Oxidative damage to mitochondrial DNA is inversely related to maximum life span in the heart and brain of mammals

GUSTAVO BARJA[1] AND ASUNCIÓN HERRERO

Department of Animal Biology-II (Animal Physiology), Faculty of Biology, Complutense University, Madrid 28040, Spain.

ABSTRACT **DNA damage is considered of paramount importance in aging. Among causes of this damage, free radical attack, particularly from mitochondrial origin, is receiving special attention. If oxidative damage to DNA is involved in aging, long-lived animals (which age slowly) should show lower levels of markers of this kind of damage than short-lived ones. However, this possibility has not heretofore been investigated. In this study, steady-state levels of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) referred to deoxyguanosine (dG) were measured by high performance liquid chromatography (HPLC) in the mitochondrial (mtDNA) and nuclear (nDNA) DNA from the heart of eight and the brain of six mammalian species ranging in maximum life span (MLSP) from 3.5 to 46 years. Exactly the same digestion of DNA to deoxynucleosides and HPLC protocols was used for mtDNA and nDNA. Significantly higher (three- to ninefold) 8-oxodG/dG values were found in mtDNA than in nDNA in all the species studied in both tissues. 8-oxodG/dG in nDNA did not correlate with MLSP across species either in the heart ($r=-0.68$; $P<0.06$) or brain ($r=0.53$; $P<0.27$). However, 8-oxodG/dG in mtDNA was inversely correlated with MLSP both in heart ($r=-0.92$; $P<0.001$) and brain ($r=-0.88$; $P<0.016$) tissues following the power function $y = a \cdot x^b$, where y is 8-oxodG/dG and x is the MLSP. This agrees with the consistent observation that mitochondrial free radical generation is also lower in long-lived than in short-lived species. The results obtained agree with the notion that oxygen radicals of mitochondrial origin oxidatively damage mtDNA in a way related to the aging rate of each species.—Barja, G., Herrero, A. Oxidative damage to mitochondrial DNA is inversely related to maximum life span in the heart and brain of mammals. *FASEB J.* 14, 312–318 (2000)**

Key Words: 8-hydroxy-deoxyguanosine · longevity · aging · free radical

WHILE THE MAJOR causes of aging remain unclear, the free radical theory of aging (1, 2), especially when focused on mitochondria (3, 4), is frequently being tested. Oxygen radicals leaking from the mitochondrial respiratory chain are thought to continuously damage important cellular macromolecules in postmitotic tissues. Among these targets, there is wide agreement that damage to DNA is of utmost importance, since repair of all the rest of the biologically relevant macromolecules is ultimately dependent on the information coded in the DNA. Although studies of variations as a function of age in tissue levels of oxidative damage in nuclear DNA (nDNA) have yielded controversial results (5–12), almost all the studies performed to date have found increases in oxidative damage in mitochondrial DNA (mtDNA) during aging (5, 6, 13–16). Furthermore, the few available studies indicate that oxidative damage is higher in mtDNA than in nDNA (5, 6, 17, 18). This is consistent with the observation that mitochondrial DNA mutations accumulate during aging (19, 20).

Different mammalian species can age at very different rates. Rapidly aging species like rodents have short maximum life spans (MLSPs: 3.5–4 years in mice and rats), whereas slowly aging species like cows and horses reach MLSPs of 30 and 46 years, respectively. In agreement with the mitochondrial free radical theory of aging, all comparative studies performed to date have found that the rate of oxygen radical production by mitochondria is inversely correlated with MLSP (21–27; see ref 28 for a review). It remains to be demonstrated, however, if the relatively lower rate of free radical generation of long-lived animals is accompanied by a lower rate of oxidative damage to their DNA. If the mitochondrial free radical-DNA damage hypothesis of aging is correct, at least the oxidative damage to mtDNA, which is placed near the main oxygen radical generator of healthy tissues, should be less in slow than in

[1] Correspondence: Departamento de Biología Animal-II, Facultad de Biología, Universidad Complutense, Madrid 28040.

0892-6638/00/0014-0312/$02.25 © FASEB

rapidly aging animal species. However, this important test has never been performed.

In this investigation, oxidative damage to both nDNA and mtDNA, estimated through the measurement of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) by high performance liquid chromatography (HPLC), was studied in the heart of eight and in the brain of six mammalian species showing more than one order of magnitude of difference in MLSP (from 3.5 years in mice to 46 years in horses). Heart and brain were selected because they are vital tissues containing postmitotic cells, the kind of tissues where aging-related changes are more readily observed. Previous descriptions of the existence of higher 8-oxodG levels in mtDNA than in nDNA are restricted to rodent liver (6, 17, 18), with a study available for human postmortem brain tissue (5). The simultaneous study of mtDNA and nDNA in the same individual animals pertaining to the abovementioned different species in the present investigation can answer the questions of whether a higher level of oxidative damage in mitochondrial than in the nuclear DNA in heart and brain is a general characteristic of mammals and whether oxidative damage to mtDNA and nDNA is related to the rate of aging.

