Fragmented Mitochondrial DNA Is the Predominant Carrier of Oxidized DNA Bases†

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ABSTRACT: Previous analyses indicated a high level of oxidative base modification in mitochondrial DNA, the extent of which raised questions about the methodological validity and biological implications. In the present study DNA was isolated from rat liver mitochondria under carefully controlled conditions, and the extent of base oxidation, DNA fragmentation, and nuclear DNA contamination were analyzed. DNA isolated from intact mitochondria treated with DNase consisted of 16.3 kilobase pairs, mostly circular, mitochondrial DNA molecules and a mixture of nuclear and mitochondrial DNA fragments, as identified by agarose gel electrophoresis and hybridization. High-performance liquid chromatography in combination with electrochemical detection confirmed that the overall level of 8-hydroxy-2′-deoxyguanosine, a marker commonly used in the analysis of base oxidation, is higher in mitochondrial than in nuclear DNA. Importantly, 8-hydroxy-2′-deoxyguanosine is relatively scarce in the 16.3 kilobase pair mitochondrial DNA molecules (0.051 pmol/μg) but is present in high levels in mitochondrial DNA fragments (0.741 pmol/μg). The fragments constitute about 18% of total mitochondrial DNA. The antitumor agent bleomycin, which binds to DNA, forms an iron complex capable of transferring electrons from Fe^{2+} to molecular oxygen. Exposure of mitochondria to bleomycin and iron resulted in nicking but not in a significant increase in base oxidation of 16.3 kilobase pair mitochondrial DNA, whereas the amount and the oxidation level of fragmented mitochondrial DNA significantly increased. These findings are relevant for a better understanding of the role of mitochondria in aging and various diseases and are consistent with the notion that despite the overall high DNA oxidation level, mitochondria can faithfully proliferate.

Mammalian mitochondrial DNA (mtDNA)[1] has very little redundancy and a high information density. It mutates much faster than nuclear DNA (nDNA), presumably because mtDNA is not covered by histones and is located near the mitochondrial respiratory chain, the richest cellular source of mutagenic reactive oxygen species (ROS) (*1*). Beginning in 1988, mtDNA mutations were linked to an ever-increasing number of diseases such as myopathies, encephalomyopathies, heart diseases, late-onset diabetes, Parkinson's, Huntington's, and Alzheimer's disease and to aging (for recent reviews, see refs *2*−*5*). The mutations comprise duplications, deletions, and base alterations, some of which may be caused by ROS (*4*, *6*, *7*).

There is a general consensus that mtDNA is subject to severe oxidative damage. This became first apparent from the analysis of 8-hydroxy-2′-deoxyguanosine (8OHdG), reported to be present in rat liver mtDNA at a level of 0.41 pmol/μg of DNA, which is about 16 times higher than that found in nDNA (*8*). Similar values were reported for other tissues (*9*−*14*). Since 8OHdG is just one out of many base modifications produced by ROS in DNA (*15*), the extent of base oxidation in mtDNA seems to be enormous. ROS can also cause DNA strand breaks (*16*). The reported severe oxidative damage to mtDNA is seemingly difficult to reconcile with the proliferation of obviously well-functioning organelles for many mitochondrial generations, and doubt concerning the validity of the mtDNA oxidation data was raised. One confounding factor may be the presence of nDNA in mtDNA preparations, which was hitherto ignored or assayed in inappropriate ways.

To resolve these uncertainties we investigated the extent of mtDNA oxidation and fragmentation under carefully controlled conditions. We find that 8OHdG is relatively scarce in circular mtDNA, but is present in high amounts in mtDNA fragments. When isolated mitochondria are subjected to oxidative stress, mtDNA is fragmented, and additional base oxidation is detected in the fragments but not in high molecular-weight mtDNA.

MATERIALS AND METHODS

Materials. All chemicals were commercial grades of highest purity. Gelase and gelase buffer were purchased from Epicenter Technologies (Madison, WI); other enzymes from Boehringer Mannheim, Germany. Low melting point agarose (LMP agarose ultrapure, Gibco BRL) was from Life Tech-

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[1] Abbreviations: BSA, bovine serum albumin; bleo/Fe, bleomycin and $FeCl_2$; DTPA, diethylenetriaminepentaacetic acid; EC, electrochemical detection; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; 8OHdG, 8-hydroxy-2′-deoxyguanosine; kb, kilobase pairs; KSH buffer, 225 mM sucrose, 10 mM KCl, and 10 mM HEPES, pH 7.4; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; ROS, reactive oxygen species; Tris, tris(hydroxymethyl)-aminomethane.

