

Normal oxidative damage to mitochondrial and nuclear DNA is extensive

(8-hydroxydeoxyguanosine/aging/cancer/mutation)

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ABSTRACT Oxidative damage to DNA can be caused by excited oxygen species, which are produced by radiation or are by-products of aerobic metabolism. The oxidized base, 8-hydroxydeoxyguanosine (oh⁸dG), 1 of ≈20 known radiation damage products, has been assayed in the DNA of rat liver. oh⁸dG is present at a level of 1 per 130,000 bases in nuclear DNA and 1 per 8000 bases in mtDNA. Mitochondria treated with various prooxidants have an increased level of oh⁸dG. The high level of oh⁸dG in mtDNA may be caused by the immense oxygen metabolism, relatively inefficient DNA repair, and the absence of histones in mitochondria. It may be responsible for the observed high mutation rate of mtDNA.

Excited oxygen species such as the superoxide radical, hydrogen peroxide, and the hydroxyl radical are formed *in vivo* during aerobic metabolism as well as during radiation (1). Although cells have developed various enzymatic and nonenzymatic systems to control excited oxygen species (2), a certain fraction escapes the cellular defense and may cause permanent or transient damage to proteins, lipids, and nucleic acids. Oxidative damage has been suggested to contribute to aging and to a host of diseases including cancer, chronic inflammation, ischemia, degenerative arterial, and autoimmune diseases (3–8).

The critical targets that may be affected by excited oxygen during aging and in diseases have not yet been identified with certainty. Since DNA plays a central role in the information transfer between generations of somatic cells, much attention has been given to its oxidative damage, particularly in relation to aging and cancer (3, 5–12). A high rate of oxidative damage to mammalian DNA has been demonstrated by measuring oxidized DNA bases excreted in urine after DNA repair (5–8). The rate of oxidative DNA damage is directly related to the metabolic rate and inversely related to life span of the organisms (7). This work has not distinguished between damage to nuclear and mitochondrial DNA (mtDNA).

Mitochondria (along with chloroplasts) occupy a unique position among cellular organelles because they possess a separate genome and all the enzymatic machinery for transcribing and translating the genetic information into proteins. Although mitochondria are also an important source of excited oxygen species (13), oxidative damage of mtDNA has not been assessed. We report here the presence of 8-hydroxydeoxyguanosine (oh⁸dG) in mtDNA and nuclear DNA (nDNA) of rat liver, a compound that has been shown to be formed in nDNA by excited oxygen species and to be easily measured (14–18).

MATERIALS AND METHODS

Isolation of Mitochondria. Mitochondria were isolated from the livers of 6-month-old female rats (≈200 g; Sprague–

Dawley) by conventional differential centrifugation as described (19).

Incubation of Mitochondria with Alloxan and Ca²⁺. Mitochondria were incubated with 60 nmol of Ca²⁺ per mg of protein and 5 mM alloxan for 15 min as described (19). After incubation, they were pelleted by centrifugation at 12,000 × *g* for 10 min at 4°C.

Incubation of Mitochondria with Iron and Alloxan. Mitochondria were incubated as described above except that the buffer also contained 5 mM MgCl₂, and Ca²⁺ was replaced by 250 μM Fe³⁺ chelated with sucrose (20). After iron uptake had proceeded for 5 min, 5 mM alloxan or buffer was added, and 15 min later mitochondria were centrifuged as described above.

γ-Irradiation of Mitochondria and Isolated DNA. Mitochondria were suspended to 6 mg of protein per ml of buffer (10 mM Tris-HCl/pH 7.4/1 mM EDTA/50 mM NaCl) and irradiated at 0°C with a ⁶⁰Co source (6000 Ci; 1 Ci = 37 GBq; Lawrence Berkeley Laboratory) at a dose rate of 1 krad/min (1 rad = 0.01 Gy). They were then concentrated by centrifugation as described above. Isolated mtDNA (25 μg/ml) was irradiated at room temperature in 40 mM Tris-HCl (pH 8.5).

