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Age-associated increase in 8-oxo-deoxyguanosine glycosylase/AP lyase activity in rat mitochondria

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ABSTRACT

The mitochondrial theory of aging postulates that organisms age due to the accumulation of DNA damage and mutations in the multiple mitochondrial genomes, leading to mitochondrial dysfunction. Among the wide variety of DNA damage, 8-oxo-deoxyguanosine (8-oxo-dG) has received the most attention due to its mutagenicity and because of the possible correlation between its accumulation and pathological processes like cancer, degenerative diseases and aging. Although still controversial, many studies show that 8-oxo-dG accumulates with age in the mitochondrial (mt) DNA. However, little is known about the processing of this lesion and no study has yet examined whether mtDNA repair changes with age. Here, we report the first study on age-related changes in mtDNA repair, accomplished by assessing the cleavage activity of mitochondrial extracts towards an 8-oxo-dG-containing substrate. In this study, mitochondria obtained from rat heart and liver were used. We find that this enzymatic activity is higher in 12 and 23 month-old rats than in 6 month-old rats, in both liver and heart extracts. These mitochondrial extracts also cleave oligonucleotides containing a U:A mismatch, at the uracil position, reflecting the combined action of mitochondrial uracil DNA glycosylase (mtUDG) and mitochondrial apurinic/apyrimidinic (AP) endonucleases. The mtUDG activity did not change with age in liver mitochondria, but there was a small increase in activity from 6 to 23 months in rat heart extracts, after normalization to citrate synthase activity. Endonuclease G activity, measured by a plasmid relaxation assay, did not show any age-associated change in liver, but there was a significant decrease from 6 to 23 months in heart mitochondria. Our results suggest that the mitochondrial capacity to repair 8-oxo-dG, the main oxidative base damage suggested to accumulate with age in mtDNA, does not decrease, but rather increases with age. The specific increase in 8-oxo-dG endonuclease activity, rather than a general up-regulation of DNA repair in mitochondria, suggests an induction of the 8-oxo-dG-specific repair pathway with age.

INTRODUCTION

Cells and organisms are continuously exposed to deleterious species generated by the partial reduction of molecular oxygen. These molecules, known as reactive oxygen species (ROS), are extremely reactive and can attack lipids, proteins and nucleic acids. The reaction of ROS, particularly hydroxyl radicals, with nucleic acids yields a wide variety of damage, including sugar modifications, strand breaks and base adducts. Among the DNA adducts induced by oxidative stress, 8-oxo-deoxyguanosine (8-oxo-dG) has received the most attention. This lesion seems to occur endogenously at high frequency and was shown to be premutagenic, because it can mismatch with adenine leading to a G to T transversion (1). Moreover, it has been postulated that there is a striking correlation between the accumulation of 8-oxo-dG and pathological and degenerative processes like cancer and aging. This adduct was found at higher levels in lung cancer tissue compared with surrounding normal cells (2), and showed a 9-fold increase in DNA from breast cancer tissues compared to normal tissue (3).

The accumulation of oxidative DNA damage, especially 8-oxo-dG, has been proposed to play a major role in the aging process (reviewed in 4). According to this hypothesis, 8-oxo-dG accumulates with age to a greater extent in the mitochondrial genome than in the nuclear genome. Many laboratories have reported changes in 8-oxo-dG content with age in a variety of different tissues, but this field is still quite controversial due to methodological differences in analytical techniques. However, using a carefully designed DNA isolation procedure and a sensitive electrochemical array detection, we detect a 3-fold increase in 8-oxo-dG content in rat liver mitochondrial (mt) DNA with age (5). It has been proposed that the accumulation of DNA damage and mutations could result in abnormal expression of the mtDNA-encoded components of the electron transport chain and of ATP synthase, ultimately leading to impaired mitochondrial function and imbalanced cellular homeostasis.

In mammalian cells, oxidative DNA damage is mainly repaired via the base excision repair (BER) pathway. BER is initiated by

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the removal of the damaged base by a glycosylase. The resulting abasic site is then further processed by apurinic/apyrimidinic (AP) endonucleases, AP lyases and a DNA polymerase, which often replaces just one nucleotide, finally filling the gap (6). Several nuclear repair activities recognizing 8-oxo-dG have been identified in mammalian cells. In addition, specific 8-oxo-dG glycosylases/AP lyases (Ogg1 proteins) have been cloned from yeast, mouse and humans (7). However, little is known about the processing of oxidative DNA damage by mitochondria. Based on early observations that mitochondria are unable to remove pyrimidine dimers from their genome, the notion has persisted that mitochondria lack DNA repair mechanisms. However, studies from this and other laboratories (7–9) have demonstrated that mitochondria repair some forms of DNA damage. These include a number of lesions that are processed by BER, such as monofunctional alkylating agents and 8-oxo-dG. Further support for the existence of BER in mitochondria comes from the observation that enzymes involved in the BER pathways, such as uracil DNA glycosylase (UDG) and AP endonucleases, have been isolated from mitochondria (7). In addition, a proficient *in vitro* repair system for DNA containing abasic sites was recently fully reconstituted solely with proteins purified from *Xenopus* oocyte mitochondria (10). Our laboratory has recently partially isolated and characterized a novel mitochondrial oxidative damage endonuclease, named mtODE, which exhibits glycosylase/AP lyase activity towards 8-oxo-dG and abasic site-containing substrates (9).