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nologies (Basel, Switzerland). Rediprime random primer labeling, Hybond-N^+ nucleic acid transfer membranes, and [α-^{32}P]dCTP were from Amersham (Buckinghamshire, U.K.).

Mitochondria. Liver mitochondria were obtained from 200 g female Wistar rats (starved overnight) by differential centrifugation (*17*). Mitochondrial protein was determined by the Biuret method with bovine serum albumin (BSA) as standard.

Incubation Conditions. All incubations were performed at 37 °C for 20 min in a buffer containing 250 mM sucrose, 20 mM KCl, 5 mM $MgCl_2$, 3 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.4, 10 mM succinate, DNase I (2.66 mg/mL), and RNase A (2.66 mg/mL) with 4 mg of mitochondrial protein/mL and oxygen blown over the surface of the suspension. Bleomycin and $FeCl_2$ (bleo/Fe), when present, were both 10 mM. The reaction was stopped with 20 mM diethylenetriaminepentaacetic acid (DTPA), pH 7.0. Mitochondria were washed twice with ice-cold incubation buffer (each centrifugation step 10 min at 10000*g*, 4 °C).

Isolation of Total DNA. Mitochondria were centrifuged for 10 min at 10000*g*, and the pellet was resuspended in KSH buffer (225 mM sucrose, 10 mM KCl, and 10 mM HEPES, pH 7.4) (200 mL/15 mg of mitochondrial protein). Solubilization was performed by addition of 5% sodium dodecyl sulfate (SDS) (200 mL/15 mg mitochondrial protein), followed by salting out with 5 M NaCl (150 mL/15 mg of mitochondrial protein). After 5 min on ice, the precipitates were removed by centrifugation for 5 min in an Eppendorf centrifuge at maximum speed, and an equal volume of preheated (50 °C) tris(hydroxymethyl)aminomethane- (Tris-) saturated phenol containing 8-hydroxyquinoline (0.1%) was added to the supernatant. After 15 min of centrifugation, the aqueous phase was transferred to a new vial, and the phenol extraction was repeated. The aqueous phase was washed with an equal volume of chloroform followed by 2 min of centrifugation. Nucleic acids in the aqueous phase were precipitated at −20 °C overnight. After centrifugation (20 min, 10000*g*) the precipitate was dissolved in doubly distilled H_2O (approximately 100 mL/100 mg of mitochondrial protein), and RNA was digested with 1 mL of RNase A (20 mg/mL) at 37 °C for 15 min.

Isolation of 16.3 Kilobase Pair mtDNA. The RNase-treated DNA was loaded onto a 0.7% agarose gel [4 mM Tris-acetate, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.0, and 0.5 mg/mL ethidium bromide] and run for approximately 30 min at 80 V. Forms I (supercoiled), II (open circular), and III (linear), still appearing as a single band, were cut out and transferred to a pristine area on the gel where a double-size piece of agarose had been cut out. The space in front of the inserted agarose slice containing the 16.3 kilobase pair (kb) forms I, II, and III was filled with l% low-melt agarose. The gel was then subjected to 200 V for 20 min. Slices containing the DNA appearing as one sharp band were cut out. To the slices, weighing 0.2−0.6 g, gelase buffer was added [40 mM bis(2-hydroxyethyl)-iminotris(hydroxymethyl)methane, pH 6.0]. The slices were kept 20 min at 70 °C for complete agarose solubilization. After temperature adaptation to 45 °C, gelase (0.2 unit/0.2−0.6 g) was added and incubated for at least 1 h. The DNA- and polysaccharide-containing solution was then subjected to a phenol and chloroform treatment as above. The salt concentration was adjusted with NaCl to 0.2 M, and 2 volumes of ethanol were added. The DNA was precipitated at −20 °C overnight.

Isolation of Fragmented DNA. After separation of high from low molecular-weight DNA on a 0.7% agarose gel (see above), a slice containing fragmented DNA was also cut out and placed in spin columns (0.5−1 g of gel/column). During centrifugation for 2 h at 3000*g*, the buffer containing the fragmented DNA was collected in Eppendorf tubes. Further purification and precipitation steps were as described above for 16.3 kb DNA.

