Free Rad. Res., Vol. 29, pp. 573–579
Reprints available directly from the publisher
Photocopying permitted by license only

Age-associated Change in Mitochondrial DNA Damage

E.K. HUDSON[a,*], B.A. HOGUE[a], N.C. SOUZA-PINTO[a], D.L. CROTEAU[b], R.M. ANSON[a], V.A. BOHR[a] and R.G. HANSFORD[a]

[a]*Laboratory of Molecular Genetics, Gerontology Research Center, NIA, NIH, 5600 Nathan Shock Dr., Baltimore, MD 21224-6823, USA;* [b]*Department of Molecular and Cellular Biology, University of California, Berkeley, CA 94720, USA*

Accepted by Prof. B. Halliwell

(Received 3 August 1998)

There is an age-associated decline in the mitochondrial function of the Wistar rat heart. Previous reports from this lab have shown a decrease in mitochondrial cytochrome c oxidase (COX) activity associated with a reduction in COX gene and protein expression and a similar decrease in the rate of mitochondrial protein synthesis. Damage to mitochondrial DNA may contribute to this decline.

Using the HPLC–Coularray system (ESA, USA), we measured levels of nuclear and mitochondrial 8-oxo-2′-deoxyguanosine (8-oxodG) from 6-month (young) and 23-month-old (senescent) rat liver DNA. We measured the sensitivity of the technique by damaging calf thymus DNA with photoactivated methylene blue for 30 s up to 2 h. The levels of damage were linear over the entire time course including the shorter times which showed levels comparable to those expected in liver. For the liver data, 8-oxodG was reported as a fraction of 2-deoxyguanosine (2-dG). There was no change in the levels of 8-oxodG levels in the nuclear DNA from 6 to 23-months of age. However, the levels of 8-oxodG increased 2.5-fold in the mitochondrial DNA with age. At 6 months, the level of 8-oxodG in mtDNA was 5-fold higher than nuclear and increased to approximately 12-fold higher by 23 months of age. These findings agree with other reports showing an age-associated increase in levels of mtDNA damage; however, the degree to which it increases is smaller. Such damage to the mitochondrial DNA may contribute to the age-associated decline in mitochondrial function.

Keywords: Mitochondrial DNA, oxidative damage, DNA repair, mitochondria, aging, liver

THE MITOCHONDRIAL GENOME AND CYTOCHROME *c* OXIDASE

Mitochondria contain their own circular genome that encodes 2 rRNAs, 22 tRNAs, and 13 polypeptides. These polypeptides are subunits of the respiratory chain complexes located in the mitochondrial inner membrane.[1] With its own genome, the mitochondrion is capable of translation, transcription, and DNA synthesis. However, all of these processes are dependent on cytosolic components of nuclear origin such as heat-shock proteins, initiation factors, elongation factors,

* Corresponding author.

transcription factors, polymerases and DNA repair enzymes[2,3] (Figure 1).

Cytochrome oxidase (COX), the terminal activity of the respiratory chain and essential for proper mitochondrial function, is composed of 13 subunits of which the three catalytic subunits are encoded by the mitochondrial genome. Therefore, a decline in mitochondrial translation or transcription of DNA with age could result in reduced in COX activity.

In addition, damage to mitochondrial DNA (mtDNA) could play a role in the decrease in COX function. One of the major theories of aging states that oxidative damage to mitochondrial macromolecules, including mtDNA, decreases mitochondrial function thereby lowering the cell's energy supply leading to cell death.[4] DNA damage could reach deleterious levels if there was a reduction in repair activity or if DNA damage levels simply overwhelmed the repair capacity of the mitochondria. Such a loss of mtDNA repair and/or increase in mtDNA damage could adversely affect COX function.

Previous reports have shown an age-associated decline in COX activity.[5,6] After confirming that there was a decrease in COX activity with age, we examined other mitochondrial processes to determine if some aspect of mitochondrial gene expression could account for this change in function. Using isolated mitochondria, we investigated both mitochondrial protein synthesis and transcription to determine if either or both played a role in the decline of COX activity.