The mitochondrial theory of aging postulates that cellular instability and aging derive from the steady accumulation of mutations in the multiple mitochondrial genomes (11). In order to achieve such accumulation one should expect that there is either an increased rate of damage formation over time or an age-dependent decline in damage removal, thus reflecting changes in DNA repair capacity. One test of this hypothesis involves the determination of whether there is a decline in mtDNA repair with aging. No such analysis has been reported so far, and we have thus investigated age-associated changes in the activity of mitochondrial enzymes involved in DNA repair. We have used mitochondrial extracts from heart and liver of young and old rats to measure the incision of duplex DNA substrates containing either an 8-oxo-dG or a U:A mismatch, thus assessing the activities of mtODE and mitochondrial uracil DNA glycosylase (mtUDG). We also measured the total mitochondrial endonucleolytic activity towards a supercoiled plasmid DNA to assess endonuclease G (Endo G) activity, which is the major mitochondrial endonuclease isolated from heart mitochondria (12).

MATERIALS AND METHODS

Materials

HEPES, benzamidine HCl, dithiotreitol (DTT), bovine serum albumin (BSA) and acrylamide/bisacrylamide (19:1) were from Sigma. Leupeptin and UDG were from Boehringer Mannheim. Isotopes were from NEN Life Science Products. G25 spin columns were from 5Prime→3Prime, Inc. T4 polynucleotide kinase was from Stratagene. Endonuclease III was purchased from Trevigen, Inc. The plasmid DNA pUC was kindly provided by Dr Gregory Dianov, LMG, NIA, NIH. All other reagents were ACS grade and from Sigma Chemical Co.

Animals

Male white Wistar rats from the Gerontology Research Center animal colony, at 6, 12 or 23 months of age, were used in this study. The animals were housed in the colony under standard conditions and fed Purina chow *ad libitum*. The animals were killed by cervical dislocation, and the liver and heart were immediately removed and processed.

Isolation of heart mitochondria

Rat heart mitochondria (RHM) were prepared by a high-speed differential centrifugation and a short protease digestion with Nagarse, as reported by Hansford (13).

Isolation of liver mitochondria

Rat liver mitochondria (RLM) were isolated using a combination of differential centrifugation and Percoll gradient centrifugation as previously described (9).

Oligonucleotides

All oligonucleotides used in this study were from Midland Certified Reagent Co. Their sequences are presented in Table 1. Oligonucleotides containing either the DNA lesion or an unmodified base were 5′-end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. To separate the unincorporated free [γ-^{32}P]ATP, the reaction mixtures were spun through a G25 column. Complementary oligonucleotides were annealed in 10 mM Tris, pH 7.8, 100 mM KCl, 1 mM EDTA, by heating the samples at 80°C for 5 min and allowing them to cool slowly to room temperature.

Table 1. Oligonucleotide substrates used

Name	Sequence										
G	5′	GAA	CGA	CTG	T **G** A	CTT	GAC	TGC	TAC	TGA	T
	3′	CTT	GCT	GAC	A **C** T	GAA	CTG	ACG	ATG	ACT	A
OG	5′	GAA	CGA	CTG	T[**OG**]A	CTT	GAC	TGC	TAC	TGA	T
	3′	CTT	GCT	GAC	A **C** T	GAA	CTG	ACG	ATG	ACT	A
UC	5′	GAA	CGA	CTG	T**T**A	CTT	GAC	TGC	TAC	TGA	T
	3′	CTT	GCT	GAC	A**A**T	GAA	CTG	ACG	ATG	ACT	A
UU	5′	GAA	CGA	CTG	T[**U**]A	CTT	GAC	TGC	TAC	TGA	T
	3′	CTT	GCT	GAC	A **A** T	GAA	CTG	ACG	ATG	ACT	A

The important features are underlined and in bold. OG, 8-oxo-dG; U, uracil. All the other bases are normal.

Measurement of 8-oxo-dG glycosylase/AP lyase activity

8-oxo-dG glycosylase/AP lyase activity in crude mitochondrial extracts was measured by an oligonucleotide incision assay, as previously described (9). Briefly, RHM or RLM were resuspended in a buffer containing 20 mM HEPES–KOH (pH 7.6), 1 mM EDTA, 2 mM DTT, 300 mM KCl, 5% glycerol and 0.05% Triton X-100. The samples were diluted to a final concentration of 25 µg/µl, with the same buffer lacking Triton X-100. The KCl concentration was then adjusted to 100 mM. Incision reactions (20 µl) containing: 40 mM HEPES–KOH (pH 7.6), 5 mM EDTA, 2 mM DTT, 75 mM KCl, 10% glycerol, 88.7 fmol of ^{32}P-labeled duplex oligonucleotide (Table 1, G or OG) and 100 µg of

mitochondrial protein, were incubated overnight at 32°C. The reactions were terminated by the addition of 0.8 µl of each 5 mg/ml Proteinase K and 10% SDS and incubation at 55°C for 15 min. The DNA was ethanol-precipitated by the addition of 1 µg of glycogen, 4 µl of 11 M ammonium acetate and 60 µl ethanol. The DNA was pelleted, dried, and resuspended in formamide dye. The samples were subjected to electrophoresis on a denaturing 20% polyacrylamide gel containing 7 M urea. After electrophoresis, the gels were subjected to autoradiography at –80°C. Quantification of results employed a Molecular Dynamics PhosphorImager and the ImageQuant NT software.

