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Aging increases mitochondrial DNA damage and oxidative stress in liver of rhesus monkeys

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ABSTRACT

While the mechanisms of cellular aging remain controversial, a leading hypothesis is that mitochondrial oxidative stress and mitochondrial dysfunction play a critical role in this process. Here, we provide data in aging rhesus macaques supporting the hypothesis that increased oxidative stress is a major characteristic of aging and may be responsible for the age-associated increase in mitochondrial dysfunction. We measured mitochondrial DNA (mtDNA) damage by quantitative PCR in liver and peripheral blood mononuclear cells of young, middle age, and old monkeys and show that older monkeys have increases in the number of mtDNA lesions. There was a direct correlation between the amount of mtDNA lesions and age, supporting the role of mtDNA damage in the process of aging. Liver from older monkeys showed significant increases in lipid peroxidation, protein carbonylations and reduced antioxidant enzyme activity. Similarly, peripheral blood mononuclear cells from the middle age group showed increased levels in carbonylated proteins, indicative of high levels of oxidative stress. Together, these results suggest that the aging process is associated with defective mitochondria, where increased production of reactive oxygen species results in extensive damage at the mtDNA and protein levels. This study provides valuable data based on the rhesus macaque model further validating age-related mitochondrial functional decline with increasing age and suggesting that mtDNA damage might be a good biomarker of aging.

1. Introduction

The aging process is characterized by cellular degeneration and impaired physiological functions. While the mechanisms of cellular aging remain uncertain, a leading hypothesis is that mitochondrial dysfunction plays a critical role in this process. The activities of the electron transport chain (ETC) protein complexes decline with age in liver, brain, and skeletal muscle of human subjects (Hsieh et al., 1994; Lesnefsky and Hoppel, 2006; Ojaimi et al., 1999; Short et al., 2005; Trounce et al., 1989; Yen et al., 1989). Moreover, aging liver, brain, heart and kidney from rodents exhibit decreased levels of ETC complexes I and IV (Benzi et al., 1992; Kumaran et al., 2005; Lenaz et al., 1997; Navarro and Boveris, 2004), whereas muscle tissue of old monkeys shows defects in complexes III, IV, and V (Muller-Hocker et al., 1996). Taken together, these data indicate that mitochondrial bioenergetics in both human and animal tissues declines with age.

Oxidative damage to proteins, lipids, and DNA is a major characteristic of aging. Accumulation of oxidized bases in the DNA, proteins, and phospholipid oxidation products increases in old animals (Beckman and Ames, 1998; Navarro and Boveris, 2004; Navarro et al., 2002; Shigenaga et al., 1994) and inversely correlates with the activities of complexes I and IV (Navarro et al., 2004, 2002), suggesting that oxidized modified proteins and lipid peroxidation products are involved in the process leading to the increased mitochondrial dysfunction. Mitochondrial DNA (mtDNA) is a sensitive biomarker for oxidant injury (Yakes and Van Houten, 1997) and the aging process causes increases in mtDNA lesions in the mouse brain (Acevedo-Torres et al., 2009a; Mandavilli et al., 2000) and mouse germ cells (Vogel et al., 2011). Consistent with an age-related decrease in the functional capacity of various antioxidant systems, a reduction in glutathione peroxidase, superoxide dismutase, and catalase has been reported (Martin et al., 2002; Muradian et al., 2002).

Abbreviations: mtDNA, mitochondrial DNA; GSH-Px, glutathione peroxidase; ETC, electron transport chain; PBMC, peripheral blood mononuclear cells.

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The liver is a key contributor to the process of aging as it integrates energy metabolism (via the synthesis and storage of carbohydrate and fatty acid intermediates), detoxification, and immunity. Although experimental data on age-dependent changes in human liver are scarce, aging of the liver is not inconspicuous and defects in the respiratory chain occur similarly in humans and rodents. For example, the expression of both mitochondrial and nuclear encoded ETC complexes and respiration rates decrease in human liver during aging (Muller-Hocker et al., 1997; Yen et al., 1989). In rat liver, the mitochondrial membrane potential, respiration, and ATP levels decrease as well (Alemany et al., 1988; Ferreira et al., 2006; Hagen et al., 1997; Modi et al., 2008; Serviddio et al., 2007). Moreover, hepatocytes from older rats showed decreased ATP levels and mitochondrial membrane potential (Sabaretnam et al., 2010; Sastre et al., 1996).

One mechanism by which aging could affect liver mitochondrial function is via oxidative damage to the mtDNA. MtDNA is a major target of reactive oxygen species (ROS) mainly due to the constant exposure to mitochondrial-generated ROS, lack of DNA protecting histones and the limited availability of DNA repair mechanisms in the mitochondria (Shigenaga et al., 1994). Oxidative mtDNA lesions may be responsible for the age-dependent defects in the ETC observed in the aged liver, that if not repaired, they may also result in mtDNA mutations (Gredilla et al., 2010). More importantly, unrepaired mtDNA lesions may lead to the generation of defective proteins that in turn may contribute to mitochondrial dysfunction (Hiona et al., 2010).

