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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.

Keywords

mitochondrial DNA; aging; mitochondria; antioxidant enzymes; rhesus monkey; liver

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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; Troughton 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 increase in old animals (Beckman and Ames, 1998; Navarro and Boveris, 2004; Navarro et al., 2002; Shigenaga et al., 1994) and inversely correlate with the activities of complex I and IV (Navarro et al., 2004; Navarro et al., 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., In Press). 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).

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

rhesis 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 (*Macaca 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 size 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 was 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 seconds at 94°C , 23 cycles of denaturation for 15 seconds at 94°C and annealing/extension at 68°C for 12 minutes, and a final extension at 72°C for 10 minutes. 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 seconds at 94°C, followed by 29 cycles of denaturation for 15 seconds at 94°C, and annealing/extension at 60°C for 45 seconds and 45 seconds at 72°C. A final extension at 72°C was performed for 10 minutes 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 $f(x) = e^{-x}/x!$ 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 $-\ln A_D/A_O$, 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.

2.5. Western blot analysis

A total of 40 µg of protein were electrophoresed using 12% SDS-PAGE and transferred to a 0.2 µm PVDF membrane. Membranes were incubated with primary antibodies against MnSOD [mouse monoclonal [2A1] to SOD₂ (1:1500) (Abcam)] and APE/REF-1 antibody (Novus) (1:15000), respectively, and a goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) secondary antibody (1:5000). Mouse monoclonal antibody [DM1A] to alpha tubulin (Abcam) was employed as control to correct for possible differences in loaded protein (1:5000). Membranes were visualized using Super Signal Western Dura Extended Duration Substrate from Pierce Biotechnology, Inc.

2.6. Determination and quantification of protein carbonylations

We employed the OxyBlot™ Protein Oxidation Detection Kit to detect protein carbonylations in PBMCs from rhesus monkeys. Aliquots containing 40 µg of protein were electrophoresed and analyzed according to the protocol provided by the manufacturer. Protein samples were treated with 2,4-dinitrophenyl hydrazine (DNPH) to derivatize the carbonyl/aldehyde groups present in the proteins to 2,4-dinitrophenylhydrazone (DNP-hydrazone), followed by electrophoresis and overnight transfer to a membrane. Membranes were incubated with the primary anti DNP antibody (1:500) that immunodetects the DNP-hydrazone or carbonyl groups and a goat anti-rabbit IgG secondary antibody (1:300). Membranes were visualized using Super Signal Western Dura Extended Duration Substrate (Pierce Biotechnology) after incubation with the secondary antibody. Protein bands were quantified using the Bio-Rad VersaDoc™ Imaging System and the Quantity One computer software. Carbonylated protein levels were corrected for differences in protein loading using the expression levels of α -tubulin (Abcam). The protein carbonylations in liver were

determined in the same monkeys used for the analyses of mtDNA lesions and mtDNA copy numbers. Levels of carbonylations were expressed as density units after correcting for variations in protein loading based on the expression of α -tubulin.

2.7. Measurements of the activity of antioxidant enzymes

Tissue homogenates were obtained by rinsing tissue with PBS in 50 mM phosphate buffer, pH 7.0, 0.1 mM EDTA.

2.7.1. Protein assays—Protein concentrations were estimated using the Coomassie dye-based protein assay Pierce BCA Protein Assay (Pierce Biotechnologies, Rockford, IL) following the manufacturer's instructions and bovine serum albumin was used as a standard. All samples were analyzed in triplicates.