Measurement of mtUDG activity

mtUDG activity in crude mitochondrial extracts was measured by an oligonucleotide incision assay. For this assay, mitochondria were suspended in a buffer containing 20 mM HEPES–KOH (pH 7.5), 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 10% glycerol and 0.05% Triton X-100. The samples were then diluted with the same buffer without Triton X-100 to the desired final protein concentrations. Reactions (20 µl) containing 70 mM HEPES–KOH (pH 7.5), 1 mM EDTA, 1 mM DTT, 75 mM NaCl, 0.5% BSA, 88.7 fmol of ^{32}P-labeled duplex oligo (Table 1, UC or UU) and either 1 or 25 µg of mitochondrial protein, for liver or heart, respectively, were incubated for 1 h at 37°C. The reactions were terminated and the DNA processed as described for the measurement of 8-oxo-dG endonucleolytic activity.

Measurement of mitochondrial endonuclease activity

The endonucleolytic activity of mitochondrial extracts was measured by the relaxation of a supercoiled DNA, as described by Houmiel *et al*. (14) using the negative supercoiled plasmid pUC. Briefly, mitochondria were suspended in a buffer containing 100 mM HEPES–KOH (pH 7.4), 100 mM NaCl, 5 mM DTT, 10% glycerol and 0.05% Triton X-100. Reactions (20 µl) containing 25 mM HEPES–KOH (pH 7.4), 0.1 mg/ml BSA, 1 mM DTT, 1 mM $MnCl_2$, 3% glycerol, 300 ng plasmid DNA and the desired amount of mitochondrial protein were incubated for 30 min at 37°C. The reactions were stopped by the addition of 1% SDS and loading buffer and loaded in a 1.5% agarose gel in TAE. The gels were run for 12 h at 1 V/cm, stained in ethidium bromide 0.1 µg/ml for 30 min, destained in water for 1 h and visualized under UV. Quantification of the results employed a Molecular Dynamics Fluorimager and the ImageQuant software.

Measurement of citrate synthase activity

Citrate synthase activity was measured spectrophotometrically by thiol release at 412 nm following the method described by Hansford and Castro (15).

Statistical analysis

The results are reported as mean ± standard deviation, of at least two different experiments, performed in duplicate. For each experiment, two different gels were run. The results are an average of at least six different mitochondrial preparations, and the number of preparations assayed for each age is presented in parenthesis. The differences among ages were analyzed by the Student's *t*-test, and a $P \leq 0.05$ was considered statistically significant.

RESULTS

Changes in 8-oxo-dG glycosylase/AP lyase activity with age

mtODE activity in crude mitochondrial extracts was measured using an oligonucleotide incision assay, with oligonucleotides containing either an unmodified guanosine or an 8-oxo-dG at position 11 (named as G for control and OG for 8-oxo-dG-containing oligonucleotides; Table 1). After incubation of these constructs with mitochondrial extracts, the products were ethanol-precipitated and resolved on a polyacrylamide–urea gel. The bands were visualized by autoradiography and PhosphorImager analysis. Figure 1A presents a typical gel of 8-oxo-dG-containing 28mer oligonucleotides incubated with increasing protein concentration, 50–200 µg (lanes 2–5), of RLM extracts. This figure shows the concentration-dependent appearance of a 10mer-radiolabeled fragment after incubation of the 8-oxo-dG oligonucleotide with RLM extracts, indicating the cleavage at the position occupied by 8-oxo-dG. This product co-migrates with the β-elimination product generated by purified mtODE (9) (not shown), indicating that the activity we measured in crude mitochondrial extracts is due to mtODE. In addition, this activity proved to be damage specific, since no incision was detected in control oligomers (not shown).

The quantification of incision activity showed a linear correlation between protein concentration and formation of the 10mer cleavage product (Fig. 1B) between 0 and 150 µg of crude mitochondrial extracts, and an apparent saturation of the cleavage activity beyond that. Using less substrate, 8.9 fmol per reaction, we were able to detect the same protein concentration dependence in the incision activity during a much shorter incubation time (90 min) (Fig. 1C). However, the total radioactivity contained in this amount of substrate was in the lower range of linearity of the PhosphorImager screens, and this could result in underestimation of incision activity due to loss of sensitivity in the quantification. Thus, we chose 18 h incubations and used the higher substrate concentration for the age comparison experiments. This way we could be assured that we would be able to detect even small changes in the percentage of incision between samples.

