea-pigs.
^{a}Significantly different ($P < 0.01$) from Low C and High C groups.
^{b}Significantly different ($P < 0.001$) from Low C and High C groups.
^{c}Significantly different ($P < 0.01$) from Low C group.
^{d}Significantly different ($P < 0.001$) from Low C group.
^{e}Values obtained between the beginning and the end of the dietary treatments. The rest of values were obtained at the end of the 5 weeks of experimentation.
Vitamin E was 15 mg/kg diet in the three groups.

Table II. Body wt, growth rate, food intake, and hepatic vitamin E and oxo^{8}dG concentration in guinea-pigs fed three diets containing different levels of vitamin E for 5 weeks

	Dietary group		
	Low E 15 mg/kg diet	Medium E 150 mg/kg diet	High E 1500 mg/kg diet
Body wt (g)	543.8 ± 27.3	575.0 ± 72.6	553.9 ± 35.3
Growth rate (g/day)^c	9.52 ± 0.80	9.94 ± 2.64	9.70 ± 0.16
Food intake (g/day)^c	45.2 ± 6.1	50.4 ± 10.2	53.3 ± 9.8
Vitamin E (μg/g)	11.9 ± 5.1	27.2 ± 7.8^a	80.4 ± 14.9^b
Oxo^{8}dG/10^5 dG	2.85 ± 0.70	2.74 ± 0.66	2.61 ± 0.92

Values are mean ± SD from seven guinea-pigs.
^{a}Significantly different ($P < 0.05$) from Low E group.
^{b}Significantly different ($P < 0.001$) from Low E and Medium E groups.
^{c}Values obtained between the beginning and the end of the dietary treatments. The rest of values were obtained at the end of the 5 weeks of experimentation.
Vitamin C was 660 mg/kg diet in the three groups.

during post-mortem internal inspection (gross pathology on dissection).

After 5 weeks of dietary treatment with different quantities of ascorbic acid or α-tocopherol, the animals of the three experimental groups in each experiment showed clearly different hepatic levels of these vitamins (Tables I and II). The vitamin C content in the liver increased significantly from group Low C to group Medium C (48-fold) and from group Low C to group High C (59-fold). Increasing the vitamin E content by one order of magnitude from group Low E to group Medium E resulted in an increase in liver vitamin E to 229% of Low E values (Table II). When dietary vitamin E was further increased by another order of magnitude from group Medium E to group High E the vitamin E hepatic content increased again to 296% of the Medium E value.

Despite the broad difference in the hepatic concentration of antioxidant vitamins neither vitamin C nor vitamin E dietary supplementation significantly modified the level of oxidative DNA damage in terms of oxo^{8}dG in the guinea-pig liver

(Tables I and II). The 95% confidence intervals of the oxo^{8}dG to dG ratio in the High C and Low C groups were 67% to 132% and 72% to 122% of the level in the Medium C group, whereas in the High E and Low E groups the confidence intervals of the oxo^{8}dG to dG ratio were 65% to 126% and 78% to 130% of the level in the Medium E group, respectively.

Discussion

Comparable levels of oxo^{8}dG were found in hepatic DNA of the three groups supplemented with different amounts of ascorbic acid and in the three groups supplemented with different levels of vitamin E, suggesting a lack of effect of these antioxidant vitamins on oxidative DNA damage in normal unstressed guinea-pigs.

The present lack of effect of the antioxidant vitamins C and E are generally in agreement with the existing literature. Thus, no significant effect of vitamin E supplementation on rat liver oxo^{8}dG (28) and on tumor incidence and oxo^{8}dG in the liver of rainbow trout (29) has been found. In intervention trials in humans no effect of α-tocopherol 200 mg/day, ascorbic acid 500 mg/day or coenzyme Q 90 mg/day for 2 months on urinary excretion of oxo^{8}dG, a repair product of oxo^{8}dG in cellular DNA, was found in male smokers (30). Similarly, a 14-week intervention trial with 20 mg β-carotene per day showed no effect on oxo^{8}dG urinary excretion (31). Moreover, the urinary excretion of the RNA damage product, 8-oxoguanosine, was not affected by the daily administration of vitamins C (1000 mg) and E (533 mg) and β-carotene (10 mg) for 1 month (32). Depletion of dietary ascorbic acid in healthy men had no effect on the level of oxo^{8}dG in lymphocyte DNA or the urinary excretion 8-oxoguanosine, whereas the level of oxo^{8}dG in sperm DNA increased substantially (33,34). Upon replenishment of ascorbate the oxo^{8}dG level in sperm returned to the initial values (34). A study performed in 10 healthy male non-smoking volunteers showed that the consumption of 300 g of Brussels sprouts as compared with non-Brassica vegetables for 12 days decreased the urinary excretion of oxo^{8}dG (35). This is in agreement with many epidemiologic studies showing that dietary intake of fruits and vegetables, particularly of the Brassica species, is inversely associated with the risk of cancer in humans (10,11,36,37). It is likely that the chemical compounds contained in fruits and vegetables responsible for these protective effects are different from vitamins C and E. Although the possibility cannot be totally discarded that subtle effects of antioxidant vitamins on oxo^{8}dG can still be observed with longer time treatments and/or a higher number of animals per group than those used in our study, the 95% confidence intervals of oxo^{8}dG indicate to what extent the statistical power of the study could have missed a small effect. Furthermore, no signs of dose-related trends were observed in any treatment.