Isolation of mtDNA. Mitochondria were suspended to 10 mg of protein per ml in 12 ml of 10 mM Tris-HCl, pH 7.4/1 mM EDTA/50 mM NaCl, lysed with 1% freshly prepared NaDodSO₄, and mixed with 1/6th vol of saturated CsCl. After standing on ice for at least 1 hr, the sample was centrifuged for 10 min at 12,000 × *g* and 4°C. The supernatant was adjusted to 1.56 g/ml with solid CsCl and propidium iodide was added to a concentration of 0.3 mg/ml. It was centrifuged overnight in a Beckman desk-top ultracentrifuge equipped with a T1 100.3 rotor at 80,000 rpm and 20°C. DNA was visualized under UV light and the mtDNA band was removed from the gradient with a syringe. Propidium iodide was then extracted with 1-butanol. The DNA solution was dialyzed overnight against Dowex (Bio-Rad) AG 50W-X8 (200–400 mesh) in 40 mM Tris-HCl (pH 8.5). When necessary, it was stored at –20°C. The yield of mtDNA was usually ≈60 μg per liver as determined spectrophotometrically, with A_{260/280} of 1.76. The mtDNA preparation was free of detectable nDNA as judged by digestion of 750 ng of DNA by the restriction enzyme *Bam*HI (Boehringer Mannheim), followed by agarose gel electrophoresis and staining with ethidium bromide.

Isolation of nDNA. nDNA was isolated from the low-speed centrifugation pellet of homogenized rat liver as described by Gupta (21).

Treatment of DNA with Cu²⁺/Ascorbate. DNA (20 μg) (calf thymus DNA purchased from Sigma, mtDNA isolated from

Abbreviations: oh⁸dG, 8-hydroxydeoxyguanosine; nDNA, nuclear DNA.

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rat liver, and pUC18 plasmid DNA) was incubated in 10 mM potassium phosphate buffer (pH 7.4) in the presence of 1 mM ascorbic acid/0.1 mM CuSO₄ for 60 min at 37°C.

Enzymatic Hydrolysis of DNA. DNA samples (10–50 μg per 0.5 ml) in 40 mM Tris-HCl (pH 8.5) containing 10 mM MgCl₂ were incubated with DNase I (Sigma) (200 units per mg of DNA), spleen exonuclease (Sigma) (0.01 unit per mg of DNA), snake venom exonuclease (Sigma) (0.5 unit per mg of DNA), and alkaline phosphatase (Boehringer Mannheim) (10 units per mg of DNA) for 2 hr at 37°C. DNA hydrolysis was complete at this time as judged by agarose gel electrophoresis and staining with ethidium bromide.

High-Performance Liquid Chromatography and Electrochemical Detection. High-performance liquid chromatography was performed with a Waters chromatograph equipped with a model 6000A solvent delivery system, a model U6K septumless injector, a Kratos (Westwood, NJ) UV/visible detector operated at 260 nm, and a BAS (West Lafayette, IN) amperometric electrochemical detector with 0.8 V and 20 nA. Chromatography was done on two C-18 Supelcosil (5 μm; 4.6 × 250 mm) columns in series. The mobile phase consisted of filtered and vacuum-degassed 50 mM phosphate buffer (pH 5.5) containing 10% methanol. Flow rate was 1.0 ml/min. Usually, 50–100 μl of DNA hydrolyzate was injected for analysis. The response of the electrode was linear between 0.2 and 50 pmol of oh⁸dG.

