

Contribution of de novo point mutations to the overall mutational burden in mitochondrial DNA of adult rats

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Received 17 November 2004; received in revised form 10 February 2005; accepted 16 February 2005
Available online 28 March 2005

Abstract

This study analyzed the incidence of point mutations in mitochondrial DNA of brain and muscle tissues from young (6-month) and old (24-month) male F344 rats. Coding sequence mutations in subunit 5 of the NADH dehydrogenase gene were detected after high-fidelity PCR amplification and cloning by denaturing gradient gel electrophoresis (DGGE) assay followed by sequencing of detected mutants. In total, almost a thousand individual clones were analyzed both in brain and muscle samples. On average, mtDNA from brain tissue showed a 66% increase with age in mutation frequencies (2.3 ± 1.9 vs. $3.8 \pm 4.5 \times 10^{-4}$ mutations/bp, mean \pm SD), which failed to reach statistical significance ($p = 0.45$). Muscle tissues yielded substantially fewer mutants with average mutant frequencies for both young and old rats almost 10 times lower than the corresponding values in the brain tissue (0.3 ± 0.4 and $0.5 \pm 0.6 \times 10^{-4}$, respectively). The difference in mutation accumulation between muscle and brain was highly significant in both the younger group (Chi-squared = 9.7, $p \leq 0.01$) and in older animals (Chi-squared = 10.9, $p \leq 0.001$). Molecular analysis of the mutated sequences revealed that almost half were identical sequences recurring in different samples and tissues. Our findings indicate that the process of mutation accumulation in mitochondria begins in the germ-line and/or during earlier stages of life, contributing up to half of the total mutational burden, whereas de novo spontaneous formation of point mutations in adulthood is far less than was anticipated.

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Keywords: Mitochondrial DNA; Point mutations; Aging; Rats; Brain; Muscle

1. Introduction

Mitochondrial theories of aging have been proposed in which senescence is attributed to a vicious cycle created by the elevated exposure of mitochondria, and in particular, mitochondrial DNA (mtDNA), to free radicals leaked from the electron transport chain (Harman, 1972; Miquel et al., 1980). A portion of oxidative DNA damage is converted into mutations leading to synthesis of defective proteins, which in turn causes ever-increasing production of oxidants. There are several lines of evidence indicating that these organelles may

be a weak link in terms of aging. Mitochondria possess several unique features setting them apart from other organelles. Functionally, mitochondria are responsible for production of ATP via oxidative phosphorylation, regulation of apoptosis, and calcium signaling. They are the main site of oxygen consumption and reactive oxygen species (ROS) production. Mitochondria harbor a second genome, which codes for key components of the electron transport system (ETS). By virtue of its close proximity to the ETS machinery and its asymmetric replication mechanism, mitochondrial DNA may be subjected to considerably higher oxidative damage (Richter et al., 1988; Mecocci et al., 1993) repaired less efficiently than similar lesions in the nuclear DNA (Croteau et al., 1999).

These factors combine to produce mutation accumulation in mitochondrial DNA (mtDNA) that is dramatically more severe than in the nucleus. A considerable fraction of the mtDNA collected from old individuals comprised of a variety of truncated molecules (Kovalenko et al., 1998).

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The incidence of specific point mutations tends to increase with age and may accrue to more than 2% of the total mtDNA pool in post-mitotic tissues (Munscher et al., 1993), although this increase is not observed (Pallotti et al., 1996). As a result, mtDNA exhibits 1000–10,000-fold higher mutation frequencies than nuclear DNA, thus affecting the performance of mtDNA-encoded components of enzymatic complexes (Cortopassi and Wong, 1999; Ferrandiz et al., 1994). That aged mitochondria contribute to the deteriorative process is suggested by microinjection experiments in which introduction of mitochondria from senescent cells resulted in the degeneration of young recipient cells (Corbisier and Remacle, 1990).