To investigate possible differences in the repair capacity between ages, we measured mtODE activity in three age groups: 6, 12 and 23 month-old rats. These groups represent young adult, middle-aged and old animals, respectively. The incision activity of the crude mitochondrial extracts was measured as the amount of oligonucleotide converted from the intact 28–10mer, in reactions containing 88.7 fmol of 8-oxo-dG, 100 µg mitochondrial protein and incubated for 18 h. For this study, we chose heart and liver mitochondria, since both organs have a high mitochondrial content, and heart, in particular, is one of the tissues with the highest oxidative metabolic rate in mammals.

The results are presented in Figure 2A. 8-oxo-dG incision activity increases significantly with age, in both liver (open bars) and heart (shaded bars) mitochondrial extracts, with the highest activity found at 12 months for both organs. RLM extracts from 6 month-old animals cleaved 25.8 ± 6.8 fmol of 8-oxo-dG oligo, while RLM from 12 and 23 month-old animals cleaved 51.96 ± 7.88 and 42.12 ± 8.4, respectively. These values represent an increase in incision activity of ~100% at 12 months and 63% at 23 months, relative to 6 months of age. No cleavage of the control oligomer was seen under these experimental conditions. The same pattern of age-associated increase in the 8-oxo-dG incision activity was found with RHM. RHM extracts from 6, 12 and

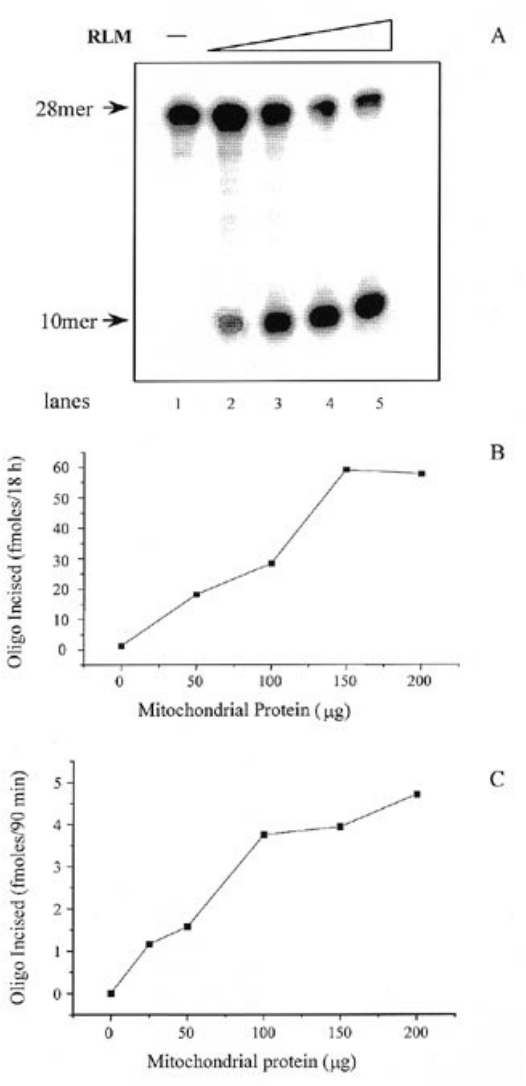

Figure 1. Cleavage of an 8-oxo-dG-containing oligonucleotide by RLM extracts. (**A**) 50–200 µg of crude RLM extracts (lanes 2–5) were incubated with 88.7 fmol of 8-oxo-dG oligo (Table 1) for 18 h at 32°C. The DNA was processed as described, the gel was exposed to a PhosphorImager screen, and the products were quantified (**B**). Control reaction (line 1) was incubated with buffer only. (**C**) 25–200 µg of RLM extracts were incubated with 8.9 fmol of 8-oxo-dG oligo for 90 min at 37°C, and the products were quantified as above. A typical gel of at least two different experiments is presented.