2.7.2. Antioxidant Enzyme Assays—Superoxide dismutase (SOD) activity was determined at room temperature by using 10 μ l of tissue added to 960 μ l carbonate buffer (0.05M, pH 10.2, 0.1 mM EDTA). Twenty (20) μ l of 30 mM epinephrine (in 0.05% acetic acid) was added to the mixture and measured at 480 nm during 4 minutes on a Hitachi U-2800 Spectrophotometer. One unit of activity is equal to the amount of enzyme that inhibits the oxidation of epinephrine by 50% per minute. Catalase activity was determined by adding 10 μ l of absolute ethanol per 100 μ l of tissue extract and then placed in an ice bath for 30 minutes. A total of 10 μ l of tissue extract was added to a cuvette containing 240 μ l phosphate buffer and 250 μ l of 0.066M H_2O_2 (in phosphate buffer). After adding 10 μ l of Triton X-100 RS, the tubes were kept at room temperature. The decrease in optical density was measured at 240 nm for one minute. The molar extinction coefficient of 43.6 M cm^{-1} was used to determine enzyme activity. One unit of activity is equal to the moles of H_2O_2 degraded $\text{min}^{-1} \text{ mg protein}^{-1}$. Data expressed as units/mg protein. Glutathione peroxidase activity was determined at 37°C. The reaction mixture consisted of 550 μ l phosphate buffer, 100 μ l 0.01M GSH (reduced form), 100 μ l 1.5mM NADPH, and 100 μ l GR (0.24 units). A total of 50 μ l of tissue extract were added to the reaction mixture and incubated at 37°C for 10 minutes. Then, 50 μ l of 12 mM t-butyl hydroperoxide was added to 450 μ l of tissue reaction mixture and measured at 340 nm for 3 minutes. The molar extinction coefficient of $6.22 \times 10^3 \text{ M cm}^{-1}$ was used to determine enzyme activity. One unit of activity is equal to the mM of NADPH oxidized $\text{min}^{-1} \text{ mg protein}^{-1}$. All samples were analyzed in duplicates and data expressed as nM of NADPH oxidized/min/mg of protein. Glutathione reductase activity was determined by using Cayman's Glutathione Reductase Assay Kit (Cayman Chemicals, Ann Arbor, MI), following company's protocol. All samples were analyzed in replicates and data were expressed as Units/mg of protein.

2.8. Measurements of lipid peroxidation

A lipid peroxidation assay was performed to determine malondialdehyde (MDA) levels using the method described by Ohkawa and colleagues (Ohkawa et al., 1979). Fifty (50) μ l of tissue homogenate were added to 50 μ l of 8.1% sodium dodecyl sulfate (SDS), vortexed, and incubated for 10 minutes at room temperature. Then, 375 μ l of 20% acetic acid (pH 3.5) and 375 μ l of thiobarbituric acid (0.6%) was added and placed in a boiling water bath for one hour. The samples were allowed to cool down at room temperature and 1.25 ml of butanol:pyridine (15:1) was added, vortexed and centrifuged at 1000 rpm for 5 minutes. Finally, 750 μ l of the organic pink layer was measured at 532 nm, generating a standard curve using five dilutions of 1, 1, 3, 3-tetraethoxypropane. Data was expressed as nM of MDA/mg of protein and performed in duplicates.

2.8. Analysis of liver histology

Formalin fixed paraffin embedded tissues were stained for hematoxylin and eosin [H&E], trichrome and Periodic Acid Schiff [PAS]. Tissues were sectioned at 4 micron thickness. At least 10 hepatic portal fields were evaluated per slide of representative area. The H&E analysis was performed for the evaluation of general morphology; trichrome was performed for the evaluation of tissue architecture and the amount of fibrosis and PAS for the determination of glycogen deposits. The sections were graded by a Board Certified pathologist for degrees of liver pathology as mild (1–10%), moderate (10–40%) and severe (>40%).

2.9 Statistics

Statistical comparisons for the analysis of mtDNA damage were made by one-way analysis of variance (ANOVA). The descriptive and inferential analyses were performed using SigmaStat. The oxidative stress data were expressed as mean $\pm$ SEM and analyzed statistically using ANOVA, followed by a post-hoc Scheffé test for comparison between groups. Pearson correlations were also determined. Statistical significance was set at $p < 0.05$ for all analyses, using SPSS (v 16.0).