Organs comprising non-dividing cells may be especially vulnerable to mitochondrial deterioration as dysfunctional cells cannot be replaced. The accumulation of mutations in mitochondrial DNA has been implicated in a range of age-dependent degenerative pathologies including Parkinson's disease (Sherer et al., 2002) and Alzheimer's disease (Castellani et al., 2002). Thus, given the possible role of mitochondria in aging, it would be of interest to know the actual mutational load in mtDNA resulting from accumulated point mutations in post-mitotic tissues.

2. Material and methods

2.1. Animals

All animal procedures were performed in accordance with institutional guidelines for the care and use of laboratory animals. Male Fisher 344 rats at 6 and 24 months of age (each $n=6$) were purchased from the National Institute on Aging and housed in accredited facilities on a 12:12 light/dark cycle with ad libitum food and water. At the time of sacrifice, rats were anesthetized with ether and exsanguinated by cardiac puncture. Brain and plantaris muscle were dissected, frozen in liquid nitrogen, and stored at -80°C until DNA isolation and analysis. Rats were assigned identification numbers YM11–YM16 for young, and OM11–OM16 for old males.

2.2. Mitochondrial DNA isolation

Total DNA was extracted from cerebral cortex (entire right hemisphere) and whole plantaris muscle using the DNeasy Tissue Kit (Qiagen, Valencia, CA). The presence of mtDNA was verified by electrophoresis on 0.8% agarose gels containing 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide. Bands corresponding to full-length mtDNA were excised and extracted using the QIAEX[®] II Gel Extraction System (Qiagen, Valencia, CA).

2.3. PCR amplification, cloning and sequencing

PCR amplifications were carried out with a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA)

using forward primer CATTCTCAACCTCCCTAG and reverse primer TTGATTAGTCCTTTTTGG (GenBank Accession # NC001665), yielding a 193 bp fragment from the 3' end of the NADH dehydrogenase gene, subunit 5 (ND5, 13282–13474). The reactions were performed in a 30 μl volume containing 100 ng of mtDNA template, 0.6 μl (1.5 units) of *Pfx* polymerase (Invitrogen, Carlsbad, CA), 25 μM nucleotide triphosphate mix, and the buffer supplied by the manufacturer. Thermocycling consisted of an initial period of denaturation at 94°C for 2 min, 30 cycles of 94°C for 30 s, 56°C for 30 s, 68°C for 2 min, followed by a final extension step at 68°C for 10 min. Approximately, 5 μl of the PCR products were loaded on a 1% agarose gel, electrophoresed, and visualized after staining with 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide. Amplified ND5 fragments were cloned using the Zero Blunt TOPO PCR[™] Cloning kit (Invitrogen). Plasmids from the individual bacterial colonies were purified using the Qiagen QIAprep Spin Miniprep kit (Qiagen) and verified for the presence of inserted fragments by electrophoresis on 1% agarose. The plasmids carrying inserts were sequenced using an SEQ8000 DNA sequencer (Beckman Coulter Inc., Fullerton, CA) according to the manufacturer's instructions.

2.4. DGGE analysis

The method used for detection of fragments carrying mutations is shown in Fig. 1. After DNA purification, aliquots from 3 to 4 individual plasmids were mixed. These mixes served as templates for the second PCR reaction with GC-clamp forward primer CGCCCGCCGCCCCGCG CCCGTCCC GCCCCCCCGCCCGCATTCTCAACCTC CCTAG using the same PCR conditions. PCR reaction products (approx. 15 μl) were loaded on denaturing gradient (0–60% urea/formamide), 8% acrylamide gels and then electrophoresed at 62°C , 80 V for 16 h. Bands were visualized using 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide under ultraviolet transillumination.

2.5. Statistical analysis

All statistical analyses were performed using the Data Analysis facility in Microsoft Excel. The Chi-squared test was used to compare the proportion of mutated fragments in brain vs. muscle for both young and old animals.