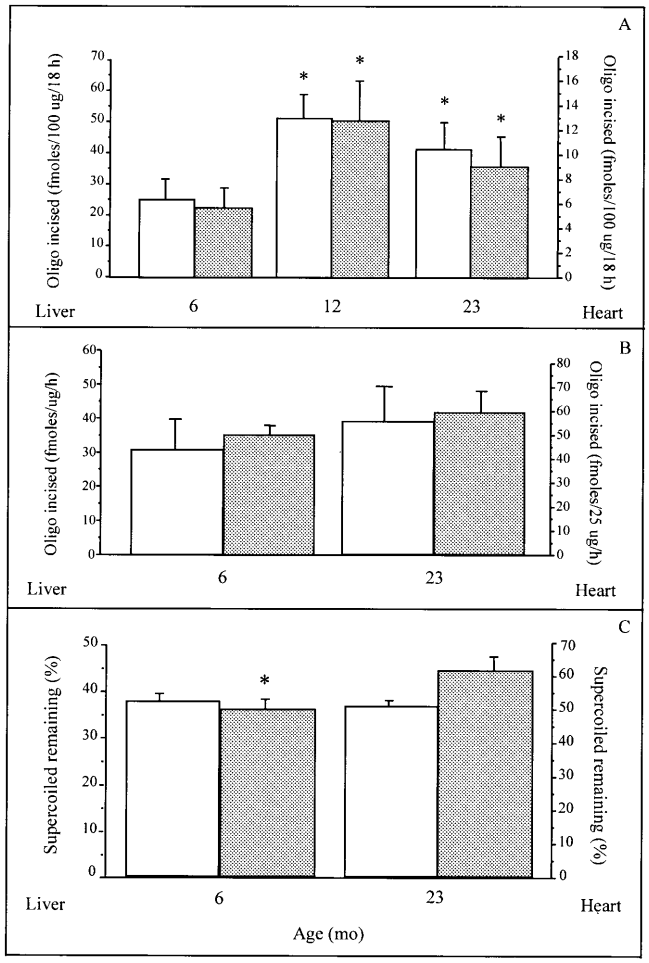

Figure 2. Age-associated changes in mtODE, UDG and Endo G activities. (**A**) 100 µg of rat liver (open bars) or heart (shaded bars) mitochondrial extracts were incubated with 88.7 fmol of 8-oxo-dG oligonucleotide (Table 1) for 18 h at 32°C. The DNA was then processed as described. The amount of oligonucleotide incised was calculated from the percentage of radioactivity in the 10mer band. (**B**) An aliquot of 1 µg of rat liver (open bars) or 25 µg of heart (shaded bars) mitochondrial extracts was incubated with 88.7 fmol of UU oligonucleotide for 1 h at 37°C. The DNA was processed as described, and the amount of oligonucleotide incised was calculated from the percentage of radioactivity in the bands correspondent to the cleavage products. (**C**) An aliquot of 0.1 µg of liver (open bars) or 0.01 µg of heart (shaded bars) mitochondrial extracts was incubated with 300 ng of plasmid DNA for 1 h at 37°C. The reactions were terminated and the products separated as described earlier. In all panels, the left *y*-axis refers to liver and the right to heart mitochondria. The values presented represent the mean ± standard deviation of two experiments performed in duplicate, with $n = 6$ for each age group. *$P \pm 0.05$.

23 month-old animals cleaved 5.87 ± 1.33, 12.96 ± 3.21 and 9.24 ± 2.44 fmol of 8-oxo-dG oligomer, respectively, representing an increase of ~120% at 12 months and 57% at 23 months, compared to the activity at 6 months of age. Activities at 12 and 23 months were both significantly higher than at 6 months, with a P value of <0.05 for both organs.

To rule out the possibility that the differences in mtODE activity were due to differential mitochondrial enrichment during the isolation procedure, the activities were normalized to citrate synthase activity. The normalized values are presented in Table 2. After normalization to citrate synthase, we again find a statistically significant increase in 8-oxo-dG incision activity at 12 and 23 months compared with 6 months in both liver and heart. For liver mitochondria, 12 and 23 month-old activities were significantly higher than those at 6 months ($P < 0.01$), and 12 months showed a slight but not significant increase ($P = 0.066$) compared to those at 23 months. For heart, however, extracts from 12 month-olds were higher than from 6 month-olds and also significantly higher ($P < 0.05$) than for 23 month-olds.

Changes in mtUDG activity with age

mtUDG activity in crude mitochondrial extracts was also measured by an oligonucleotide incision assay, using an oligonucleotide containing a U:A mismatch at position 11, oligo UU

Table 2. Normalization of mtODE, mtUDG and mtEndo G activities by citrate synthase

	Liver			Heart		
Age (mo)	6	12	23	6	12	23
Enzyme Activity						
MtODE[1]	219.8±54.9 (n=5)	482.8±106.9 (n=5)*	418.1±96.3 (n=5)*	2.9 ± 0.9 (n=9)	7.3 ± 1.2 (n=6)*@	4.14 ± 0.9 (n=6)*
MtUDG[2]	257.2±85.9 (n=6)	_	261.9±65.4 (n=6)	26.4 ± 3.4 (n=6)	_	33.7 ± 4.2 (n=6)*
mtEndo G[3]	323.25±46.48 (n=6)	_	279.39±39.11 (n=6)	233.98±43.77 (n=6)	_	311.23±31.19 (n=6)*

[1]Data presented as (% incision/100 µg protein)/(µmol citrate/mg protein/min).
[2]Data presented as (% incision/µg protein)/(µmol citrate/mg protein/min) for liver and (% incision/25 µg protein)/(µmol citrate/mg protein/min) for heart.
[3]Data presented as (% supercoiled remaining/0.1 µg protein/h)/(µmol citrate/mg protein/min) for liver and (% supercoiled remaining/0.01 µg protein/h)/(µmol citrate/100 µg protein/min) for heart.
*P < 0.05 versus 6 months; @P < 0.05 versus 23 months.

(Table 1). Figure 3 shows the dose-dependent conversion of the intact 28mer into a 10mer fragment, after the incubation of 88.7 fmol of UU oligo with increasing protein concentration of RLM extracts, from 0.05 to 2 µg (lanes 4–8). The cleavage products obtained under these conditions co-migrate with the product of the incubation of UU oligo with purified UDG protein plus fapyglycosylase (FPG) protein (lane 3), indicating that the mitochondrial extracts specifically recognize and cleave at the position containing the U:A mismatch. Under these experimental conditions, no cleavage was detected in a control oligo, containing an unmodified T:A base pair. Since UDGs do not possess an associated AP lyase activity, the specific cleavage at the position containing the uracil residue probably reflects the combined activities of mtUDG and mtAP endonucleases. This is likely since co-incubation of the UU oligo with mitochondrial extract plus purified FPG protein or Endo III protein does not result in an additional 10mer product formation compared to the mitochondrial extract alone (data not shown). These results indicate that under these experimental conditions the mtAP endonuclease activity is not a rate-limiting step.