MATERIALS AND METHODS

Tris, isoamyl alcohol, phosphoric acid, sodium hydroxide, sodium perchlorate, and $ZnCl_2$ were purchased from Merck (Rahway, N.J.). Sodium-EDTA, RNase, and alkaline phosphatase were from Boehringer Mannheim (Mannheim, Germany). Ethanol, methanol, and isopropanol were purchased from Sharlau (Barcelona, Spain). Chloroform and acetonitrile were from Fisher (Pittsburgh, Pa.), agarose from Fluka (St. Louis, Mo.), and the molecular weight size DNA standard from Bio-Rad (Hercules, Calif.). All other reagents and solvents, unless otherwise stated, were obtained from Sigma (St. Louis, Mo.).

Animals and tissue samples

Male mice, rats, guinea pigs, and rabbits were killed at the laboratory by decapitation. Male sheep, pigs, cows, and horses were killed at the abattoir. The mean age of the animals was 8 months (mice), 11 months (rat), 1.4 years (guinea pig), 1.5 years (rabbit), 1 year (pig), and 1.5–2.5 years (sheep, cow, and horse). The selection of these ages allows a comparison among young adults of all the species while maintaining a compromise between the choice of similar chronological or biological ages for interspecies comparison. The maximum longevities of the selected species are well known (29) and vary progressively from 3.5 to 46 years: mouse (*Mus musculus*, MLSP=3.5 years), rat (*Rattus norvegicus*, MLSP=4 years), guinea pig (*Cavia porcellus*, MLSP=8 years), rabbit (*Oryctolagus caniculus*, MLSP=13 years), sheep (*Ovis aries*, MLSP=20 years), pig (*Sus scrofa*, MLSP=27 years), cow (*Bos taurus*, MLSP=30 years), and horse (*Equus caballus*, MLSP=46 years). All the animals were in good health according to routine veterinary controls at the abattoir and no animal was obese or scraggy. Heart samples were taken from ventricles and brain samples were taken from occipital cortex (cow and horse) or were whole brains (rodents). The samples were obtained in all animals at the same time after death (5 min), they were cut in small pieces, immediately frozen in liquid nitrogen, and transferred before 2 h to a −80°C freezer for storage to be used later for isolation of nuclear and mitochondrial DNA and analysis of 8-oxodG and deoxyguanosine (dG).

Isolation of DNA

Nuclear DNA was isolated, after sodium dodecyl sulfate treatment of heart samples, by chloroform extraction and ethanol precipitation following the method of Loft and Poulsen (30) as described previously (31). Mitochondrial DNA was isolated by the method of Latorre et al. (32), following the procedure of Asunción et al. (15) exactly. The mtDNA preparations were free of nuclear DNA as tested by agarose gel electrophoresis and staining with ethidium bromide. Before digestion, the amount of mtDNA was determined in a small aliquot with high sensitivity by measuring the fluorescence after binding of the Hoechst 33258 dye (Pharmacia; Piscataway, N.J.).

DNA digestion

The isolated nuclear and mitochondrial DNAs were digested to deoxynucleoside level by incubation at 37°C with 5 U of nuclease P1 (in 20 μl of 20 mM sodium acetate, 10 mM $ZnCl_2$, 15% glycerol, pH 4.8) for 30 min and 1 U alkaline phosphatase (in 20 μl of 1 M Tris-HCl, pH 8.0) for 1 h (30).

8-oxodG and dG assays

The concentrations of 8-oxodG and deoxyguanosine (dG) were measured by HPLC with on-line electrochemical and ultraviolet detection, respectively. For analysis, the deoxynucleoside mixture was injected into a reverse-phase Beckman Ultrasphere ODS column (5 μM, 4.6 mm × 25 cm) eluted with 2.5% acetonitrile in 50 mM phosphate buffer pH 5.5. The amount of deoxynucleosides injected in the HPLC was similar in all species. The mean amounts of heart mtDNA (determined by the Hoechst method) used for injection in the HPLC after digestion were 7.5 μg (mouse), 16.5 μg (rat), 16.5 μg (guinea pig); 17.4 μg (rabbit), 15.0 μg (sheep), 12.0 μg (pig), 13.3 μg (cow), and 14.4 μg (horse). In the case of brain mtDNA, the mean amounts used for injection were 12.0 μg (mouse), 20.1 μg (rat), 26.1 μg (guinea pig), 16.6 μg (rabbit), 14.0 μg (cow), and 15.2 μg (horse). These amounts were not significantly correlated with the MLSP of the donor species according to the criteria stated in the Statistical analysis section (see below). A Waters 590 pump at 1 ml/min was used. 8-oxodG and dG were detected with an ESA Coulochem II electrochemical coulometric detector (ESA, Inc., Bedford, Mass.) with a high sensitivity 5011 analytical cell run in the oxidative mode (E1=0 mV, E2=200 mV) and a Bio-Rad model 1806 UV detector at 254 nm. For quantification, peak areas of dG standards and three level calibrations with pure 8-oxodG standards (Sigma) were analyzed during each HPLC run.