3. Results

3.1. DGGE analysis of mtDNA from brain and muscle samples

A novel aspect of our approach is that we pre-screened pools of mtDNA clones—representing a portion of the coding sequence of NADH dehydrogenase, subunit 5—by denaturing gradient gel electrophoresis (DGGE), and then

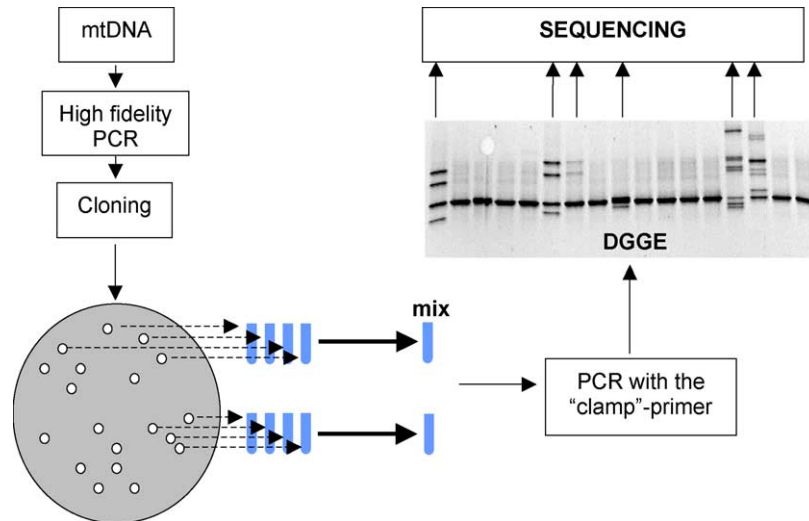


Fig. 1. Assay for detection and analysis of mutations in mtDNA. Fragments were amplified using high-fidelity polymerase, cloned into PCR-Blunt II-TOPO vector and plated. Kanamycin resistant colonies were collected and grown overnight in LB medium. Aliquots from every three or four individual colonies were mixed and resulting mixes were used for a second PCR reaction to add a G:C-rich clamp to the fragment. The resulting product was loaded on 0–60% denaturant 8% acrylamide gel and electrophoresed at 62 °C, 80 V for 16 h. The individual colonies that composed mixes with mutation patterns were sequenced. The ethidium bromide stained gel pictures part of the mixed clones from OM14 brain sample.

analyzed apparent mutations by PCR sequencing. In brain tissue, 968 fragments were analyzed altogether (Table 1 and Fig. 2). The DGGE analysis of 443 plasmids from six younger animals (443 plasmids) revealed 13 mutations. The calculated mutant frequencies varied about 10-fold among animals in this group, ranging from 0.6 to $5.6/10^4$ nucleotides, which corresponds to approximately 1–9 mutations per full-length mtDNA. Similarly, in the 525 plasmids from six older rats, 21 mutants were found. In one of the samples (OM11), no mutations could be detected even in a double complement of screened plasmids. The remaining samples, however, displayed an even greater variability in mutant frequencies that ranged from 0.6 to 10×10^{-4} , corresponding to 1–16 mutations per mtDNA. On average, the mtDNA extracted from the brains of the older rats showed a statistically insignificant 66% ($p=0.45$) increase in their mutant frequencies (2.3 ± 1.9 vs. $3.8 \pm 4.5 \times 10^{-4}$). A similar analysis conducted on a comparable collection of plasmids obtained from the plantaris muscle yielded substantially fewer mutants. In 504 fragments from young animals, only two carried mutations, whereas in older rats only four mutants out of 495 fragments were found in three samples. The average mutant frequencies for young and old groups were almost an order of magnitude lower than corresponding values in the brain and comprised 0.3 ± 0.4 and $0.5 \pm 0.6 \times 10^{-4}$, respectively (Chi-squared $p \approx 0.001$ and 0.01, respectively).