To investigate the age-associated changes in mtUDG activity, 88.7 fmol of UU oligo were incubated with liver or heart mitochondrial extracts for 1 h. mtUDG activity was measured as the percentage of radioactivity in the two cleavage products co-migrating with the product from the incubation of UU oligo plus UDG and FPG proteins (Fig. 3, lane 3). Figure 2B shows that 1 µg of RLM extracts (open bars) from 6 month-old animals cleaved 31.34 ± 9.2 fmol and extracts from 23 month-olds cleaved 39.47 ± 10.1 fmol of UU oligo. Although slightly higher at 23 months, there was no significant difference in mtUDG between the ages. Similarly, incubation of UU oligo with 25 µg of RHM extracts (shaded bars) showed a slightly higher activity in older animals, but no significant change in mtUDG activity with age. Mitochondria from 6 and 23 month-old animals cleaved 50.92 ± 3.93 and 59.98 ± 8.77 fmol, respectively.

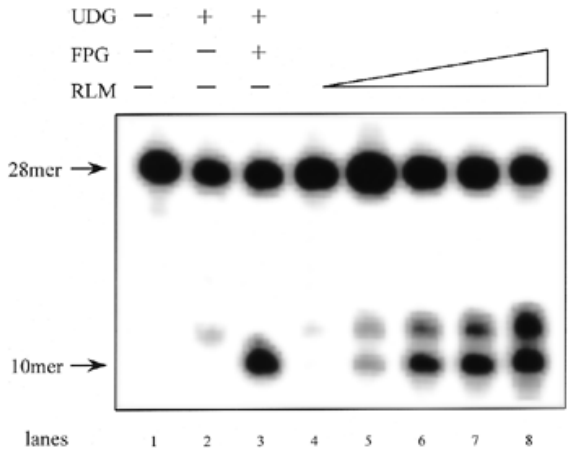

Figure 3. Cleavage of a U:A-containing oligonucleotide by RLM extracts. Aliquots of 0.05–2 µg of crude RLM extracts (lanes 4–8) were incubated with 88.7 fmol of UU oligo (Table 1) for 1 h at 37°C. The DNA was precipitated and resolved as described. Control reactions containing 0.01 U of UDG (lane 2) or UDG plus 0.01 U of FPG (lane 3) are presented. A typical gel of at least two different experiments is presented.

mtUDG activity was also normalized to citrate synthase activity. After normalization, no change in mtUDG with age was found in liver extracts. However, a significant increase from 6 to 23 months of age (P = 0.01) was found in heart mitochondria (Table 2).

Changes in mitochondrial endonuclease activity with age

The total endonucleolytic activity of crude mitochondrial extracts was measured by assaying the conversion of a supercoiled plasmid DNA, pUC, to the open circular and linear forms, after

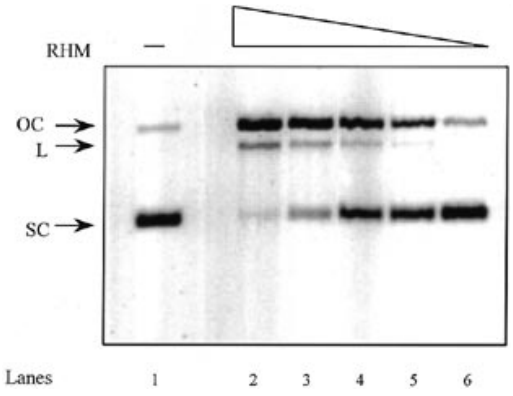

Figure 4. pUC plasmid DNA relaxation by RHM extracts. 300 ng of supercoiled plasmid DNA were incubated for 1 h at 37°C, with increasing concentration (0.001–0.1 µg) of RHM extracts. The reaction was then terminated by adding loading buffer and the samples separated in a 1.5% agarose gel. A typical gel of two independent experiments is presented.

incubation with mitochondrial extracts. Figure 4 shows the dose-dependent conversion of the supercoiled plasmid to circular and linear forms after incubation with increasing concentrations (0.001–0.1 µg) of RHM (lanes 2–6). The relaxation of supercoiled plasmid into open circular DNA reflects the formation of one or more single strand breaks, while the conversion to the linear form is due either to one double strand break or two distinct single strand breaks closely positioned. This endonucleolytic activity was dependent on divalent cations, since activity was completely blocked in presence of 1 mM EDTA (not shown), suggesting that it is mainly due to Endo G (12).