We sought to investigate how the aging process contributes to oxidative damage and pathology of the hepatic tissue in the rhesus monkey (*Macaca mulatta*) model system. To date, no studies have measured mtDNA damage in the liver of rhesus at different ages. The rhesus monkey is the most highly used nonhuman primate in biological research and aging research in particular (Roth et al., 2004). Rhesus monkeys and humans have extraordinarily similar aging profiles (Roth et al., 2004), and die from similar age-related diseases, including diabetes, cardiovascular disease and cancer (Roth et al., 2004). To test the hypothesis that mtDNA damage and altered oxidative stress state may be used as markers of liver aging in rhesus monkeys, we studied young, middle age, and old rhesus monkeys and show that increasing age correlates with an increase in oxidative stress, oxidative damage to the mtDNA, and lipid peroxidation.

2. Materials and methods

2.1. Tissue harvesting

Liver tissue was harvested at the time of necropsy from male rhesus monkeys (*M. mulatta*) housed at the Caribbean Primate Research Center at Sabana Seca, University of Puerto Rico. All monkeys used for harvesting liver samples died of unnatural causes and were otherwise healthy based on their individual medical files. Monkeys suffering from hepatic or gastroenterological conditions or with abdominal masses were not included in the study. Tissue samples were immediately frozen in liquid nitrogen and subsequently stored at −80 °C until processed for the various assays. Samples were separated into three age groups: 0.6–8 year-old (young), 9–17 year-old (middle age) and >19 years of age (old). In the liver studies, the levels of mtDNA lesions, the activity of antioxidant enzymes, levels of protein carbonylations and the tissue histological analyses were performed using the same cohort of animals. Table 1 (Supplementary material) shows the number of animals employed for each assay. Tissue harvesting and blood withdrawals (below) were performed according to the protocol approved by the Institutional Animal Care and Use Committee of the University of Puerto Rico Medical Sciences Campus.

2.2. Blood samples

Blood withdrawals were performed from healthy rhesus monkeys. Blood samples were obtained from the femoral vein with the animals under anesthesia using ketamine HCl (12 mg/kg). Peripheral blood mononuclear cells (PBMCs) were obtained and used for DNA and protein isolation. Monkeys were separated into three age groups: 6–7 year-old (young), 10–19 year-old (middle age) and >22 year-old (old). The sizes of the age groups analyzed for mtDNA lesions in PBMCs were 11 young, 10 middle age and 4 old monkeys. For the APE1 and MnSOD expression and the analysis of protein carbonylations, 5 young, 2 middle age, and 4 old monkeys were employed. The monkeys used in the studies with PBMCs are not the same utilized for the liver analyses. The number of animals used is shown in Table 1 (Supplemental material).

2.3. Mitochondrial DNA damage analysis and relative copy number by quantitative polymerase chain reaction (QPCR)

Detailed descriptions of DNA isolation, quantitation, and DNA lesion analysis were performed as described (Acevedo-Torres et al., 2009a). Briefly, DNA was isolated and the integrity of the genomic DNA samples examined prior to DNA damage (QPCR) analysis. All our samples exhibited high molecular weight genomic DNA without evidence of degradation products. The amplification of a 10 kb mtDNA fragment was used to detect DNA lesions using MasterAmp™ Extra-long PCR reagents (Epicentre), following an initial denaturation for 45 s at 94 °C, 23 cycles of denaturation for 15 s at 94 °C and annealing/extension at 68 °C for 12 min, and a final extension at 72 °C for 10 min. The primer nucleotide sequences for the amplification of the 10 kb mtDNA amplicon are the following: 5′-AGGCCAATTAGCGCGCACAC-3′(forward) and 5′-TGCAATGGGGGCTTCGACAT-3′ (reverse). Amplification of a small mtDNA amplicon (100 bp) was used to detect fluctuations in mtDNA steady-state levels and to normalize the amplification of the 10 kb mtDNA fragment for possible changes in mtDNA abundance. Because the probability of introducing a lesion into such a small fragment is low, the amplification of the 100 bp fragment is independent of the presence of lesions thus providing an accurate measure of mtDNA molecules/abundance. For the amplification of the 100 bp rhesus mitochondrial fragment we performed an initial denaturation for 45 s at 94 °C, followed by 29 cycles of denaturation for 15 s at 94 °C, and annealing/extension at 60 °C for 45 s and 45 s at 72 °C. A final extension at 72 °C was performed for 10 min at the completion of the profile. The primer nucleotide sequences used are the following: 5′-GAAGCCTTTGCTTCAAAACG-3′ (forward) and 5′-AGGGTGGTTCTTCGAATGTG-3′ (reverse). The relative copy numbers were calculated as the relative amplification of the young, middle age and old monkeys compared to the infant individuals.

2.4. Calculation of DNA lesion frequencies

Lesions were calculated using the Poisson equation as previously described (Ayala-Torres et al., 2000; Santos et al., 2006). We assumed a random distribution of lesions as damage is introduced in an evenly fashion into the DNA. We used the Poisson equation [defined as $f(x) = e^{-\lambda}\lambda^x/x!$ for the zero class molecules; $x = 0$ (molecules exhibiting no damage)], where amplification is directly proportional to the fraction of undamaged DNA templates. Therefore, the average lesion frequency per DNA strand can be calculated as $\lambda = -\ln A_D/A_O$, where A_D represents the amount of amplification of the damaged template and A_O is the amount of amplification product from undamaged DNA. The results are expressed as a relative amplification ratio (A_D/A_O) and, using the Poisson equation, as lesion frequency per strand. Levels of mtDNA lesions in liver were calculated comparing young, middle age and old animals to the infants whereas mtDNA lesions in PBMCs were calculated comparing middle age and old animals to the young.