Isolation of nDNA. The pellet obtained by the low-spin centrifugation of the liver homogenate (*17*) was washed (resuspended and centrifuged at 1000*g*) five times with 20 times the volume of the pellet. The final pellet (2 mL) was diluted with 3.4 mL of 10 mM Tris, pH 8.0, and 1 mM EDTA. After addition of 600 μL of 10% SDS and 1.8 mg of proteinase K, the sample was left for 3 h at 37 °C. This was followed by the DNA purification steps described above.

Hybridization of DNA. Total DNA, purified 16.3 kb mtDNA forms, and purified fragmented DNA were loaded onto an agarose gel (0.7%) and run at 80 V for 1 h. The DNA was transferred to a positively charged nitrocellulose membrane (Hybond-N+) and cross-linked to the membrane by UV light (312 nm, 2 min). With the Rediprime labeling system, gelase-purified, *Alu*I-cut mtDNA was labeled with [α-^{32}P]dCTP and diluted in hybridization buffer (0.5 M sodium phosphate, pH 7.0, 7% SDS, and 1% BSA). Nuclear DNA, isolated from rat liver and *Eco*RI-cut, was labeled and diluted in the same way. Hybridization of the immobilized DNA to either the mtDNA or nDNA probe was performed overnight at 60 °C. Washing of the membranes was done in 0.3 M NaCl, 0.03 M sodium citrate, pH 7.4 (2× SSC) and 0.1% SDS, followed by 0.1× SSC and 0.1% SDS, both at 60 °C for 10 min. After exposure of the membranes to a phosphoimager plate (exposure time depending on the specific radioactivity of the probe), the radiolabeled DNA was quantified (Image Quant, Molecular Dynamics) by using the signal intensities of the mtDNA and nDNA probes quantified by high-performance liquid chromatography (HPLC) in combination with UV detection.

Hydrolysis of DNA. DNA was denatured at 95 °C for 5 min, chilled, and then hydrolyzed at 60 °C for 10 min with nuclease P1 (6.7 mg for up to 5 mg of DNA) in 20 mM sodium acetate buffer, pH 5.0, containing 0.1 mM $ZnCl_2$, followed by alkaline phosphatase treatment (7.5 units) at pH 8 (adjusted with 1 M Tris, pH 8.6) at 37 °C for 1 h.

HPLC Analysis of Nucleosides. Nucleoside separation was performed on a Nucleosil 120-5618 reversed-phase column (Macherey and Nagel, Oensingen, Switzerland) with 50 mM potassium phosphate, pH 5.6, containing 10% methanol as running buffer at a flow rate of 1 mL/min. Nucleoside elution was followed by absorption measurement at 256 nm. Calibration was done with deoxyguanosine.

Detection of 8-Hydroxy-2′-deoxyguanosine. 8OHdG was analyzed with an electrochemical detector (Bioanalytical Systems, West Lafayette, IN) consisting of a working electrode set at 600 mV and an Ag/AgCl reference electrode. Calibration was done with 8OHdG obtained by oxidation of deoxyguanosine (0.5 mM) in the presence of ascorbate (1.5 mM), $CuSO_4$ (0.18 mM), and H_2O_2 (1%). The purity of

Table 1: DNA Species Obtained from Rat Liver Mitochondria

	control mitochondria			bleomycin/Fe^{2+}-treated mitochondria		
	mean	SEM	*n*	mean	SEM	*n*
total DNA[a]	15.9 μg^b	2.67	5	11.8 μg^b	2.01	5
mtDNA[c]	12.4 μg^b	2.29	5	10.0 μg^b	1.49	5
nDNA[c]	3.5 μg^b (22% of total DNA)	0.31	5	1.7 μg^b (14.1% of total DNA)	0.24	5
low molecular-weight mtDNA[d]	2.3 μg^b (18.5% of total mtDNA)[e]	0.21	5	2.1 μg^b (21.2% of total mtDNA)[e]	0.23	5
mtDNA[d] (% of total low molecular-weight DNA)	42.10%	3.35	5	56.90%	4.17	5

[a] Determined by HPLC/UV analysis. [b] Per 100 milligram of mitochondrial protein. [c] Calculated with the data obtained from Southern blotting and HPLC/UV analysis of total DNA. [d] Calculated with the data obtained from Southern blotting and HPLC/UV analysis of low molecular-weight DNA. [e] $p < 0.009$.