3. Results

3.1. Age-dependent increases in mtDNA damage in liver and in peripheral blood mononuclear cells (PBMCs) of rhesus monkeys

In this study we used a quantitative gene-specific PCR (QPCR) assay to measure oxidative damage to the mtDNA. We have successfully applied the QPCR assay to measure DNA damage in yeast (Acevedo-Torres et al., 2009b) and mice (Acevedo-Torres et al., 2009a), yet, this is the first time the QPCR technique is used to measure mtDNA damage in tissues and blood cells from rhesus macaque monkeys. To measure mtDNA damage, we amplified a 10 kb mtDNA fragment that represents ~61% of the rhesus mitochondrial genome (Brown et al., 1979; Gokey et al., 2004). The presence of certain oxidative lesions in the mtDNA fragment such as base modifications, single and double strand breaks, apurinic/apyrimidinic sites can stall the thermostable DNA polymerase resulting in decreased amplification of the 10 kb mtDNA fragment (Ayala-Torres et al., 2000). Thus, amplification is inversely proportional to the presence of damage.

We analyzed liver obtained from young (0.6–8 year old), middle age (9–17 year old), and old (>19 years of age) rhesus monkeys and found significant age-dependent increases in mtDNA damage (Figure 1). In middle age and old monkeys, for example, amplification of a 10 kb mtDNA fragment was reduced 15% and 30%, respectively (Figure 1, panel A). These results were corrected for the significant 12 and 16% decrease in mtDNA abundance detected in middle age and old rhesus macaques, respectively, after amplification of a 100 bp mtDNA fragment that, because of its small size, is independent of the presence of lesions and thus, provides an accurate control determination of steady-state levels of mtDNA molecules (Figure 1, panel B). Consequently, our results indicate that a decrease in mtDNA copy number in old monkeys also correlates with increases in mtDNA lesions with age.

The frequency of mtDNA lesions in liver from middle age and old rhesus macaques were calculated using the Poisson equation (as described in Section 2.4) and we show that liver mtDNA from both age groups exhibit significantly higher levels of mtDNA lesions compared to young monkeys (Figure 1, panel C). Moreover, levels of mtDNA lesions were significantly higher in old monkeys compared to middle age animals (0.41 lesions/10 kb/strand versus 0.27 lesions/10 kb/strand, respectively) (Figure 1, panel C). Regression analysis of the frequency of mtDNA lesions versus age generated a statistically significant

linear relationship ($p = 0.043$) (Supplemental material-Figure 2), strongly suggesting the role of mtDNA damage in the process of aging.

We also examined levels of oxidative lesions in mtDNA from PBMCs obtained from young, middle age and old monkeys. MtDNA from PBMCs of old monkeys showed a 30% reduction relative to levels in young monkeys (Figure 2, panel A) and a significant increase in the number of mtDNA lesions (Figure 2, panel B). PBMCs from middle age monkeys showed an age-dependent increase in amplification of the mtDNA fragment of 25%, representing a compensatory mechanism as shown by the loss of oxidative mtDNA lesions (-0.20 lesions/10kb; Figure 2, panel B). However, this compensatory response was not sustained in mitochondria from old monkeys, resulting in a significantly higher number of mtDNA lesions than in young monkeys.

3.2. Age-dependent increases in lipid peroxidation and in protein carbonylations in liver and peripheral blood mononuclear cells (PBMCs) from rhesus monkeys

Next we measured malondialdehyde (MDA), a lipid peroxidation product that is formed by the action of ROS on polyunsaturated lipids. MDA is a reactive aldehyde and considered one of several reactive electrophile species that cause toxic stress in cells by forming covalent protein adducts known as advanced lipo-oxidation end products. In our analyses, we found a significant 51 and 36% increase in the levels of MDA in liver from middle age and old monkeys, respectively, when compared to young monkeys ($p < 0.001$ and $p < 0.01$) (Figure 3, panel A). Moreover, we found significant increases in the levels of carbonylated proteins in liver from middle age and old monkeys compared to young animals ($p < 0.005$ and $p < 0.0005$, respectively) and an approximately 2-fold significant increase in oxidized proteins in old monkeys compared to middle age animals ($p = 0.01$) (Figure 3, panel B and supplemental material). Taken together, these data suggest that hepatic aging in our cohort of rhesus monkeys is associated with the accumulation of lipid peroxidation products and oxidized/carbonylated proteins.