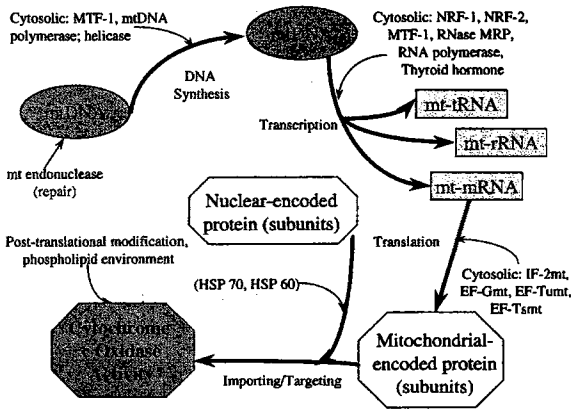
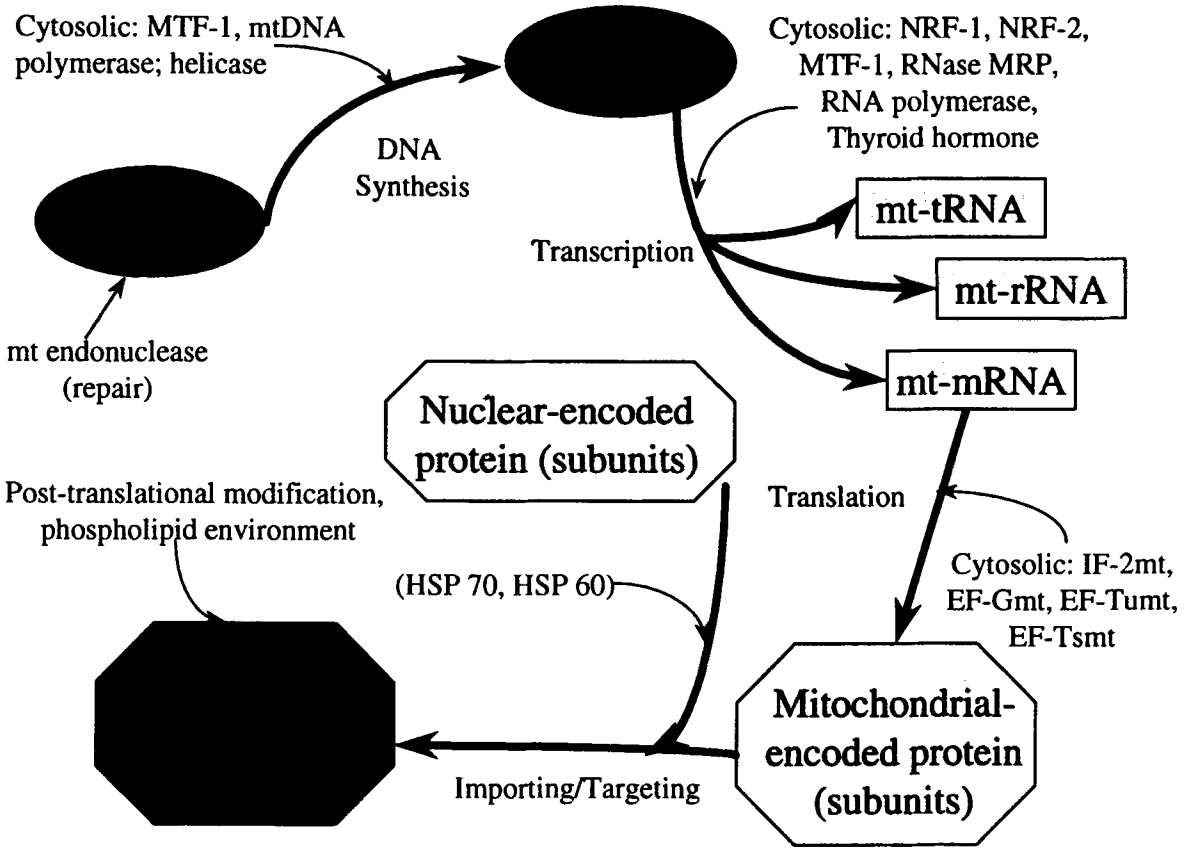


FIGURE 1 *The process of mitochondrial biogenesis.* This flow chart illustrates the complexity of the interaction between the nuclear and mitochondrial genomes required for mitochondrial biogenesis and function. The "backbone", shown with the thick black lines, represents the events that take place within the mitochondrion. Those activities and proteins of non-mitochondrial origin are labeled in blue and are required at numerous points in the pathway as indicated by the small arrows. They include transcription, initiation, and elongation factors as well as the heat shock proteins needed for mitochondrial biogenesis. In addition, numerous nuclear encoded subunits, such as those of cytochrome oxidase and the ATP synthase, and proteins are required for proper mitochondrial function. (See Color plate I at the end of this issue.)