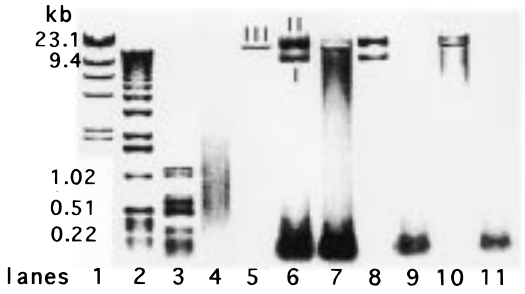

FIGURE 1: Agarose gel electrophoresis of DNA isolated from rat liver mitochondria. I, supercoiled mtDNA; II, open circular mtDNA; III, linear mtDNA; lanes 1 and 2, molecular-weight markers; lane 3, purified mtDNA (*Alu*I-cut); lane 4, nuclear DNA (*Eco*RI-cut); lane 5, mtDNA linearized with *Cla*I; lane 6, total DNA isolated from control mitochondria; lane 7, total DNA isolated from bleo/Fe-treated mitochondria; lanes 8 and 9 and lanes 10 and 11, the material of lanes 6 and 7, respectively, after separation. Both lanes 6 and 7 contain 400 ng of DNA and, in the low molecular-weight range some incompletely digested RNA, as shown by HPLC analysis.

8OHdG was checked by its absorption at 245 and 293 nm. 8OHdG in total DNA and low molecular-weight DNA was analyzed in material obtained from a total of 3 livers, and 8OHdG in high molecular-weight DNA was analyzed in material obtained from a total of 8−9 livers.

Statistical analyses were done by using standard errors of the mean (SEM), and the significance of the results was estimated with Student's *t*-test.

RESULTS

The mitochondria, prepared from rat liver by differential centrifugation, were tightly coupled, as judged by oxygen uptake measurements. To reduce the amount of contaminating nDNA these intact mitochondria were incubated with DNase I, thereafter washed extensively, and lysed under neutral conditions. DNA extracted from the lysed mitochondria consisted of circular and linear molecules with a size of about 16.3 kb and of fragments with a size of 0.5−0.1 kb, as shown by agarose gel electrophoresis and ethidium bromide staining (Figure 1, lane 6). It should be noted that ethidium bromide stains both DNA and RNA. These high and low molecular-weight DNA species could be separated from each other (Figure 1, lanes 8 and 9), and were extensively purified (see Materials and Methods). Southern blot analysis showed that the high molecular-weight DNA is exclusively mtDNA (Figure 2, top panel, lanes 6 and 8), whereas the low molecular-weight DNA is a mixture of mtDNA (Figure 2, top panel, lanes 6 and 9) and nDNA (Figure 2, bottom panel, lanes 6 and 9). When mitochondria were oxidatively stressed with 10 μM bleo/Fe, the same DNA species were found as in controls, except that some of the high molecular-weight mtDNA was nicked and "smeared" (Figure 1, lanes 7 and 10; Figure 2, top panel, lane 7 and lanes 10 and 11, and bottom panel, lanes 7 and 11). Lanes 8−11 in Figures 1 and 2 show the successful separation into the different nucleic acid species and cannot be compared in a quantitative manner to lanes 6 and 7.