3.2. Sequencing

Table 2 summarize the sequencing analysis of individual plasmid inserts from mixes demonstrating mutant patterns on DGGE. Some mutations were found on more than one

occasion, within different age groups and/or tissues, and thus are assumed to originate from the germ-line. For example, an A:T→T:A transversion (position 13398) was found in muscle samples from two different aged rats (OM12 and OM13), whereas a single-nucleotide deletion in an A₆ homopolymeric run (–A, 13336–13341) was detected in muscle samples from three young rats (YM11, YM13, and YM14), and an A:T→C:G transversion (13409) in two samples from younger animals (YM12 and YM13). A transition, A:T→G:C (13303), on the other hand, was present in both datasets from brain and muscle tissues (OM13—brain, OM14—muscle).

The recovered mutational spectrum was dominated by events involving A:T base pairs. These types of modifications accounted for 74% of the mutations, comprising A:T→G:C transitions (41%), and both A:T→T:A (15%) and A:T→C:G (18%) transversions. The remaining mutations consisted of single-nucleotide deletions within homopolymeric runs (15%) and G:C→A:T transversions (11%). Homopolymeric A:T runs appear to be especially vulnerable to mutagenesis as, apart from frameshifts, about a third of all point mutations (7/23) were located within three A₄ stretches.

4. Discussion

A common formulation of a mitochondrial theory of aging postulates that oxidant leakage from the ETS causes oxidative damage to mtDNA and thus promotes the generation of mutations in adult individuals. We now report that the accumulation of de novo mutations in aging mtDNA during the span of 18 months is very modest

Table 1
Mutants detected in mtDNA from brain and plantaris muscle of young (6-month) and old (24-month) male rats

Sample	Fragments screened	DNA (bp)	Mutations (DGGE)	Sequence confirmed	MF ($\times 10^{-4}$ /bp)	MF ^a corrected
<i>Brain</i>						
YM11	60	8940	2	1	2.2	1.1
YM12	65	9685	1	1	1.0	0
YM13	60	8940	5	5	5.6	3.4
YM14	60	8940	3	3	3.4	2.2
YM15	120	17880	1	0	0.6	0.6
YM16	78	11622	1	0	0.9	0.9
Total	443	57961	13	9	2.3 \pm 0.8	1.4 \pm 0.5
OM11	144	21456	0	0	0.0	0.0
OM12	60	8940	2	1	2.2	1.1
OM13	60	8940	8	7	9.0	5.6
OM14	60	8940	9	7	10.1	9.0
OM15	120	17880	1	0	0.6	0.6
OM16	81	12069	1	2	0.8	0.8
Total	525	78225	21	17	3.8 \pm 1.8	2.8 \pm 1.5
<i>Muscle</i>						
YM11	84	12516	1	1	0.8	0.8
YM12	60	8940	0	0	0.0	0.0
YM13	92	13708	0	0	0.0	0.0
YM14	92	13708	0	0	0.0	0.0
YM15	84	12516	1	1	0.8	0.0
YM16	92	8940	0	0	0.0	0.0
Total	504	70328	2	2	0.3 \pm 0.4	0.1 \pm 0.1
OM11	51	7599	0	0	0.0	0.0
OM12	92	13708	1	1	0.7	0.0
OM13	60	8940	1	1	1.1	0.0
OM14	92	13708	0	0	0.0	0.0
OM15	92	13708	0	0	0.0	0.0
OM16	108	16092	2	2	1.2	1.2
Total	495	73755	4	4	0.5 \pm 0.6	0.2 \pm 0.2

MF, mutation frequency ($\times 10^{-4}$ mutants/bp).

^a Mutation frequencies corrected for shared mutations.

(no more than 2-fold) for both the brain and the muscle of rats. This indicates that with regard to mtDNA, the combined effect of antioxidant defenses and repair systems may be sufficient, in adult animals, to preclude extensive mutagenesis based on oxidative damage. On the other hand, values observed in young individuals are quite high and hint at a significantly different situation at the earlier stages of life.