CHANGES IN MITOCHONDRIAL PROTEIN SYNTHESIS AND GENE EXPRESSION WITH AGE

To verify that there was an age-associated decrease in COX function in our model, we isolated rat heart mitochondria and compared cytochrome oxidase activities in young, 6–7-month old rats and old, 23–24-month old rats. In agreement with previous reports, there was a 30% decrease in COX activity with age.[5,6] Citrate synthase failed to show an age-associated change in activity suggesting that the decline in COX activity was the result of the reduced specific activity of the enzyme and did not reflect differential contamination of the preparation. The decrease in COX activity was associated with a decrease in the protein levels of the COX subunits and a lower rate of mitochondrial protein synthesis with age.[7] There was a similar decrease in the mRNA expression of the COX subunits and a reduction in the rate of mitochondrial transcription.[7] Combined, these decreases in mitochondrial function could account for the decrease in COX activity with age. However, we wanted to determine if damage to mtDNA could also play a role in this decline.



Color Plate 1 (see page 574, figure 1) *The process of mitochondrial biogenesis.* This flow chart illustrates the complexity of the interaction between the nuclear and mitochondrial genomes required for mitochondrial biogenesis and function. The "backbone", shown with the thick black lines, represents the events that take place within the mitochondrion. Those activities and proteins of non-mitochondrial origin are labeled in blue and are required at numerous points in the pathway as indicated by the small arrows. They include transcription, initiation, and elongation factors as well as the heat shock proteins needed for mitochondrial biogenesis. In addition, numerous nuclear encoded subunits, such as those of cytochrome oxidase and the ATP synthase, and proteins are required for proper mitochondrial function.

OXIDATIVE DAMAGE TO OTHER CELLULAR COMPONENTS

The free radical theory of aging has become part of the fabric of the scientific community.[8] Because they are one of the major sources of reactive oxygen species in the cell, mitochondria are believed to play a role in the aging process.[4] It has been shown that other cellular macromolecules such as proteins, phospholipids, and nucleic acids suffer oxidative damage.[9–11] We examined damage to mitochondria by measuring lipid peroxidation in isolated mitochondria using the thiobarbituric acid reactive species (TBARS) assay.[12] This spectrophotometric assay measures levels of malondialdehyde (MDA), a secondary product of lipid peroxidation.

The results show no change in mitochondrial TBARS levels indicating no increase in lipid peroxidation in the aging rat heart. However, the sensitivity of this spectrophotometric assay has been brought into question. The problem is that other aldehydes may react with TBA giving spurious results. It is possible that the best method for measuring lipid peroxidation may be the separation of other aldehydes in the sample by HPLC followed by a spectrophotometric analysis or electrochemical analysis of the lipid peroxidation product 4-hydroxynonenal.[13] The increased sensitivity of these methods may show a difference in lipid peroxidation levels.

CHANGES IN MITOCHONDRIAL DNA PROCESSING WITH AGE

Our lab recently characterized a protein called mitochondrial oxidative damage-specific endonuclease (mtODE).[14] We have purified and defined an endonuclease with 8-oxoguanine specificity that was located within the mitochondria. We thought mtODE could provide some insight into age-associated changes in mtDNA processing and/or mtDNA repair. Due to reports showing an increase in mtDNA damage with age, we expected to find a decrease in mtODE activity.[15–17] Using a radiolabeled oligo containing 8-oxoguanine, we tested mitochondrial preparations from young and old rat hearts for mtODE activity.[18]

We found there to be a 40% *increase* in mtODE activity with age.[18] This lead us to speculate that an increase in DNA damage somehow led to an increase in the expression or activity of DNA processing enzymes. To test this hypothesis, we began isolating DNA from rat heart to confirm that there were higher levels of oxidative DNA damage with age. Efforts to determine the levels of oxidative damage to mtDNA were hampered by the inability to isolate appropriate amounts of mtDNA for HPLC–EC analysis from two rat hearts. For reasons not yet understood, most of the heart mtDNA failed to precipitate out of the ethanol. We are currently trying to modify and optimize the method using the smallest number of rats possible.