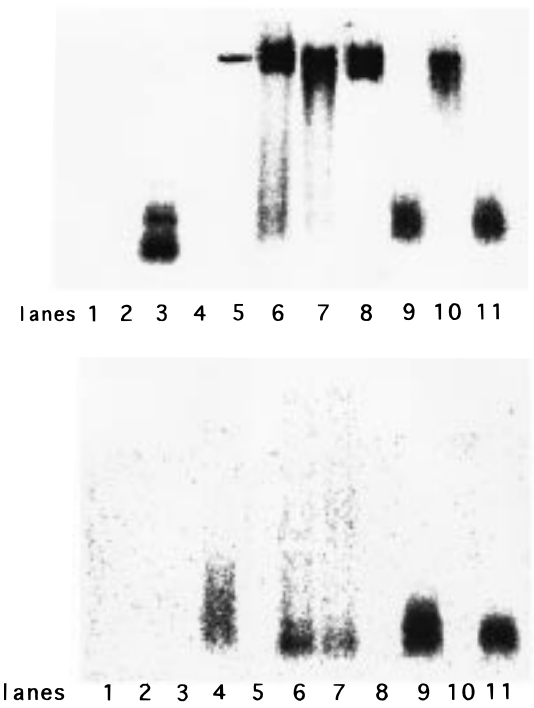

FIGURE 2: (Top panel) Southern blot analysis with a mitochondrial DNA probe. The gel shown in Figure 1 was processed. The probe was prepared as described in Materials and Methods DNA was hybridyzed against mtDNA (*Alu*I-cut). The lanes are the same as in Figure 1. (Bottom panel) Southern blot analysis with a nuclear DNA probe. The gel shown in Figure 1 was processed. The probe was prepared as described in Materials and Methods. DNA was hybridyzed against nDNA (*Eco*RI-cut). The lanes are the same as in Figure 1.

The yields of the various DNA species obtained from the intact liver mitochondria are summarized in Table 1. HPLC analysis revealed 15.9 μg of DNA/100 mg of mitochondrial protein for control mitochondria and 11.8 μg of DNA/100 mg for bleo/Fe-treated mitochondria. The smaller yield can be explained by a loss of DNA due to breakage of mitochondria during oxidative treatment. Next, Southern blot analysis was done to qualitatively and quantitatively evaluate mtDNA and nDNA. The amount of nDNA was 22.2% in the isolate obtained from control mitochondria and 14.1% in that from oxidatively stressed mitochondria. This differ-

Table 2: 8-Hydroxy-2′-deoxyguanosine (8OHdG) in DNA Species Obtained from Rat Liver Mitochondria[a]

	control mitochondria			bleomycin/Fe^{2+}-treated mitochondria		
	mean	SEM	*n*	mean	SEM	*n*
total DNA	0.185	0.0096	3	0.246	0.027	3
low molecular-weight DNA (mtDNA + nDNA)	0.320	0.0340	5	0.482	0.051	5
high molecular-weight DNA (mtDNA)	0.051[b]	0.0057	3	0.057[b]	0.007	3
low molecular weight mtDNA	0.741[c]			0.837−0.789[d]		
nDNA	0.014	0.0015	4	0.077[e]	0.003	3
total mtDNA	0.178			0.223−0.212[d]		

[a] The values are given in picomoles 8OHdG per microgram of DNA. The detection limit of the HPLC/EC-system is 0.1 pmol of 8OHdG. Between 6.15 and 9.12 μg of DNA were analyzed. No inverse correlation between the amount of DNA analyzed and the level of 8OHdG was detected (not shown). [b] $p = 0.171$. [c] Calculated with 0.014 pmol of 8OHdG/μg for nDNA. [d] We were not able to separate the low molecular-weight nDNA and mtDNA material. We therefore used the oxidation level of nDNA (0.014 pmol/μg for control DNA, 0.077 pmol/μg for oxidized DNA), the oxidation level of the low molecular-weight DNA (nDNA plus mtDNA), and the relative abundance of these species to calculate the oxidation level of low molecular-weight mtDNA. If the nDNA component of the low molecular-weight DNA were damaged to the low level seen in total nDNA isolated independently, then it would mean that the remainder of the low molecular-weight DNA, i.e., the fraction of it that is mtDNA, would be damaged to an extent of 0.837 or 0.789 pmol of 8OHdG/μg, respectively. [e] Oxidation level obtained with nDNA in the presence of bleomycin and Fe^{2+}, both at 10 μM; conditions and buffers as for mitochondria (see Materials and Methods).