Statistical analyses

The statistical relationships between MLSP and 8-oxodG in nDNA or mtDNA were studied first by linear regression (equation $y=a+b\cdot x$), where y is 8-oxodG and x is MLSP. When the correlation was found to be significant, adjustment by nonlinear regression to the power function $y = a \cdot x^b$

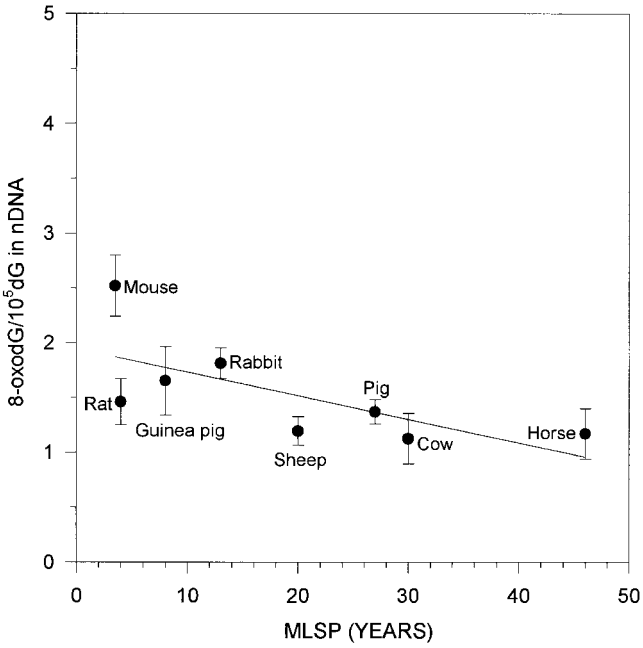

Figure 1. Lack of significant relationship between heart 8-oxodG/10^5dG in nuclear DNA (nDNA) and maximum life span (MLSP) in eight mammalian species. 8-oxodG/10^5dG values were plotted as a function of MLSP, and data were fitted to a straight line by linear regression ($r=-0.68$, $P<0.059$). Values are means ± SE. The number of animals per species was 10 mice, 7 rats, 4 pigs, and 5 guinea pigs, rabbits, sheep, cows, and horses.

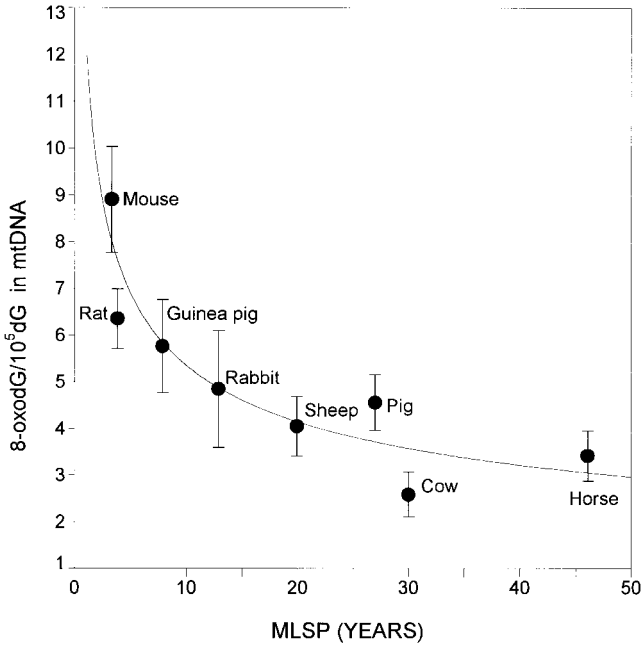

Figure 2. Inverse correlation between heart 8-oxodG/10^5dG in mitochondrial DNA (mtDNA) and maximum life span (MLSP) in eight mammalian species. 8-oxodG/10^5dG values were plotted as a function of MLSP and data were fitted to the power function $y = a \cdot x^b$, where y is 8-oxodG in mtDNA and x is MLSP ($r=-0.92$, $P<0.001$). Values are means ± SE. The number of animals per species was 8 mice and rats, 6 guinea pigs and pigs, and 7 rabbits, sheep, cows, and horses.

(where y is 8-oxodG and x is MLSP) was performed with Table-Curve software (Jandel Scientific). The correlations were analyzed using the Pearson correlation coefficient (r) selecting 0.05 as the point of minimum statistical significance.

RESULTS

When the values of heart 8-oxodG in nDNA of the eight mammalian species studied in this investigation were plotted as a function of their MLSP, a linear trend to show lower levels in long-lived species was observed (**Fig. 1**). However, this trend did not reach statistical significance ($r=-0.68$, $P<0.059$). A different situation was observed for heart mtDNA. In this case, a significant inverse correlation between 8-oxodG values and MLSP was found (**Fig. 2**; $r=-0.92$, $P<0.001$). This relationship followed the power function $y = a \cdot x^b$, where y is 8-oxodG in mtDNA and x is MLSP.