We next analyzed total proteins obtained from PBMCs to determine whether aging results in protein oxidation. We found that levels of carbonylated proteins were significantly higher in middle age monkeys, whereas old monkeys showed levels similar to those of young animals (Figure 3, panel C and supplemental material). Middle age monkeys exhibited 35-fold more carbonylated proteins than young monkeys, suggesting that levels of oxidative stress are significantly higher in middle age as compared to young age.

3.3. Age-dependent changes in the activity of antioxidant enzymes

To determine if aging modulates the activity of the hepatic antioxidant system in rhesus, we determined levels of various antioxidant enzymes in liver extracts from young, middle age and old monkeys. We found that glutathione peroxidase (GSH-Px), the antioxidant enzyme that reduces lipid hydroperoxides to their corresponding alcohols and hydrogen peroxide to water, presents a significant negative correlation with age (0.714 ; $p < 0.01$). By comparing the GSH-Px activity between the three ages groups, there was a 16 and 65% reduction in middle age and old monkeys, respectively ($p < 0.05$) (Figure 4, panel A). However, the activity of glutathione reductase did not show significant changes ($p > 0.05$) in the older group, when compared to young or middle-age monkeys (data not shown). Catalase activity increased a significant 39%, ($p < 0.05$) in old monkeys when compared to young monkeys and showed no significant difference with middle age monkeys (Figure 4, panel B). The results also showed a significant positive correlation ($r = 0.667$, $p = 0.025$) of catalase activity with age. There was a 46% reduction in total SOD activity when comparing young and middle age versus old monkeys that was statistically significant only between the middle age and older group ($p < 0.05$) (Figure 4, panel C). Middle age monkeys showed significant

increased levels of the mtDNA repair enzyme APE1 and of the mitochondrial antioxidant protein MnSOD (Figure 4, panel D). Interestingly, expression levels of APE1 showed a significant ($p<0.05$) 30% reduction in PBMCs from old monkeys compared to young animals (Figure 4, panel D).

3.4. Age-associated changes in liver histology in rhesus monkeys

We performed histological analyses of liver tissue obtained from 2-, 10- and 23-year-old normal rhesus monkeys for evidence of age-dependent liver pathology. Figure 5, panels A and C show liver sections from a 10-year-old monkey with normal vascular hepatic space/sinusoids and no evidence of fibrosis. Similar observations were evidenced in livers from 2-year-old rhesus monkeys (data not shown). Liver sections from an old, 23-year-old rhesus exhibit obliterated sinusoids and moderate central congestion (panel B). Areas of mild to moderate centrilobular steatosis (lipid deposits) are also evident in the liver from the old individuals (panel D). Moreover, Panel D shows piecemeal necrosis and portal fibrosis extending out from the portal space as well as the loss of limiting plate. Panels E and F show liver sections from an old (20-year-old) rhesus exhibiting mild cell inflammation, mild to moderate steatosis, and the presence of binucleated hepatocytes. Taken together, these results show the presence of degenerative lesions in liver from aged healthy rhesus monkeys.

4. Discussion

Although numerous studies have shown the aging liver suffers mitochondrial dysfunction in human and murine models, data in aging rhesus or the cause of this mitochondrial failure is unknown. Here we show that aging rhesus monkeys have increased levels of mtDNA damage and oxidative stress in the liver in conjunction with enzymatic changes supporting a dysfunction of oxidative clearance mechanisms as a potential cause for increases in mtDNA damage. Our results suggest that mtDNA damage may represent an early change contributing to liver age-associated mitochondrial dysfunction and an excellent biomarker of aging in humans.