Ascorbic acid inhibits oxo^{8}dG formation in calf thymus DNA and V79 Chinese hamster cells exposed to ultraviolet radiation (38,39). Glutathione, cysteine and vitamin C (but not vitamin E) also protected against increases in renal oxo^{8}dG caused by the kidney carcinogen potassium bromate in rats (40), although tissue levels of ascorbate and glutathione, which are synthesized by rat tissues, were not measured in that study. On the other hand, vitamin E and ellagic acid, but not vitamin C, inhibited the increase in liver oxo^{8}dG induced by the hepatocarcinogen 2-nitropropane in rats (41). All the above results taken together suggest that antioxidant vitamins can be protective against increases in oxo^{8}dG induced in some target

organs with oxidative damage that has arisen from specific exogenous sources, whereas in general they have little influence on the basal level of oxidative damage to DNA.

Oxidative DNA damage is considered to be site-specific and, in the case of nuclear DNA, it would occur at sites of iron or copper binding to DNA (42). Mechanistically, it is possible that under normal conditions, the close vicinity between places of endogenously controlled basal ROS generation at DNA on the one hand, and their targets at these sites on the other side, would give little opportunity for the interception of free radicals by antioxidant vitamins before they reach these nearby targets. In contrast, antioxidants would easily intercept for instance increases in H_2O_2 generated further away (e.g. by metabolism of xenobiotics in the cytoplasm) before it can diffuse to the nucleus and damage the DNA. This would explain the capacity of vitamins C and E to decrease some target organ/cell specific induction of oxidative DNA damage. Obviously, much further research is needed to clarify if those or other speculative possibilities are correct or not. In any case, a role for antioxidants, including vitamins C and E, as protectors against increases in oxidative stress above that normally generated endogenously in basal conditions, would be highly relevant for human health through prevention against disease during aging, since humans live in an stressful environment of pollutants, infections, xenobiotics, drugs, radiation, smoking and excess calories.

Other possible reason for the lack of effect of vitamins C and E on levels of oxidative DNA damage in most studies might be variations in the activity of oxo^8dG-repairing enzymes. Steady-state levels of oxo^8dG in DNA reflect the balance between rates of oxidative damage and repair of dG. The presence of oxo^8dG repairing enzymes has been demonstrated in *Escherichia coli* (43) and eukaryotic cells (44,45). Repair of oxo^8dG is rapid in mice liver after high doses of X-ray irradiation (46) or in rat kidney after treatment with the kidney-specific carcinogen potassium bromate (47).

Guinea-pigs must receive 0.5 mg of vitamin C/day in order to avoid the development of scurvy. The time and dose of vitamin C treatment, growth rates and food intake, tissue levels of ascorbate, and absence of any signs of scurvy in Low C animals indicate that they are representative of a state of marginal deficiency. Also, the hepatic concentration α-tocopherol reached in group Low E (11.9 μg/g tissue) shows that the animals in this group were not vitamin E deficient. On the other hand, the lower growth rates of High C animals in relation to Medium C ones are indicative, which is in agreement with previous results concerning fatty acid composition (19), of the presence of some negative effects of the vitamin C megadose. We have previously shown that exactly the same time-doses of antioxidant vitamins used here decrease *in vivo* protein carbonyls and sensitivity to *in vitro* lipid peroxidation (19,20,22,23) in tissues (including liver) of normal unstressed guinea-pigs. Gradually increasing dietary vitamin E (5, 50 or 250 mg/kg) also progressively decreased *in vivo* liver phosphatidylcholine and phosphatidylethanolamine hydroperoxides measured by HPLC with chemiluminescence detection in ODS rats, which do not synthesize ascorbate (48). Differing from their effects on protein and lipid peroxidation, a lack of capacity of vitamins C and E to decrease oxidative DNA damage below basal levels in normal unstressed animals would be in agreement with their lack of effect on maximum life span of mammals, although antioxidants can increase mean life span (49–54). This would be consistent with an important role of endogenous DNA damage in determining the intrinsic aging rate and maximum life span, since these are species-specific genetically determined traits that cannot be strongly influenced by environmental external conditions.

In conclusion, even very large variation in the content of the antioxidant vitamins C and E in the diet and liver had no influence on the steady-state level of oxidative damage to guanine in the liver DNA of normal unstressed guinea-pigs. Although antioxidant vitamins may be protective in some specific target organs or cells, they appear to have little influence on oxidative damage to DNA, at least under normal unstressed conditions.

Acknowledgements

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