Our findings echo the results obtained in two studies on mutational load in different regions of human brain. In a study conducted on temporoparietal/pre-frontal polymodal association cortex from 14 younger (1–31 years) and 27 older (53–87 years) individuals, the mean aggregate mutational burden (corrected for PCR-generated artifacts) for the young group was $1.21 \pm 0.57 \times 10^{-4}$ mutations/bp, whereas mutation frequencies in the elderly did not exceed $1.75 \pm 1.59 \times 10^{-4}$ mutations/bp, despite a difference of more than 50 years in median ages (Lin et al., 2002). Furthermore, a remarkable constancy of mitochondrial mutational load was seen, between young (1–22 years) and old (65–86) individuals in the substantia nigra, in a second, similarly designed experiment (Simon et al., 2004). These findings led to speculation that the high burden

observed in younger individuals could be either inherited or acquired at earlier stages of life. The lack of accumulation of mutations in older subjects was suggested to be due to elimination of cells in which mutational burden reached a level incompatible with life (Simon et al., 2004).

Our results may bear on one or more of these hypotheses. The relatively high mutation frequencies observed in young

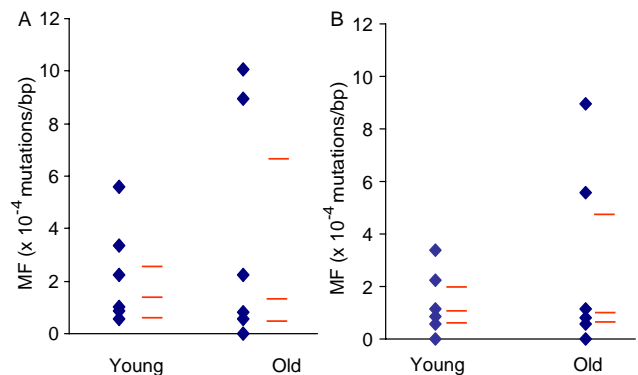


Fig. 2. Scatter plot of mutant frequency values for brain samples from young and old animals. Medians and quartiles are presented as horizontal lines: (A) total values; (B) values after subtracting identical mutations found in different animals and in different tissues (see Table 2).

Table 2
Mutations found in mitochondrial DNA (ND subunit 5) from brain and plantaris muscle of young (6-month) and old (24-month) rats