To investigate possible age-associated changes in the total mitochondrial endonucleolytic activity, 300 ng of plasmid DNA were incubated with 0.1 µg of protein from RLM or 0.01 µg of protein from RHM from 6 and 23 month-old animals. The percentage of intact supercoiled DNA remaining after 1 h incubation was measured using ImageQuant software. The results are presented in Figure 2C. We did not find any change with age in liver extracts (open bars), but a decrease in endonucleolytic activity in heart extracts from older animals (shaded bars). RLM extracts from young animals relaxed by 49.8 ± 4.3% of the initial amount of DNA, with 50.2% remaining in the intact supercoiled form. Extracts from older animals relaxed 52.8 ± 3.67%, with 47.2% remaining in the supercoiled form. In contrast, heart extracts from young rats relaxed 49.7 ± 3.1%, with 50.3% supercoiled remaining, and extracts from older animals converted 38.1 ± 3.2, with 61.93% remaining in the intact supercoiled form. After normalization of these results by citrate synthase activity (Table 2), the same pattern was found. Liver mitochondria showed a slight increase in activity from 6 to 23 months old, which was, however, not significant. Heart mitochondria, on the other hand, showed a significant ($P < 0.05$) decrease in endonuclease activity from 6 to 23 months of age.

DISCUSSION

A large body of experimental evidence supports the existence of a relationship between genomic instability, DNA damage and aging. In recent years, various attempts have been made to measure DNA repair capacity changes with age. Despite initial results demonstrating a linear correlation between the logarithm of lifespan and the DNA repair capacity in cells from different mammalian species (16), the results from further studies have differed and there is no consensus on this matter (17). Moreover, this subject was further complicated by the heterogeneity in repair between different regions of the genome. Recently, Guo *et al.* (18) reported that the removal of UV-induced damage in actively transcribed regions is lower in hepatocytes isolated from old than from young rats, suggesting a decrease in transcription coupled repair with age. Studies on DNA repair changes with aging have, so far, only been carried out in nuclear DNA or in total cellular DNA. There has been no such analysis of DNA repair changes in the mtDNA with age, despite the suggestion that the damage accumulation with age is remarkably higher in the mtDNA than in nuclear DNA (5,19).

In this paper, we have investigated the age-associated changes in the activities of mitochondrial enzymes involved in the processing of DNA damage. It has been the general consensus that the DNA repair capacity declines with age, and that this contributes to damage accumulation in the mitochondrial genomes. Contrary to this notion, we find a significant increase in mtODE activity with age in liver and heart mitochondria. The incision activity of crude mitochondrial extracts towards 8-oxo-dG doubles from 6 to 12 months of age, followed by a slight decrease at 23 months in both organs (Fig. 2A; Table 2). Although we measured cleavage of an 8-oxo-dG-containing substrate by whole mitochondrial extracts, it is very likely that this activity reflects the activity of mtODE, which we have previously isolated from RLM (9). Support for this comes from results showing that the activity exhibited by the whole mitochondrial extracts displays the same properties as mtODE. It recognizes 8-oxo-dG in double strand but not in single strand DNA substrates, the cleavage activity is insensitive to EDTA in the reaction buffer and exhibits the same salt dependence as mtODE (9) (data not shown). These results suggest that, contrary to the common notion, mitochondrial oxidative DNA damage repair does not decline with age. In fact, it increases. If we assume that our conditions mimic *in vivo* physiology, the accumulation of oxidative lesions with age in mtDNA is not due to deficiency in repair, but might rather be due to increased damage formation.

Due to the low levels of this activity in crude mitochondrial extracts, the incision assay used for 8-oxo-dG-containing oligomers required a long incubation (18 h) to detect the products, and this could suggest that we were working under saturating conditions. However, the percentage of cleavage increased linearly with protein concentration (Fig. 1B) showing that the amount of product formed depends on enzyme activity and/or abundance. Further support for this comes from the observation that the same turnover rate can be observed in shorter reactions where we have decreased the initial substrate concentration (Fig. 1C). Thus, it is clear that changes in the amount of product generated by different mitochondrial extracts reflect actual differences in relative activity of mtODE.

Because mitochondrial preparations are not homogeneous, the possibility should be considered that the differences in activity we observed could be due to contamination by nuclear proteins. However, it is important to point out that during the isolation of heart mitochondria, the heart tissue is subject to protease digestion with Nagarse, a wide spectrum protease, in order to release the mitochondria from the myofibrillar net. Therefore, any cytosolic protein should be digested and the final mitochondrial

pellet is free of contaminating cytosolic activities. In addition, Croteau *et al*. (9) found the same mtODE activity in extracts from liver mitochondria previously treated with proteases, as well as in mitoplasts, as in untreated samples. Since the same isolation procedure has been used for liver mitochondria in both studies, these results exclude the presence of contaminating 8-oxo-dG incising activity in our liver mitochondria suspension.