The results obtained in the brain were qualitatively similar to those obtained in the heart for both kinds of DNA, although lower correlation coefficients and statistical significances were observed in this case. Brain 8-oxodG in nDNA did not show a correlation with MLSP (**Fig. 3**; $r = 0.53$, $P<0.27$). However, similar to what happened in the heart, brain 8-oxodG levels in mtDNA were inversely correlated with

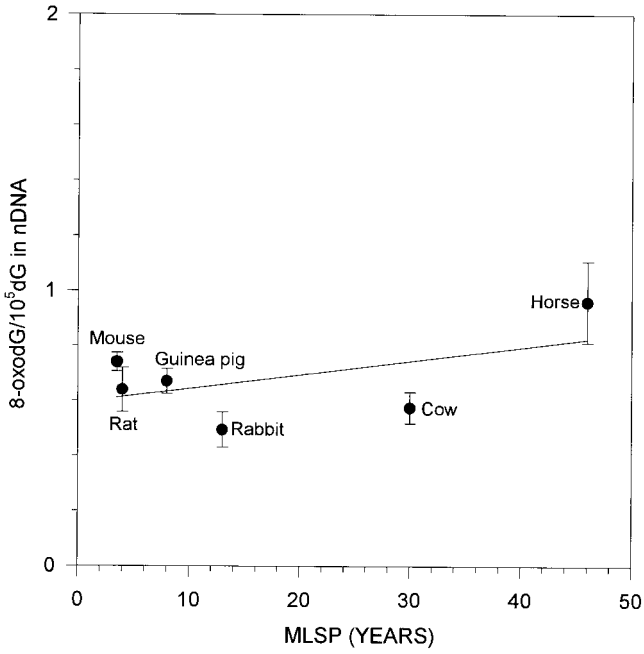

Figure 3. Lack of significant relationship between brain 8-oxodG/10^5dG in nuclear DNA (nDNA) and maximum life span (MLSP) in six mammalian species. 8-oxodG/10^5dG values were plotted as a function of MLSP and data were fitted to a straight line by linear regression ($r = 0.53$, $P<0.27$). Values are means ± SE. The number of animals per species was 12 mice, 7 rats, and 6 guinea pigs, rabbits, cows, and horses.

the species-specific MLSP in the mammals studied (**Fig. 4**; power equation $y = a \cdot x^b$; $r = -0.88$; $P < 0.016$).

8-oxodG values in mtDNA were also compared with those in nDNA in both tissues when both values were available in the same individual animal (**Table 1**). In all species and tissues, 8-oxodG values were significantly higher in mtDNA than in nDNA. Since heart 8-oxodG values tended to decrease with increases in MLSP in both nDNA and mtDNA (Figs. 1 and 2), the ratio '8-oxodG in mtDNA/8-oxodG in nDNA' tended to be similar in all the species, ranging from 3.2-fold in pigs and rabbits to 5.5-fold in mouse, with a mean value of 4.1-fold for all the mammals studied (Table 1). Since brain 8-oxodG was inversely correlated with MLSP in mtDNA (Fig. 4) whereas the nonsignificant trend of the correlation with MLSP was positive in the case of brain nDNA (Fig. 3), the ratio '8-oxodG in mtDNA/8-oxodG in nDNA' in the brain was inversely correlated with MLSP ($r = -0.84$; $P < 0.025$), i.e., the difference in 8-oxodG between mtDNA and nDNA (always higher in mtDNA) tended to be lower in long-lived than in short-lived mammals. This difference was around 9-fold in mice and rats, 5- to 6-fold in guinea pigs, rabbits and cows, and 3-fold in horses, with a mean value of 6.6-fold for all the species (Table 1).

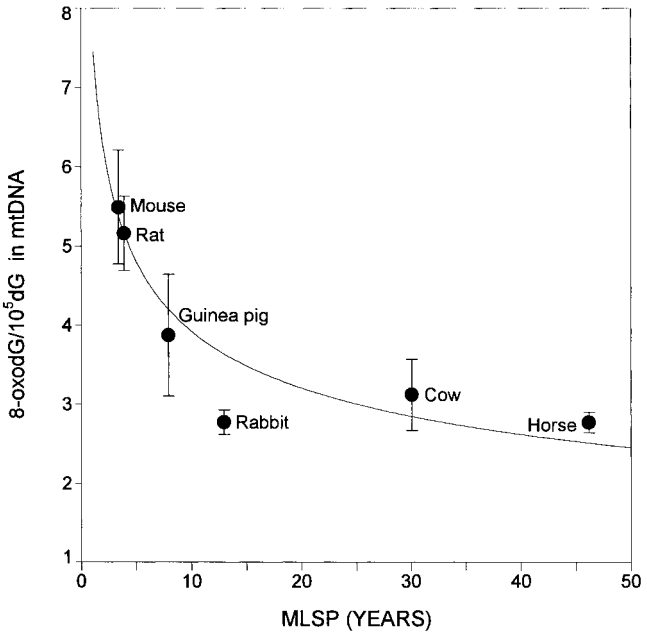

Figure 4. Inverse correlation between brain 8-oxodG/10^5dG in mitochondrial DNA (mtDNA) and maximum life span (MLSP) in six mammalian species. 8-oxodG/10^5dG values were plotted as a function of MLSP and data were fitted to the power function $y = a \cdot x^b$, where y is 8-oxodG in mtDNA and x is MLSP ($r = -0.88$, $P < 0.016$). Values are means ± SE. The number of animals per species was 9 mice and rats, 5 rabbits, and 7 guinea pigs, cows, and horses.