To test our hypothesis, we began using isolated rat liver mitochondria and began the process of characterizing liver mtODE activity hoping it would have the same increase in activity as that seen in heart. As was the case with heart, liver mtODE activity increased 40% with age.[18] So we felt confident that we could test our theory and began the isolation of both nuclear and mitochondrial DNA from rat liver.

PREPARATION AND ANALYSIS OF LIVER DNA

Liver mitochondria were isolated by differential centrifugation for the preparation of mtDNA, and the pellet of the first low speed spin was used for the preparation of nuclear DNA. The DNA was isolated using the standard RNAase A, proteinase K, and phenol chloroform procedure with some modifications which generated enough DNA for analysis (200–500 μg).[19] Nucleosides were prepared enzymatically

from 100 µg of DNA using nuclease P1 and alkaline phosphatase then filtered through a 0.22-micron filter and a 30 kD cutoff spin column.[20]

To determine the levels of DNA damage, we monitored levels of 8-oxodG, the oxidative product of 2-dG, in the nuclear and mitochondrial DNA samples.[21] This electrochemically active DNA adduct was measured using an ESA four-channel coularray with two channels set at low potentials for the detection of 8-oxodG and two channels set at higher potentials for the detection of 2-dG. The midpoint potentials of the two compounds, 8-oxodG and 2-dG, differed by 400 mV simplifying identification (Figure 2). The levels of 2-dG were too high to be measured in the same run, so the samples were diluted 1:100 in mobile phase then analyzed in a separate run for 2-dG. All samples were analyzed twice.

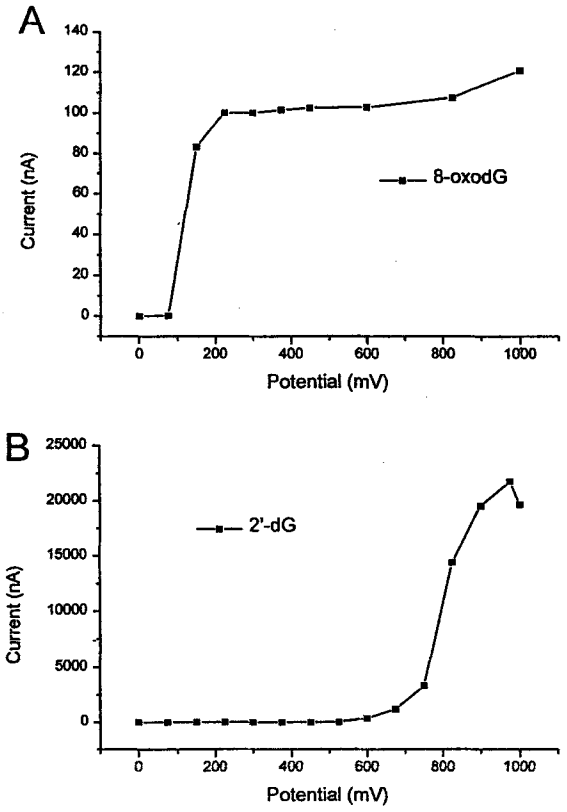


FIGURE 2 *Hydrodynamic voltammograms (HDVs) of 8-oxodG and 2-dG.* The HDV is a current–voltage curve that indicates the specific voltage at which a compound is oxidized and produces a current. Using standards for 8-oxodG and 2-dG, we found the optimum potential for 2-dG to be around 950 mV (panel A) and for 8-oxodG it was around 300 mV (panel B). This allows accurate identification and separation of the two DNA adducts.

The mobile phase was composed of 100 mM sodium acetate, pH 5.15 and 5% methanol. The nucleosides were separated isocratically using a C-8 column at a flow rate of 1 ml/min for 30 min to avoid spillover to the successive run. Nucleoside standards were run first and had approximately a 70:30 ratio over the two channels. The concentrations of the standards were determined spectrophotometrically. The peaks were identified in the samples according to the retention time and ratio accuracy. For our biological samples, the 8-oxodG was expressed per 10^5 2-dG.

To illustrate the sensitivity and accuracy of the system, calf thymus DNA was damaged with photoactivated methylene blue for up to 2 h then analyzed for 8-oxodG content.[22,23] Damage increased in a linear fashion over the entire 2-h time course, and we were able to detect low levels of damage with only a 30 s of light exposure. More importantly, we were able to confidently detect levels in the fmol range needed for biological samples (Figure 3).