Our results showed a dramatic increase in oxidative mtDNA lesions in livers of middle age and old monkeys, indicative of significant increases in oxidative stress in this tissue. To our knowledge, this is the first report showing an age-dependent increase in mtDNA damage in liver from rhesus monkeys. Moreover, we also demonstrated that mtDNA abundance significantly decreases with age. It is well established that aging induces the loss of mitochondrial function in liver of rodents and humans (Alemany et al., 1988; Ferreira et al., 2006; Hagen et al., 1997; Modi et al., 2008; Muller-Hocker et al., 1997; Sabaretnam et al., 2010; Serviddio et al., 2007; Yen et al., 1989). It is possible that in the rhesus aging liver, the observed augmentation in levels of mtDNA damage, in parallel with the detected mtDNA depletion, may be responsible for the impairment of mitochondrial respiratory capacity and ATP synthesis. Our data are consistent with aging hepatocytes having mtDNA damage as being more vulnerable to dysfunction and apoptosis. Similarly, other studies consistent with our hypothesis show the attenuation of the age-associated increases in plasma glucose and insulin resistance in rhesus macaques by caloric restriction (CR) (Colman et al., 2009). CR is the only intervention that increases the lifespan of many different organisms (Gredilla and Barja, 2005; Masoro, 1993), possibly in part via reduction of oxidative stress to DNA and proteins (Gredilla et al., 2001; Lopez-Torres et al., 2002) (Forster et al., 2000; Lass et al., 1998; Zainal et al., 2000).

The increased levels of mtDNA damage are consistent with the age-dependent increases in lipid peroxidation and GSH-Px as observed in rhesus livers. Interestingly, mitochondria represent about one third of total GSH-Px activity in the liver (Chance et al., 1979)

suggesting that mitochondria may account for a large portion of the age-dependent decline in GSH-Px activity in rhesus liver. Similarly, other studies have reported that both old rats and mice showed decreased GSH-Px activity in liver (Iantomasi et al., 1994). Indeed, a deficiency in GSH-Px activity is associated with increased levels of mitochondrial generated ROS and lipid peroxidation in the liver and with decreased liver mitochondrial function, supporting the notion that GSH-Px may protect from oxidative stress via the inhibition of mitochondrial ROS generation (Esposito et al., 2000). Our data are consistent with this interpretation based on the age-dependent decreased levels of GSH-Px activity in aging monkeys, coupled with increased levels of lipid peroxidation intermediates and increased levels of carbonylated proteins. The observed increases in lipid peroxidation and carbonylated proteins in old animals are also in accordance with the age-associated accumulation of lipofuscin in the liver, an end product of oxidized protein and lipid peroxidation and a marker for aging (Brunk and Terman, 2002; Ikeda et al., 1985). Thus, the decline in GSH-Px activity may lead to the accumulation of ROS resulting in mtDNA damage, increased lipid peroxidation and protein oxidation in the aging liver. The increased catalase activity that was detected may represent a compensatory response to the increased accumulation of oxidative stress and decreased activity of GSH-Px we observe in middle age and old monkeys. Interestingly, histopathological changes including steatosis, inflammation, and fibrosis, all of which are associated with oxidative stress and mitochondrial dysfunction (Bradbury, 2006; Pessayre et al., 2002), were present in liver of old monkeys.

Consistent with our results, PBMCs from old monkeys also exhibit increased levels of mtDNA damage compared to young monkeys. Interestingly, levels of damage were lower in PBMCs of middle age monkeys compared to old animals. It is possible that only those PBMCs with low levels of mtDNA lesions are maintained in the circulation. Moreover, levels of the mitochondrial protein MnSOD and carbonylated proteins were increased in PBMCs from middle age monkeys, further supporting the presence of high levels of oxidative stress. Similarly, plasma antioxidant levels and nuclear DNA repair rates have been shown to be induced in lymphocytes from old subjects (Humphreys et al., 2007).

The observed increase in expression of the base excision repair enzyme APE1 in PBMCs from middle age rhesus monkeys is also representative of increased oxidative stress. The induced expression of APE1 has been shown in both in vivo and in vitro studies to occur after oxidative stress (Grosch et al., 1998; Grosch and Kaina, 1999; Pines et al., 2005; Ramana et al., 1998) and APE1 induction is consistent with increased rates of DNA repair and/or with redox-dependent effects on gene regulation (Izumi et al., 2005). Taken together these results suggest that PBMCs from middle age and old monkeys exhibit high levels of oxidative stress that reflect the mitochondrial damage observed in the liver and thus, may represent a surrogate tissue to measure aging in rhesus monkeys.