It has been suggested that the mitochondrial population in samples from old animals is more heterogeneous than in young counterparts. In fact, Hagen *et al*. (20) recently showed that three distinct hepatocyte populations could be obtained from livers from old rats based on their average membrane potential, indicating considerable heterogeneity in the mitochondrial population. Since a differential loss of damaged mitochondria can occur, especially when isolating them from old animals (21), it is possible that the purified mitochondrial fraction may not be representative of the *in vivo* mitochondrial population. In order to account for possible differences in mitochondrial content in our preparations, we normalized our values to citrate synthase activity (Table 2). This enzyme has been widely used as a marker for mitochondrial content, based on results showing that its activity (15), as well as the message level (22) did not change with age. The same age-associated increase in mtODE activity was found after normalization to citrate synthase (Table 2), indicating that variations in mitochondrial content did not account for the differences in activity observed. Although this does not exclude differential enrichment in populations during isolation, it assures us that leaky mitochondria, which could be contaminated by cytosolic proteins, did not contribute to the final mitochondrial suspension. These mitochondria would have lower citrate synthase content, and would consequently reduce the normalized values.

In order to verify whether the changes in 8-oxo-dG cleavage activity with age reflect an overall age-associated increase in DNA repair proteins, we measured the activities of two other well-characterized mitochondrial enzymes, mtUDG and mtEndo G. The significant increase in cleavage activity with age, in both liver and heart mitochondria, strongly contrasts with the other enzyme activities. mtUDG activity did not change in liver mitochondria, but showed a discrete but significant increase with age in heart mitochondria, when normalized to citrate synthase activity (Fig. 2B; Table 2). The total endonuclease activity also showed no change with age in liver but, in contrast to mtUDG, was less active in heart mitochondria from old animals (Fig. 2C; Table 2).

Taken together, these results suggest that the mtODE activity may be specifically up-regulated during the aging process. In bacteria (23) and in mammals (24), the glycosylase activity towards 8-oxo-dG can be induced by low doses of DNA damaging agents. More specifically, in rats treated with potassium bromate, a selective renal carcinogen, 8-oxo-dG glycosylase activity increases in whole cell extracts from kidneys but does not change in liver (24), suggesting a relationship between DNA damage formation and glycosylase induction. Although it has long been held that DNA repair enzymes are not induced in mammalian cells, there are recent reports finding that BER enzymes can be induced in response to DNA damage. Chen *et al*. (25) reported an up-regulation of polymerase β after oxidative stress, and this correlated with enhanced BER in nuclear DNA and enhanced cellular tolerance to higher doses of DNA damaging agents. Furthermore, Ramana *et al*. (26) showed that ROS generating agents could induce the overexpression of APE, the major nuclear AP endonuclease in mammalian cells. Thus, it is possible that a DNA damage responsive element, or even an element responsive to oxidative stress itself, could trigger an adaptive response, which would lead to an increased rate of damage removal by the up-regulation of enzymes involved in BER. In agreement with this possibility, Le *et al*. (27) showed that pre-exposure of human lung carcinoma cells to a low dose of γ irradiation enhanced the initial rate of repair of thymine glycol, one of the main forms of base damage induced under these conditions. Furthermore, Driggers *et al*. (28) reported that a rat insulinoma cell line showed an elevated removal of oxidative damage from the mitochondrial genome 4 h after the removal of the damaging agent, suggesting the existence of such an adaptive response.

In this study, we found no changes in mtUDG activity with age in liver (Fig. 2B), but a significant increase in the citrate syntase normalized activity in heart mitochondria (Table 2). In contrast to mtODE, this change in mtUDG activity is organ specific, and may reflect other changes in signal transduction and gene expression in heart cells rather than age-associated changes.

We also found organ-specific changes in Endo G activity. To assess Endo G activity, we used the relaxation of a negatively supercoiled plasmid DNA. Although this assay measures the total endonucleolytic activity in a given extract, the activity was sensitive to EDTA and dependent upon the presence of magnesium or manganese in the reaction buffer (not shown). These properties resemble those of isolated Endo G (29). Moreover, the plasmid relaxation assay correlates well with other assays used for Endo G activity such as acid solubilization of labeled DNA and the use of sequencing gels to assess sequence-specific cleavage (12). Although the physiological role of this enzyme is still uncertain, it has been proposed that it plays a role in DNA repair, maybe in degradation of damaged templates. This suggestion is supported by results showing that during rat cardiac development, the levels of this endonuclease showed a positive correlation with the oxidative status of the tissue, implying a role in the repair of oxidative damaged mitochondrial genomes (30). Further support of this also comes from the observation of preferential cutting by Endo G at sites containing local distortions in the duplex DNA introduced by adducts (31).

An interesting observation that arises from our results is the great difference in the activity of these three enzymes between heart and liver mitochondria. While mtODE and mtUDG activities are 5–20 times higher in liver extracts, Endo G is about 10 times more active in heart mitochondria. Although very little is known about mtDNA repair, and no study has specifically addressed the question of organ-specific differences, these results suggest that different organs may rely on different repair pathways. It is possible that a difference in damage accumulation accounts for such response. There are no available studies to clarify these differences in tissue specificity at this time.

In conclusion, our results show an increased 8-oxo-dG endonuclease activity in older animals suggesting increased repair of 8-oxo-dG and induction of this activity during the aging process. This suggests that the accumulation of 8-oxo-dG in the mtDNA with age may be due to an increased formation of these lesions rather than a decline in DNA repair. It has been suggested that the rate of free radical production is the determinant factor in the rate of aging (32), and it is possible that damage formation surpasses the cell's protective mechanisms, including DNA repair, leading to damage accumulation and ultimately loss of function.

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