To confirm that our peak of interest was indeed 8-oxodG, we damaged rat liver DNA with methylene blue and light, then split the sample in two and treated one-half with Fpg to remove 8-oxodG.[24] We analyzed our peak of interest and found that Fpg reduced the signal by 90% thus confirming our peak was 8-oxodG.[25] We then analyzed our liver DNA.

AGE-ASSOCIATED INCREASE IN mtDNA DAMAGE

Our results show an approximate 2.5-fold increase in mitochondrial 8-oxodG and no change in nuclear levels with age. At 6 months of age,

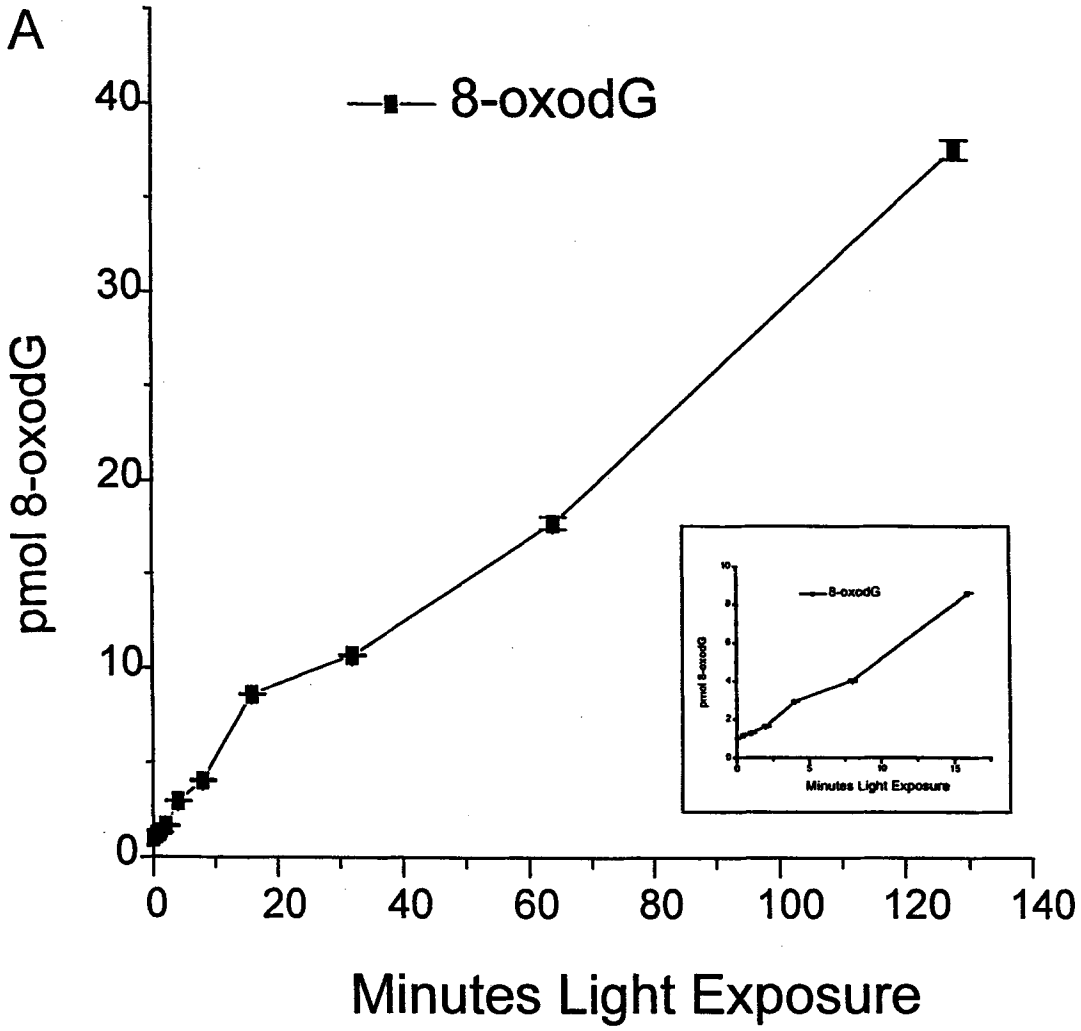


FIGURE 3 *HPLC–Coularray detection of 8-oxodG at physiological damage levels.* Photoactivated methylene blue (MB) was used to induce the lesion, 8-oxodG, in calf thymus DNA. DNA was exposed to MB for 0–120 min, then submitted blind for HPLC analysis. Channels 1 and 2 were set to detect 8-oxodG and channels 3 and 4 were set for 2-dG. The induction of damage remained linear up to 120 min (panel A) and was able to detect damage at physiological levels (Inset).