Clone	Mutation	Sequence context	Position	Codon change	AA change
<i>Brain</i>					
YM11-14	-A	TTCCTC...A5·CTCTAA	13336-41	Frame	
YM12-44	A→C	AATCCC...C...AAATCA	13409	CCA→CCC	pro→pro
Ym13-4	T→G	CCCGAA...G...TATTCC	13327	ATT→AGT	ile→ser
YM13-6	-A	TTCCTC. A5·CTCTAA	13336-41	Frame	
YM13-26	A→C	AATCCC...A...AAATCA	13409	CCA→CCC	pro→pro
YM13-27	C→T	TTAAAC...T...TACTAG	13374	CTA→TTA	leu→leu
	T→C	GAATTA...C...TCCTCA	13330	ATT→ACT	ile→thr
YM13-28	A→G	CTACCC...G...CCAATT	13310	CCA→CCG	pro→pro
YM13-55	A→G	AACCTC...G...ATCACC	13421	TCA→TCG	ser→ser
YM14-59	-A	TTCCTC...A5·CTCTAA	13336-41	Frame	
	A→G	CTAGCT...G...CAAATT	13357	TAC→TGC	tyr→cys
OM12-47	A→T	TCAGAA...T...AGACAA	13398	AAG→TAG	lys→ter*
OM13-2	T→A	CCCAAC...A...ATCTAA	13438	CTA→CAA	leu→gln
OM13-8	-A	GATCAG...A3·GACAAT	13396-9	Frame	
OM13-16	A→T	TCAGAA...T...AGACAA	13398	AAG→TAG	lys→ter*
OM13-25	A→G	AAAGAC...G...ATCCCA	13403	ACA→ACG	thr→thr
OM13-26	A→G	TAGGCT...G...CTACCC	13303	TAC→TGC	tyr→cys
OM13-40	A→C	TCTAAA...C...TAATAT	13446	ATA→CTA	met→leu
OM13-41	C→T	ATCTGA...T...TAGAAA	13392	CTA→TTA	leu→leu
OM14-1	A→G	CCTACT...G...GACCTA	13379	CTA→CTG	leu→leu
OM14-15	T→C	TCTAAA...C...TCTAGC	13349	AAT→AAC	asn→asn
OM14-27	A→C	CCAAC...C...TCTAAA	13439	CTA→CTC	leu→leu
OM14-40	A→G	ATCCCA...G...AATCAA	13410	AAA→GAA	lys→glu
OM14-41	A→G	CAGAAA...G...GACAAT	13399	AAG→AGG	lys→ter*
OM14-44	A→T	TCCCAA...T...ATCAAC	13411	AAA→ATA	lys→met
OM14-59	A→G	TAGGCT...G...CTACCC	13303	TAC→TGC	tyr→cys
OM16-69	CC→TT	TACCCA...TT.AATTAT	13311	CCA→TTA	pro→leu
	A→G	CCTCAA...G...AAACTC	13338	AAA→GAA	lys→glu
<i>Plantaris</i>					
YM11-32	T→C	ACTATC...C...AAAATA	13442	TCT→TCC	ser→ser
YM15-17	-A	GATCAG.A3...GACAAT	13396-9	Frame	
OM13-34	A→C	AATCCC...C...AAATCA	13409	CCA→CCC	pro→pro
OM12-43	A→C	AATCCC...C...AAATCA	13409	CCA→CCC	pro→pro
OM16-23	C→T	TAAATT...T...TAGCTA	13351	TCT→TTT	ser→phe
OM16-97	A→G	AAAATC...G...ACCTCA	13415	TCA→TCG	ser→ser

Shaded cells indicate identical mutation found in different individuals and groups. Table includes data from clones that generated unambiguous sequences.

rats ($2.3 \pm 1.9 \times 10^{-4}$ mutations/bp) are suggestive of disparities in the rates of mutation accumulation in early and late stages of life, although sources and mechanisms of this disparity are not entirely clear. We speculate that there is high likelihood for elevated mutation formation during organogenesis in the second half of gestation. In rats, changes in mitochondrial morphology (Shepard et al., 1998) as well as a switch from glycolysis to oxidative phosphorylation (Tanimura and Shepard, 1970) are observed starting from gestational day 11–12. During this period, a significant increase in oxygen consumption occurs, and it is accompanied by inhibited expression of several genes in DNA repair pathways (Vinson and Hales, 2002). In addition, brain seems to be especially vulnerable due to higher superoxide production (Fantel and Person, 2002) and depressed activity of SOD compared to other organs in embryos and adults (Mackler et al., 1973). It should be noted that in our previous analysis of mtDNA mutational load in mouse livers, older mice (22 months) exhibited

mutation frequencies comparable to values observed in older (24 months) rat brains (2.5 ± 7.8 vs. $3.8 \pm 4.5 \times 10^{-4}$ mutation/bp), whereas in younger mice (2 months) no mutations were detected (Khaidakov et al., 2003). This apparent contradiction may reflect a difference in developmental dynamics and metabolic rates (Wetter et al., 1999) between proliferating and post-mitotic tissues.

The striking difference in mutational load in the brain and the muscle tissues both in younger and older animals (7–8-fold) observed in this study could be at least partially attributed to much greater metabolic activity in brain than in relatively sluggish plantaris muscle (Wetter et al., 1999). In addition, we found a considerable number of identical mutations both in brain and muscle tissues of young and old animals (Table 2), shared between individuals, tissues and age groups, which exhibited similar brain vs. muscle ratio. Multiple mutations of the same sequence are not likely to be coincidental. One of the possible sources of such mutations could be hotspot mutagenesis. They also could be found

within one tissue sample due to replication of altered mtDNA and of cells during development, in different tissues through early embryonic or (to a limited extent) germ-line mutations, and in different individuals as inherited germ-line mutations—a distinct possibility for inbred animals.