RESULTS

DNA isolated from rat liver mitochondria contains 0.41 (0.06 SD; *n* = 12) pmol of oh⁸dG per μg, as analyzed by high-performance liquid chromatography of deoxynucleosides in combination with electrochemical detection (Fig. 1 and Table 1). Besides oh⁸dG, the chromatogram shows several electrochemically responsive compounds whose identities are presently unknown. In nDNA assayed from the same rat livers, we found a 16 times lower level of oh⁸dG (0.025 pmol/μg; 0.004 SD; *n* = 4). Since mtDNA comprises only ≈1% of total cellular DNA and our recovery of mtDNA is ≈70%, the maximal possible contamination of mtDNA in nDNA, 0.3%, could not account for the oh⁸dG in nDNA.

Several control experiments ascertained the validity of the results obtained with mtDNA. The mitochondrial origin of the DNA was confirmed by agarose gel electrophoresis and restriction enzyme digestion. Artifacts of oxidation of mtDNA during isolation and analysis was ruled out in two ways: (i) the presence of the antioxidant butylated hydroxy-

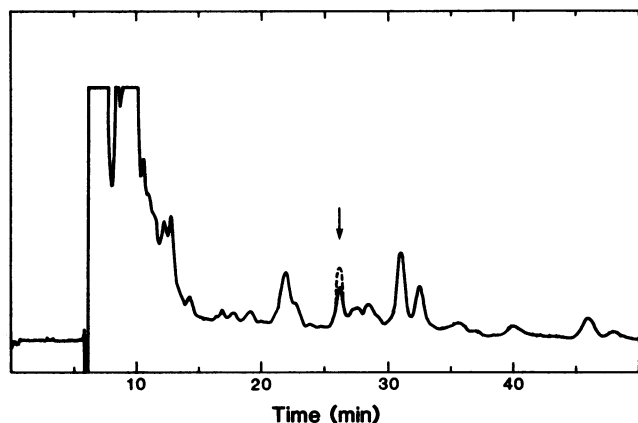


FIG. 1. Determination of oh⁸dG in mtDNA. DNA (40 μg) isolated from rat liver mitochondria was enzymatically hydrolyzed and subjected to high-performance liquid chromatography in combination with electrochemical detection. The position of oh⁸dG in the chromatogram is indicated by the arrow. Dashed line represents a sample spiked with 10 pmol of oh⁸dG as standard.

Table 1. Formation of oh⁸dG in mtDNA by prooxidants

Conditions	oh ⁸ dG pmol per μg of mtDNA
Control	0.41
Alloxan	2.10
Alloxan/Ca ²⁺	1.67
Fe ³⁺	1.26
Alloxan/Fe ³⁺	1.94
γ-Irradiation (15 krad)	
Mitochondria	0.94
mtDNA	2.70

toluene (0.1%) during the isolation did not change the level of oh⁸dG; (ii) in pUC18 plasmid DNA added to isolated mitochondria and carried through the DNA isolation procedure, no oh⁸dG was detected.

When isolated rat liver mtDNA, calf thymus nDNA, and pUC18 plasmid DNA were incubated with Cu²⁺/ascorbate, approximately equal amounts of oh⁸dG (2.7–3.1 pmol per μg of DNA) were formed, indicating that mtDNA is not inherently more sensitive to oxidative damage under these conditions.

If oh⁸dG were formed in mtDNA by excited oxygen species, it should be possible to increase its level by oxidative stress. To test this, we treated isolated rat liver mitochondria with prooxidants (Table 1). Alloxan, which is reduced by pyridine nucleotides and stimulates the intramitochondrial formation of oxygen radicals (19), increases the level of oh⁸dG in mtDNA. When the alloxan-induced oxygen radical formation is limited by Ca²⁺-dependent intramitochondrial pyridine nucleotide hydrolysis (19), less oh⁸dG is formed than in the absence of Ca²⁺. Iron ions, known to catalyze the formation of hydroxyl radicals from hydrogen peroxide (22), also stimulate the formation of oh⁸dG. Furthermore, DNA in mitochondria is oxidized by γ-rays. About 0.53 pmol of oh⁸dG per μg of mtDNA was formed at a dose of 15 krad. This dose increased oh⁸dG in nuclei obtained from the same livers from 0.020 to 0.088 pmol per μg of DNA (result not shown in Table 1). The lower susceptibility in nDNA may be due to shielding proteins. Irradiation of isolated mtDNA yielded ≈2.3 pmol of oh⁸dG per μg of DNA.