TABLE 1. *Ratio between 8-oxodG/10^5 dG in mitochondrial DNA and 8-oxodG/10^5 dG in nuclear DNA*[a]

	Heart	Brain
Mouse	5.5 ± 2.5 (6)	8.7 ± 1.6 (8)
Rat	4.5 ± 0.5 (6)	9.2 ± 1.4 (7)
Guinea pig	4.6 ± 1.2 (5)	5.7 ± 2.3 (6)
Rabbit	3.2 ± 0.8 (5)	6.3 ± 0.6 (5)
Sheep	4.1 ± 0.3 (5)	—
Pig	3.2 ± 0.6 (3)	—
Cow	3.3 ± 0.9 (5)	6.7 ± 1.5 (6)
Horse	4.1 ± 1.1 (5)	3.1 ± 0.3 (6)

[a] Values are means ± SEM, with the number of animals in parenthesis. Data were calculated dividing the 8-oxodG/10^5 dG value found in the mtDNA by the 8-oxodG/10^5 dG value observed in the nuclear DNA when both values were available in the same animal; mean ± SEM values were computed in the different species. 8-oxodG/10^5 dG values were significantly higher in mtDNA than in nDNA in all tissues and species (Student's *t* test).

DISCUSSION

In this investigation, we describe for the first time that the steady-state concentration of the oxidative damage marker 8-oxodG in mitochondrial DNA is inversely correlated with MLSP in the heart and brain of mammals, i.e., slowly aging mammals show lower 8-oxodG levels in mtDNA than rapidly aging ones. Furthermore, this inverse relationship is restricted to mtDNA, since 8-oxodG levels in nuclear DNA were not significantly correlated with MLSP. The correlations between 8-oxodG and MLSP were better in the heart than in the brain, possibly in part because the heart is composed mainly of postmitotic cells, the ones where aging-related changes are best observed, whereas both neurons (postmitotic) and glial cells (which show capacity for division) are present in the brain. It has been described by other authors that 8-oxodG in liver nuclear DNA is negative correlated with MLSP in mammals (33, 34). This disparity with the nature of our nuclear DNA results could be due to methodological differences, especially since the values reported in those investigations were high for rodent liver (8.5 per 10^5 dG) in relation to present day values, suggesting artifactual 8-oxodG formation; however, this cannot be ascertained since those data were included in review studies (33, 34) without stating the methodological details used and were not published as original contributions. Tissue differences could also be involved, although in principle we would not expect to see aging-related differences in a nonpostmitotic tissue (liver) or in partially or totally postmitotic ones (brain and heart).

The main oxygen radical generator of the intensely respiring heart and brain cells, the inner mitochondrial membrane, is situated near mtDNA (not near nDNA), and the most reactive free radicals (like $OH^{\cdot}$) cannot diffuse far away and will react

unspecifically with macromolecules situated near their sites of generation. Therefore, it is logical to think that oxygen radicals generated by mitochondria can be a main cause of the 8-oxodG(mtDNA)-MLSP inverse relationship found in this investigation. In strong agreement with a such a causal relationship, all studies performed to date (including those carried out in heart and brain) have consistently shown that the rate of mitochondrial oxygen radical generation is also inversely related to MLSP (21–28). The higher mtDNA 8-oxodG levels of short-lived animals in comparison with long-lived ones would thus be caused, at least in part, by their higher rates of mitochondrial oxygen radical generation—the reverse being true in long-lived species.

Oxidative attack due to free radicals of mitochondrial origin are among the primary possible causes of mtDNA damage. Recent measurements using total detection polymerase chain reaction-based assays show that multiple deleterious modifications (fragmentations, deletions, point mutations, etc.) accumulate in the mtDNA with age to such an extent that the percentage of full-length wild-type mtDNA decreases even down to 11% (19) or to undetectable or trace amounts (20) in muscle or heart of old individuals. Others, however, have recently questioned (35) the results describing accumulations of mutations amounting to 90% or more of mtDNA during aging without ruling out the possibility that a low overall level of mtDNA damage could have a strong physiological significance due to possible phenomena like clonal expansion of defective mtDNA molecules (36, 37). This would explain the presence of mosaics in postmitotic tissues containing cells heavily loaded with mutant mtDNA molecules (38). With one exception(14), almost all studies of 8-oxodG in mtDNA (although scarce) have found (5, 6, 13, 15, 16) higher values in old than in young animals of the same species; in the case of nDNA, controversial results have been reported for age-related variations of 8-oxodG in heart (6–8), brain (5–12), and other tissues. However, those age-related increases cannot be the result of a lifetime accumulation of oxidative damage in mtDNA. The levels of 8-oxodG found in the mtDNA of old individuals, which are between 2- and 15-fold higher than those of young ones according to the different reports (5, 6, 13, 15, 16), as well as those found in the mtDNA of short-lived species in this investigation, would be quickly reached in the absence of repair systems. Mitochondria lack repair systems for some forms of DNA damage like nucleotide excision repair or repair of pyrimidine dimers (39). But contrary to previous beliefs, recent investigations show that, analogous to what happens for nDNA, repair of oxidative damage is very active in mtDNA (39, 40). Since mitochondrial free radical generation is known to be higher in short-lived than in long-lived species (21–28), we expect the same for the repair of endogenous oxidative damage to mtDNA (although this still has not been investigated); otherwise, the damage would soon rise to unbearable levels. Nevertheless, a higher level of oxidative attack to the mtDNA of short-lived animals compared with long-lived ones also suggested that higher 8-oxodG levels can help to explain the quicker rate of accumulation of other multiple mtDNA deleterious modifications during aging (19, 20), which are repaired with more difficulty or not at all.