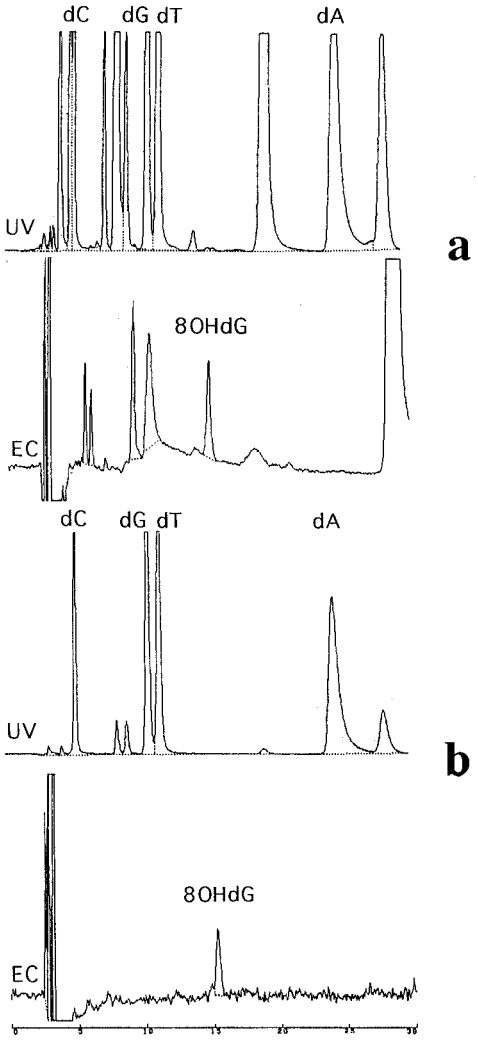

FIGURE 3: HPLC/EC analysis. UV traces show the position of the DNA nucleosides deoxycytidine (dC), deoxyguanosine (dG), thymidine (dT), and deoxyadenosine (dA). Unlabeled peaks represent hydrolytic products of RNA and unidentified compounds. On the EC traces the position of 8-hydroxydeoxyguanosine (8OHdG) is indicated. (a) Low molecular-weight DNA (4.3 μg) isolated from rat liver mitochondria; 8OHdG content 1.3 pmol. (b) Highly purified high molecular-weight mtDNA (4.0 μg); 8OHdG content 0.2 pmol.

ence is explained by the fact that bleo/Fe at the concentrations used here strongly degrades nDNA (results not shown), facilitating the digestion by DNase I. Incompletely digested nDNA seems to be associated with the outer mitochondrial membrane, because in mitoplasts (mitochondria freed of the outer membrane) the relative amount of contaminating nDNA is reduced (results not shown). Southern blot analysis further showed that mtDNA obtained from control mitochondria is fragmented to some extent (18.5%). The amount of mtDNA fragments is significantly increased to 21.1% in mitochondria incubated with bleo/Fe.

Figure 3 represents a typical result of the HPLC/EC analysis of DNA fragments (panel a) and of highly purified high molecular-weight mtDNA (panel b). The oxidation levels measured as 8OHdG of the various DNA species are summarized in Table 2. The amount of 8OHdG in total DNA isolated from bleo/Fe-treated mitochondria is 1.3 times higher than that obtained from control mitochondria. The oxidation level of the fragments alone, containing mtDNA and nDNA, is higher than that of total DNA and differs by a factor of 1.5 in the material isolated from control and bleo/Fe-challenged mitochondria. The high molecular-weight mtDNA molecules, however, show upon oxidative challenge only a minor (from 0.051 to 0.057 pmol/μg) and insignificant ($p = 0.17$) increase in 8OHdG. The increase in oxidative damage seen in total DNA must, therefore, be attributed to a higher oxidation level in the low molecular-weight DNA, as also revealed by the values obtained for the sum of low molecular-weight mtDNA and nDNA.

Rat liver nDNA isolated and purified in this laboratory contains 0.014 pmol of 8OHdG/μg. This value was used in the calculation of the amount of 8OHdG in low molecular-weight mtDNA. For control mitochondria an oxidation level of 0.741 pmol of 8OHdG/μg of DNA is thereby obtained. In bleo/Fe-treated mitochondrial preparations a possible oxidation of nDNA has to be taken into account. We, therefore, determined in isolated nDNA the amount of 8OHdG detectable after exposure to 10 μM bleo/Fe, which turned out to be 0.077 pmol/μg of nDNA. The oxidation level of mtDNA, calculated with the lowest (0.014 pmol/μg) and the highest possible value (0.077 pmol/μg) for nDNA, is 0.837 and 0.789 pmol/μg of mtDNA, respectively. In both situations the 8OHdG level in the low molecular-weight mtDNA obtained from bleo/Fe-challenged mitochondria is significantly ($p < 0.009$) increased compared to that

obtained from control mitochondria (0.741 pmol/μg of mtDNA).