DISCUSSION

Oxidants, such as radiation, can cause various types of damage to DNA, such as strand breaks and oxidation of sugar and base residues (23–27). The C-8 position of deoxyguanosine in DNA is hydroxylated to yield oh⁸dG (28), which can be measured with high sensitivity by high-performance liquid chromatography in combination with electrochemical detection and is thus a useful indicator for oxidant-induced DNA damage (14–18, 28). We also find a variety of other electrochemically active adducts in mtDNA (Fig. 1), some of which appear to be oxidative adducts. The identity of these adducts is under investigation.

oh⁸dG has so far been measured in nDNA of mouse liver, HeLa cells, human granulocytes, and rat kidney (16–18). The reported levels range from 0.006 to 0.015 pmol/μg in untreated cells, values similar to those we find in the nDNA of rat liver. In contrast, a much higher level of oh⁸dG is found in mtDNA. Assuming 1 × 10⁷ Da for mtDNA and 1 × 10⁴ mtDNA copies (29), it can be calculated that ≈4.1 × 10⁴ oh⁸dG residues are present in the mitochondrial genome of a rat liver cell. An analogous calculation yields 1.4 × 10⁵ oh⁸dG residues for the nuclear genome.

As oh⁸dG is but 1 of ≈20 known primary DNA damage products caused by radiation (23–27), the other products are presumably also being formed (5–7), as well as single and double strand breaks. Thus, oh⁸dG might represent only 5%

of the total oxidative DNA damage (23–27). This proportion cannot be determined from a steady-state level and would depend on both yield and repair. The efficiency of repair might be related to the toxicity of each adduct. Since oh^8dG does not stop replication, it might be relatively benign, although it does cause misreading at the oh^8dG residue itself and at neighboring bases (30).

There are probably several reasons for the high steady-state oxidation level in mtDNA. First, mitochondria consume >90% of the cell's oxygen, and the mitochondrial respiratory chain is the source of a continuing flux of oxygen radicals (1). Second, since mtDNA is not covered extensively by proteins such as histones, it may be more susceptible to the attack by excited oxygen than nDNA. Finally, mitochondria may be less efficient in repairing DNA damage and replication errors than the nucleus (31–34). These organelles lack nucleotide excision repair and recombinational DNA repair (35, 36). They do, however, possess three uracil DNA glycosylases (37–39), two endonuclease activities specific for apurinic/aprimidinic sites, endonucleases that act at lesions introduced by high UV doses (A. E. Tomkinson, personal communication), and a DNA ligase (40). It is reasonable to suppose that these enzymes participate in DNA repair, although some of them may have a role in eliminating damaged DNA molecules. If repair does indeed occur to an appreciable extent, the oh^8dG levels found in mtDNA would be indicative of a copious flux of excited oxygen species in mitochondria. It may also be of interest to note in this context that the mitochondrial genome is also much more susceptible than the nuclear genome to alkylation (41–46).

In vertebrates, mtDNA evolves 5–10 times faster than nDNA of the same organism (47). The mean rate of divergence over the whole mtDNA molecule is $\approx 2\%$ per 10^6 years (48). The main reason for the high rate of evolution of mtDNA could be the high oxidative stress shown in this report in combination with the relative inefficiency of replication error and DNA damage repair (49). Another reason seems to be relaxed constraints on components of the mitochondrial translation apparatus (50).

In summary, mtDNA is much more oxidatively damaged than nDNA, although the damage of nDNA also appears to be very high. Both types of DNA damage may be relevant for an understanding of the molecular mechanisms underlying aging, certain diseases, and certain types of cancer.

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