In agreement with the existence of a higher turnover of oxidatively damaged DNA bases in short-lived than in long-lived species, the rate of urinary excretion of 8-oxodG and thymine and thymidine glycol is known to be higher in rats and mice than in monkeys and humans (41, 42). On the other hand, the data obtained in this investigation show that the equilibrium between oxidative attack and repair in mtDNA is reached at higher 8-oxodG steady-state levels in short-lived than in old animals. The same is true for old when compared to young individuals (5, 6, 13, 15, 16). Since 8-oxodG is mutagenic (43, 44), the relatively higher steady-state 8-oxodG levels observed in short-lived species and in old individuals would constantly impose a higher risk of mutations in their mtDNA. The presence of a higher oxidative attack and higher 8-oxodG levels in the mtDNA of short-lived in comparison with long-lived animals can help to explain why mtDNA mutations accumulate faster in rodents (in 2–3 years) than in slow-aging animals (90 or more years in humans).

Our results should be considered within the context of current gerontological facts and theories. The maximum difference in mtDNA 8-oxodG levels between short- and long-lived animals (4-fold) was smaller than their difference in longevity (13-fold between mouse and horse). The same occurred in all of our previous studies comparing rates of mitochondrial oxygen radical generation among animals showing different longevities (24–27). These observations do not contradict the free radical theory of aging. On the contrary, they are consistent with the widely accepted concept that aging is due to more than one single major cause. Aging is thought to be due to relatively few causes with major effects (the most interesting ones to study) plus many causes with minor effects. Besides, even if oxidative stress were the main cause of endogenous DNA damage, it would not be its only basis. Other causes that are of minor importance when considered individually could nevertheless have a relevant role collectively, taking into account all their effects. A single mechanism cannot explain all the variance of longevity between species, whereas the quantitative differ-

ences in oxidative damage to mtDNA are consistent with a multicausal origin of aging.

Previous investigations have consistently found that 8-oxodG concentration is higher in mtDNA than in nDNA. Very few studies are available, however, and those that are are limited to rodent liver (6, 17, 18), with a single study available in postmortem human brain (5). Other reports describing high 8-oxodG levels in mtDNA (13, 15, 16) are not useful for this kind of discussion because 8-oxodG in nDNA was not simultaneously measured in those investigations, and 8-oxodG values are known to vary depending on the laboratories and methods of measurement used. This scarcity of data and other considerations have led to doubts that oxidative damage is in fact higher in mtDNA than in nDNA (2, 39). In the present investigation we have found that this difference between the two types of DNA exists in the heart and brain of all the mammals studied, using exactly the same method for mtDNA and nDNA in all the steps in which it is possible to do it: DNA digestion and chromatographic separation of 8-oxodG and dG. 8-oxodG levels were around fourfold higher in mtDNA than in nDNA in the heart of all mammals and between three- and ninefold higher in the case of the brain, the highest difference being observed in the brain of short-lived mammals. Thus, our results confirm earlier reports from rodent liver (6, 17, 18) and human brain after autopsy (5), extending them to the heart and brain of mammals in general. Since it is now known that the rate of repair of 8-oxodG is rapid in mitochondria, similar to what happens in the nucleus (39), the higher steady-state 8-oxodG levels observed in mtDNA in relation to nDNA must be due to the higher oxidative attack caused by the higher rate of oxygen radical generation present in the organelles. This higher oxidative attack suffered by mtDNA can help explain the accumulation of mtDNA mutations in postmitotic tissues during aging of all kinds of animal species (short-lived or long-lived) at the end of their life span (19, 20).

In summary, the results obtained here show that levels of the oxidative damage marker 8-oxodG are higher in mitochondrial than in nuclear DNA in the heart and brain of a wide range of mammalian species. Furthermore, the steady-state 8-oxodG concentration in mtDNA is higher in rapidly aging mammalian species than in those that age slowly, whereas 8-oxodG levels in nuclear DNA do not correlate with mammalian longevity. Taken together, these results are consistent with the predictions of a free radical theory of aging focused on mitochondria (3, 4, 28, 45, 46). FJ

The authors are grateful to Drs. J. Sastre, J. Viña, and collaborators for their valuable technical help. The authors acknowledge to Nicolas López, Jose Luis del Pozo, and the rest of the veterinary staffs of the Madrid Norte and Villaviciosa de Odon abattoirs for facilitating the access to the tissue samples from mammals of large body size. This work was supported by grants from the National Research Foundation of the Spanish Ministry of Health (FISss no. 96/1253 and 99/1049).