DISCUSSION

Oxidative damage of mtDNA has received growing attention since its discovery a decade ago because mtDNA damage is the cause of or contributes to many human diseases and to aging. There is a general consensus that oxidative damage is much higher in mtDNA than in nDNA, but doubts were raised about the reported absolute values.

The present investigation shows that DNA isolated from carefully prepared and extensively DNase-treated rat liver mitochondria consists of 16.3 kb mtDNA (forms I, II, and III) and a mixture of low molecular-weight mtDNA and nDNA fragments, as shown by agarose gel electrophoresis in combination with Southern blotting. The presence of mtDNA fragments and contaminating nDNA in mtDNA preparations has been ignored in the majority of reports, and the uncertainties introduced by the contamination have been discussed (*14*). This issue asked for a detailed, differential analysis of oxidative damage in DNA isolated from mitochondria. This was achieved in the present study by separating low from high molecular-weight DNA molecules and analyzing them separately.

The oxidation level of total DNA (the sum of high and low molecular-weight mtDNA and nDNA) found here is in the range of previously published values (*14*). Two important results emerge from the analysis of DNA after separation of high from low molecular-weight mtDNA. First, the former shows an oxidation level only slightly (3.7-fold) above that of nDNA. Second, the mtDNA fragments, which constitute about 18% of the total mtDNA, are highly oxidized (15 times the value of the 16.3 kb mtDNA).

To support and extend the notion that the oxidation and fragmentation of mtDNA found in control mitochondria are related to oxidative stress, we looked for the possibility to intentionally increase it in vitro. This was afforded with bleo/Fe offered to respiring mitochondria, a procedure that nicked high molecular-weight mtDNA, decreased the amount of nDNA (due to fragmentation), and raised the amount of mtDNA fragments. The level of 8-OHdG in all the isolated DNA species was elevated. The smallest increase was found in high molecular-weight mtDNA, and the highest in fragmented DNA. To be able to calculate the oxidation level of mtDNA fragments, the possibility that nDNA might be oxidized prior to uptake of bleo/Fe into mitochondria has to be considered. To this end we treated purified nDNA with bleo/Fe in the absence of mitochondria. This enabled us to calculate the oxidation level in mtDNA fragments obtained from bleo/Fe-treated mitochondria, which was strongly elevated compared to the controls.

Despite high oxidative damage of mtDNA, it is faithfully replicated for many mitochondrial generations. In control and oxidatively challenged mitochondria we find most of the oxidized bases in mtDNA fragments and not in the 16.3 kb mtDNA molecules, which are the templates for replication. Thus, the previously published number of on average four modified bases per molecule (*8*) is reduced to about one-half in the 16.3 kb mtDNA molecule, leaving a good chance for faithful replication. These findings also indicate the existence of an efficient mtDNA repair or degrading system. Repair endonucleases are indeed present in mitochondria. Hegler et al. (*18*) detected and quantified incision sites of repair endonucleases. Croteau and Bohr (*19*) were the first to partially purify and characterize a mitochondrial oxidative damage endonuclease of rat liver. Furthermore, Shen et al. (*20*) showed that in human cells 80% of the damage in mtDNA induced by bleomycin was repaired after 2 h. Exposure of a human fibroblast cell line to hydrogen peroxide for 15 min resulted in the same repair activity for mtDNA as for nDNA, whereas longer exposure led to persistent mtDNA damage (*21*). From these studies it can be deduced that some of the small mtDNA molecules are degradation products. Mitochondria, however, respond to oxidative stress by synthesizing new DNA[2] and thereby deliver any size of mtDNA replication products. Thus, some of the fragments may be DNA newly formed in response to bleo/Fe.

In summary, total DNA isolated from liver mitochondria has an oxidation level in the previously published range, but this level represents the sum of the three DNA species (16.3 kb mtDNA, mtDNA fragments, and nDNA fragments). Most important is the finding that, in both control and stress conditions, the 16.3 kb mtDNA molecules appear oxidized only to a moderate extent and that the big share in oxidized bases is detected in the mtDNA fragments. Future investigations of oxidative mtDNA damage must take into account the extent of mtDNA fragmentation and nDNA contamination.

[2] P. B. Walter and C. Richter, unpublished observation.

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