An interesting feature of recurring mutations detected in our study is that in both tissues their frequencies increased proportionally to the age-associated increase of the overall mutational load. This phenomenon may have several explanations, including metabolic activity associated hotspot mutagenesis. Alternatively, the germ-line origin of such mutations cannot be completely ruled out, even though it appears that a total fraction of germ-line events should not increase with age. Computer modeling based on random partitioning of mitochondrial genomes between daughter cells, or random drift in post-mitotic tissues, demonstrated that specific mutations could be overrepresented in the mtDNA of individual cells even in the absence of selective pressure (Coller et al., 2002; Elson et al., 2001). In the bulk mtDNA preparations such hetero- or homoplasmies should not be detectable as these mechanisms are bi-directional, supposedly random and, therefore, mutually canceling in the cell population. At the same time, there may be other contributing factors. For example, these essentially non-selective mechanisms may be modulated by a lysosomal bias with regard to the size of recyclable mitochondria. Recent publications indicate a lysosomal preference for smaller organelles (Terman et al., 2004), which may reduce turnover of dysfunctional or enlarged mitochondria. Because neurons have greater metabolic activity, mitochondrial turnover and the cumulative effect of lysosomal bias may be much more pronounced in the brain. In other words, mutations (including germ-line events) might not be selected against in the muscle, but rather not selected for, or, at least, not to the same degree as in the brain.

In view of the relatively low or negligible difference in de novo point mutation load between young and old subjects, it appears that the contribution of de novo point mutations to a total mutational load in mtDNA is relatively small. While it is clear that mtDNA pools are compromised in old animals, other types of mutations arising through ROS-independent pathways or mutation independent mechanisms may be responsible for a rapid increase in the overall mutational burden. For example, data on age-related accumulation of deletions in mtDNA, using long-PCR methods, demonstrate a consistent pattern of decreasing relative amounts of full-length DNA and a concomitantly increasing fraction of truncated mtDNAs, both in terms of number of copies and diversity (Kovalenko et al., 1998). The overwhelming majority of detected deletions are flanked by direct repeats (Wallace et al., 2004), abundant in mtDNA, either implying a looping-out excision or slippage-misalignment mechanism for their formation. There does not seem to be a strong connection between oxidative damage and deletions, since the addition of non-enzymatic antioxidants to the medium, while effective in reducing spontaneous point mutations,

does not protect against either spontaneous, or X-ray-induced, deletions (Klein, 2004). Another mutation independent and potentially most efficient mechanism for accelerated deterioration of mtDNA pools is clonal expansion of mutated sequences resulting in the development of hetero- and homoplasmies, which is a common occurrence in all cell types including post-mitotic tissues (Fayet et al., 2002; Nekhaeva et al., 2002).

Based on our findings and data available from the literature, we conclude that a significant portion of the mtDNA mutational burden (on average 30–40%) originates early in life and that oxidative damage in adult animals is probably sufficient only to double the mutational load in mtDNA attributable to point mutations. Our data also suggest that there is a disparity in the rate of mutagenesis between early and later stages of life and that approximately half of de novo mutations in mtDNA in older animals are acquired within the first few months of life. If this, indeed, is the case, it may help to understand why several studies on intervention in mitochondrial antioxidant enzymes expression levels failed to produce appreciable changes in longevity, especially in long-lived strains (Orr et al., 2003; Spencer et al., 2003).

Acknowledgements

This research was supported by a grant-in-aid from the American Heart Association—Heartland Affiliate (to RHK), by a grant for Methylation, Epigenetics and Longevity from Life Extension Foundation (to CAC) and by NIA/NIH P01AG20641 (to RJSR).

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