REFERENCES

1. Sohal, R. S., and Weindruch, R. (1996) Oxidative stress, caloric restriction, and aging. *Science* **273,** 59–63
2. Beckman, K. B., and Ames, B. N. (1998) The free radical theory of aging matures. *Physiol. Rev.* **78,** 547–581
3. Harman, D. (1972) The biological clock: the mitochondria? *J. Am. Geriatr. Soc.* **20,** 145–147
4. Barja, G. (1998) Mitochondrial free radical production and aging in mammals and birds. *Ann. N.Y. Acad. Sci.* **854,** 224–238
5. Mecocci, P., MacGarvey, U., Kaufman, A. E., Koontz, D., Shoffner, J. M., Wallace, D. C., and Beal, F. (1993) Oxidative damage to mitochondrial DNA shows age-dependent increases in human brain. *Ann. Neurol.* **34,** 609–616
6. Sohal, R. S., Agarwal, S., Candas, M., Forster, M. J., and Lal, H. (1994) Effect of age on DNA oxidative damage in different tissues of C57BL/6 mice. *Mech. Ageing Dev.* **76,** ,215–224
7. Sohal, R. S., Agarwal, S., and Sohal, B. H. (1995) Oxidative stress and aging in the Mongolian gerbil (*Meriones unguiculatus*). *Mech. Ageing Dev.* **81,** 15–25
8. Kaneko, T., Tahara, S., and Matsuo, M. (1996) Non-linear accumulation of 8-hydroxy-2′-deoxyguanosine, a marker of oxidized DNA damage, during aging. *Mutat. Res.* **316,** 277–285
9. Fraga, C. G., Shigenaga, M. K., Park, J. W., Degan, P., and Ames, B. N. (1990) Oxidative damage to DNA during aging: 8-hydroxy-2′-deoxyguanosine in rat organ DNA and urine. *Proc. Natl. Acad. Sci. USA* **87,** 4533–45537
10. Sai, K., Tagaki, A., Umemura, T., Hasegawa, R., and Kurokawa, Y. (1992) Changes of 8-hydroxydeoxyguanosine levels in rat organ DNA during the aging process. *J. Exp. Pathol. Toxicol. Oncol.* **11,** 139–143
11. Hirano, T., Yamaguchi, R., Asami, S., Iwamoto, N., and Kasai, H. (1996) 8-hydroxyguanine levels in nuclear DNA and its repair in rat organs associated with age. *J. Gerontol.* **51A,** B303–B307
12. Koppele, J. M. T., Lucassen, P. J., Sakee, A. N., Van Asten, J. G., Ravid, R., Swaab, D. F., and Van Bezooijen, C. F. A. (1996) 8OHdG levels in brain do not indicate oxidative DNA damage in Alzheimer's disease. *Neurobiol. Aging* **17,** 819–826
13. Ozawa, T. (1995) Mitochondrial DNA mutations associated with aging and degenerative diseases. *Exp. Gerontol.* **30,** 269–290
14. Muscari, C., Giaccari, A., Stefanelli, C., Viticchi, C., Giordano, E., Guarnieri, C., and Caldarera, C. M. (1996) Presence of a DNA-4236 bp deletion and 8-hydroxy-deoxyguanosine in mouse cardiac mitochondrial DNA during aging. *Aging Clin. Exp. Res.* **8,** 429–433
15. Asunción, J. G., Millan, A., Pla, R., Bruseghini, L., Esteras, A., Pallardo, F. V., Sastre, J., and Viña, J. (1996) Mitochondrial glutathione oxidation correlates with age-associated oxidative damage to mitochondrial DNA. *FASEB J.* **10,** 333–338
16. Sastre, J., Millán, A., Asunción, J. G., Plá, R., Juan, G., Pallardó, F. V., O'Connor, E., Martín, J. A., Droy-Lefaix, M. T., and Viña, J. (1998) A gingko biloba extract (EGb 761) prevents mitochondrial aging by protecting against oxidative stress. *Free Rad. Biol. Med.* **24,** 298–304
17. Richter, C., Park, J. W., and Ames, B. N. (1988) Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proc. Natl. Acad. Sci. USA* **85,** 6465–6467
18. Chung, M. H., Kasai, H., Nishimura, S., and Yu, B. P. (1992) Protection of DNA damage by dietary restriction. *Free Rad. Biol. Med.* **12,** 523–525
19. Ozawa, T. (1998) Mitochondrial DNA mutations and age. *Ann. N.Y. Acad. Sci.* **854,** 128–154
20. Kovalenko, S. A., Kospidas, G., Kelso, J., Rosenfeldt, F., and Linnane, A. W. (1998) Tissue-specific distribution of multiple mitochondrial DNA rearrangements during human aging. *Ann. N.Y. Acad. Sci.* **854,** 171–181