the 8-oxodG levels in the mtDNA were roughly four times higher than that in the nucleus. However, due to the age-associated increase in mtDNA damage, there was approximately 10 times more mitochondrial than nuclear DNA damage by 23 months.[25] The magnitude of the increase in mtDNA damage was in agreement with previous reports, however the absolute values were ten times lower.[15,25]

SUMMARY

The decrease in heart cytochrome oxidase activity is due to a decrease in mitochondrial protein synthesis and gene expression. Mitochondrial gene expression may in turn decrease as a result of mtDNA damage. mtODE activity increases with age in both rat heart and liver. In the liver, mitochondrial 8-oxodG content increases 2.5-fold

while there is no change in the level of damage in the nuclear DNA with age.

DISCUSSION

The measurement of oxidative damage in DNA has become an area of intense interest, and there is a portion of the scientific community that has dedicated itself to determining the most accurate method for the measurement of oxidative damage. We have used the HPLC combined with the electrochemical array to measure 8-oxodG as an indicator of the degree of oxidative damage. The relative changes in the levels of mtDNA damage are evident. There are numerous design advances that make the array the preferred method of analysis. The flow through property of the electrochemical cells allows complete oxidation of the sample and an accurate measurement. The coularray allows precise identification of peaks based on both retention time and the ratio of the current across multiple channels. This makes peak identification and quantitation more exact than single channel EC detectors. The advantage of HPLC–EC over GC–MS is the ease and relative mildness of the sample preparation for HPLC analysis reducing the likelihood of damage produced from handling and derivatization for GC–MS. However, it lacks the ability to measure a wide array of adducts.

The levels of 8-oxodG that we measured were approximately 10 times lower than those reported by other laboratories.[15,17] This is in large part due to the advances that Ames and his colleagues have made in the field of DNA isolation and nucleoside preparation.[20] This, in conjunction with the EC array technology, has served to "lower the bar" of DNA damage detection.

An exhaustive search of the literature produced relatively few original reports regarding the increased occurrence of mitochondrial DNA damage with age. The mitochondrial free radical theory of aging, for the most part, lacks extensive experimental support.[4] These results presented here can add to the inventory of mtDNA damage reports, but a comprehensive and long-ranged study in humans is needed to determine the true impact of free radical damage to mitochondria in aging.

References

[1] S. Anderson, A. Bankier, B. Barrell, M. De Bruijn, A. Coulson, J. Drouin, I. Eperon, D. Nierlich, B. Roe, F. Sanger, P. Schreier, A. Smith, R. Staden and I. Young (1981) Sequence and organization of the human mitochondrial genome. *Nature*, **290**, 457– 465.

[2] D. Clayton (1991) Replication and transcription of vertebrate mitochondrial DNA. *Annual Reviews of Cell Biology*, **7**, 453–478.

[3] G. Attardi, and G. Schatz (1988) Biogenesis of Mitochondria. *Annual Reviews of Cell Biology*, **4**, 289–333.

[4] J. Miquel, A. Economos, J. Fleming and J. Johnson, Jr. (1980) Mitochondrial role in cell aging. *Experimental Gerontology*, **15**, 575–591.

[5] R. Hansford and F. Castro (1982) Age-linked changes in the activity of enzymes of the tricarboxylate cycle and lipid oxidation, and of carnitine content, in the muscles of the rat. *Mechanisms of Ageing and Development*, **19**, 191–201.

[6] M. Takasawa, M. Hayakawa, S. Sugiyama, K. Hattori, T. Ito and T. Ozawa (1993) Age-associated damage in mitochondrial function in rat hearts. *Experimental Gerontology*, **28**, 269–280.

[7] E. Hudson, N. Tsuchiya and R. Hansford (1998) Age-associated changes in mitochondrial mRNA expression and translation in the Wistar rat heart. *Mechanisms of Ageing and Development*, **103**, 179–193.

[8] D. Harman (1956) Aging: a theory based on free radical and radiation chemistry, *Journal of Gerontology*, **11**, 298–300.

[9] P. Starke-Reed and C. Oliver (1989) Protein oxidation and proteolysis during aging and oxidative stress. *Archives of Biochemistry and Biophysics*, **275**, 559–567.

[10] Y. Wei, S. Kao and H. Lee (1996) Simultaneous increase of mitochondrial DNA deletions and lipid peroxidation in human aging. *Annals of the New York Academy of Sciences*, **786**, 24–43.

[11] C. Fraga, M. Shigenaga, J-W Park, P. Degan and B. Ames (1990) Oxidative damage to DNA during aging: 8-hydroxy-2′-deoxyguanosine in rat organ DNA and urine. *Proceedings of the National Academy of Sciences of the USA*, **87**, 4533–4537.

[12] H. Ohkawa, N. Ohishi and K. Yagi (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, **95**, 351–358.

[13] P. Berejo, P. Gomez-Serranillos, J. Santos, E. Pastor, P. Gil and S. Martin-Aragon (1997) Determination of malonaldehyde in Alzheimer's disease: a comparative study of high-performance chromatography and thiobarbituric acid test. *Gerontology*, **43**, 218–222.

[14] D. Croteau, C. ap Rhys, E. Hudson, G. Dianov, R. Hansford and V. Bohr (1997) An oxidative damage-specific endonuclease from rat liver mitochondria. *Journal of Biological Chemistry*, **272**, 27338–27344.

[15] B. Ames, M. Shegenaga and T. Hagen (1993) Oxidants, antioxidants, and the degenerative diseases of aging. *Proceedings of the National Academy of Sciences of the USA*, **90**, 7915–7922.

[16] P. Mecocci, U. MaeGarvey, A. Kaufman, D. Koontz, J. Shoffner, D. Wallace and F. Beal (1993) Oxidative damage to mitochondrial DNA shows marked age-dependent increases in human brain. *Annals of Neurology*, **34**, 609–616.

[17] J. de la Asuncion, A. Milan, R. Pla, L. Bruseghini, A. Esreras, F. Pallardo, J. Satre and J. Vina (1996) Mitochondrial glutathione oxidation correlates with age-associated oxidative damage to mitochondrial DNA. *FASEB Journal*, **10**, 333–338.

[18] N. Souza-Pinto, D. Croteau, E. Hudson, R. Hansford and V. Bohr Age-associated in 8-oxo-deoxyguanine endonuclease activity in rat mitochondria (manuscript submitted).

[19] M. Hayakawa, S. Sigiyama, K. Hattori, M. Takasawa and T. Ozawa (1993) Age-associated damage in mitochondrial DNA in human hearts. *Molecular and Cellular Biochemistry*, **119**, 95–103.

[20] M. Shigenaga, E. Aboujaoude, Q. Chen and B. Ames (1994) Assays of oxidative DNA damage biomarkers 8-oxo-2′-deoxyguanosine and 8-oxoguanine in nuclear DNA and biological fluids by high-performance liquid chromatography with electrochemical detection. *Methods in Enzymology*, **234**, 16–33.

[21] H. Kasai and S. Nishimura (1984) Hydroxylation of deoxyguanosine at the C-8 position by ascorbic acid and other reducing agents. *Nucleic Acids Research*, **12**(14), 2137–2145.

[22] R. Anson, D. Croteau, R. Stierum, C. Filburn, R. Parsell and V. Bohr (1998) Homogenous repair of singlet oxygen-induced DNA damage in differentially transcribed regions and strands of human mitochondrial DNA. *Nucleic Acids Research*, **26**, 662–668.

[23] H. Czeczot, B. Tudek, B. Lambert, J. Laval and S. Boiteux (1991) *Escherichia coli* Fpg protein and UvrABC endonuclease repair DNA damage induced by methylene blue plus visible *in vivo* and *in vitro*. *Journal of Bacteriology*, **173**(11), 3419–3424.

[24] S. Boiteux, T. O'Conner and J. Lava (1987) Formamidopyrimidine- DNA glycosylase of *Escherichia coli*: cloning, and sequencing of the fpg structural gene and overproduction of the protein. *EMBO Journal*, **6**(10), 3177–3183.

[25] E. Hudson, B. Hogue, N. Souza-Pinto, D. Croteau, R. Anson, V. Bohr and R. Hansford Age-associated increase in mitochondrial DNA damage (manuscript in preparation).