21. Sohal, R. S., Svensson, I., and Brunk, U. T. (1990) Hydrogen peroxide production by liver mitochondria in different species. *Mech. Ageing Dev.* **53,** 209–215
22. Ku, H. H., Brunk, U. T., and Sohal, R. S. (1993) Relationship between mitochondrial superoxide and hydrogen peroxide production and longevity of mammalian species. *Free Rad. Biol. Med.* **15,** 621–627
23. Ku, H. H., and Sohal, R. S. (1993) Comparison of mitochondrial pro-oxidant generation and antioxidant defenses between rat and pigeon: possible basis of variation in longevity and metabolic potential. *Mech. Ageing Dev.* **72,** 67–76
24. Barja, G., Cadenas, S., Rojas, C., Pérez-Campo, R., López-Torres, M. (1994) Low mitochondrial free radical production per unit O_2 consumption can explain the simultaneous presence of high longevity and high aerobic metabolic rate in birds. *Free Rad. Res.* **21,** 317–328
25. Herrero, A., and Barja, G. (1997) Sites and mechanisms responsible for the low rate of free radical production of heart mitochondria in the long-lived pigeon. *Mech. Ageing Dev.* **98,** 95–111
26. Barja, G., and Herrero, A. (1998) Localization at Complex I and mechanism of the higher free radical production of brain non-synaptic mitochondria in the short-lived rat than in the longevous pigeon. *J. Bioenerg. Biomembr.* **30,** 235–243
27. Herrero, A., and Barja, G. (1998) H_2O_2 production of heart mitochondria and aging rate are slower in canaries and parakeets than in mice: sites of free radical generation and mechanisms involved. *Mech. Ageing and Dev.* **103,** 133–146
28. Barja, G. (1999) Mitochondrial free radical generation: sites of production in states 4 and 3, organ specificity and relationship with aging rate. *J. Bioenerg. Biomembr.* In press
29. Altman, P., and Dittmer, P. (1972) Life spans: animals. In *Biology Data Book,* pp. 229–235, Federation of American Societies for Experimental Biology, Bethesda, Md.
30. Loft, S., and Poulsen, H. E. (1999) Markers of oxidative damage to DNA: antioxidants and molecular damage. *Methods Enzymol.* **300,** 166–184
31. Cadenas, S., Barja, G., Poulsen, H. E., and Loft, S. (1997) 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. *Carcinogenesis* **18,** 2373–2377
32. Latorre, A., Moya, A., and Ayala, A. (1986) Evolution of mitochondrial DNA in *Drosophila suboscura. Proc. Natl. Acad. Sci. USA* **83,** 8649–8653
33. Cutler, G. (1991) Antioxidants and aging. *Am. J. Clin. Nutr.* **53,** 373S–379S
34. Cutler, G. (1991) Human longevity and aging: possible role of reactive oxygen species. *Ann. N.Y. Acad. Sci.* **621,** 1–28
35. Lightowlers, R. N., Jacobs, H. T., and Kajander O. A. (1999) Mitochondrial DNA—all thing's bad? *Trends Genet.* **15,** 91–93
36. Yoneda, M., Chomyn, A., Martinuzzi, A., Horko, O., and Attardi, G. (1992) Marked replicative advantage of human mtDNA carrying a point mutation that causes a the MELAS encephalomyopathy. *Proc. Natl. Acad. Sci. USA.* **89,** 11164–11168
37. de Grey, A. D. N. J. (1998) A mechanism proposed to explain the rise in oxidative stress during aging. *J. Anti-aging Med.* **1,** 53–65
38. Brierley, E. J., Johnson, M. A., Lightowlers, R. N., James, O. F. W., and Turnbull D. M. (1998) Role of mitochondrial DNA mutations in human aging: implications fro the central nervous system and muscle. *Ann. Neurol.* **43,** 217–223
39. Anson, R. M., Croteau, D. L., Stierum, R. H., Fliburn, C., Parsell, R., and Bohr, V. A. (1998) Homogeneous repair of singlet oxygen-induced DNA damage in differentially transcribed regions and strands of human mitochondrial DNA. *Nucleic Acids Res.* **26,** 662–668
40. Driggers, W. J., Holmquist, G. P., LeDoux, S. P., and Wilson, G. L. (1997) Mapping frequencies of endogenous oxidative damage and the kinetic response to oxidative stress in a region of rat mtDNA. *Nucleic Acids Res.* **25,** 4362–4369
41. Ames, B. N. (1989) Endogenous oxidative DNA damage, aging, and cancer. *Free Rad. Res. Commun.* **7,** 121–128
42. Adelman, R., Saul, R. L., and Ames, B. N. (1988) Oxidative damage to DNA: Relation to species metabolic rate and life span. *Proc. Natl. Acad. Sci. USA* **85,** 2706–2708
43. Shibutani, S., Takeshita, M., and Grollman, A. P. (1991) Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxo-dG. *Nature (London)* **349,** 431–434
44. Cheng, K. C., Cahill, D. S., Kasai, H., Nishimura, S., and Loeb, L. A. (1992) 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G-T and A-C substitutions. *J. Biol. Chem.* **267,** 166–172
45. Shigenaga, M. K. H., and Ames, B. N. (1994) Oxidants and mitochondrial decay in aging. In *Natural Antioxidants in Health and Disease,* pp. 63–106, Academic Press, New York
46. Miquel, J. (1988) An integrated theory of aging as a result of mitochondrial-DNA mutation in differentiated cells. *Arch. Gerontol. Geriatr.* **12,** 99–117

Received for publication April 9, 1999.
Revised for publication September 28, 1999.