Our results indicate that increased mtDNA damage, decreased mtDNA molecules and increased oxidative stress levels accompany liver aging in rhesus monkeys. We propose a model (Figure 6) in which mitochondrial dysfunction due to increased damage to mtDNA and oxidative stress may lead to changes in liver that may predispose aged tissue to disease, decreased immune functions, and impaired drug clearance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

mtDNA	mitochondrial DNA
GSH-Px	glutathione peroxidase
ETC	electron transport chain
PBMC	peripheral blood mononuclear cells

References

- Hsieh R, Hou J, Hsu H, Wei Y. Age-dependent respiratory function decline and DNA deletions in human muscle mitochondria. *Biochem Mol Biol Int.* 1994; 32:1009–1022. [PubMed: 8061617]
- Lesnefsky E, Hoppel C. Oxidative phosphorylation and aging. *Ageing Res Rev.* 2006; 5:402–433. [PubMed: 16831573]
- Ojaimi J, Masters C, Opeskin K, McKelvie P, Byrne E. Mitochondrial respiratory activity in the human brain as a function of age. *Mech Aging Dev.* 1999; 111:39–47. [PubMed: 10576606]
- Short K, Bigelow M, Kahl J, Singh R, Coenen-Schimke J, Raghavakaimal S, Nair K. Decline in skeletal muscle mitochondrial function with aging in humans. *Proc Natl Acad Sci USA.* 2005; 102
- Trounce I, Byrne E, Marzuki S. Decline in skeletal muscle mitochondrial respiratory chain function: possible factor in ageing. *Lancet.* 1989; 1:637–639. [PubMed: 2564459]
- Yen T, Chen Y, King K, Yeh S, Wei Y. Liver mitochondrial respiratory functions decline with age. *Biochem. Biophys. Res. Commun.* 1989; 165:944–1003.
- Benzi G, Pastoris O, Marzatico F, Villa R, Dagani F, Curti D. The mitochondrial electron transfer alteration as a factor involved in the brain aging. *Neurobiol Aging.* 1992; 13:361–368. [PubMed: 1320745]
- Kumaran S, Subathra M, Bahu M, Panneerselvam C. Supplementation of L-carnitine improves mitochondrial enzymes in heart and skeletal muscle of aged rats. *Exp Aging Res.* 2005; 31:55–67. [PubMed: 15842073]
- Lenaz G, Bovina C, Castelluccio C, Fato R, Formiggini G, Genova M, Marchetti M, Pich M, Pallotti F, Parenti C, Biagini G. Mitochondrial complex I defects in aging. *Mol Cell Biochem.* 1997; 174:329–333. [PubMed: 9309707]
- Navarro A, Boveris A. Rat brain and liver mitochondria develop oxidative stress and lose enzymatic activities on aging. *Regul Integr Comp Physiol.* 2004; 287:R1244–R1249.
- Muller-Hocker J, Schafer S, Link T, Possekkel S, Hammer C. Defects of the respiratory chain in various tissues of old monkeys: acytochemical-immunocytochemical study. *Mech Aging Dev.* 1996; 86:197–213. [PubMed: 8733113]
- Beckman K, Ames B. The free radical theory of aging matures. *Physiol Rev.* 1998; 78:547–581. [PubMed: 9562038]
- Navarro A, Sanchez-Pino M, Gomez C, Peralta J, Boveris A. Behavioral dysfunction, brain oxidative stress, and impaired mitochondrial electron transfer in aging mice. *Am J Physiol Regul Integr Comp Physiol.* 2002; 282:R985–R992. [PubMed: 11893601]
- Shigenaga M, Hagen TM, Ames BN. Oxidative damage and mitochondrial decay in aging. *Proc. Natl. Acad. Sci. USA.* 1994; 91:10771–10778.
- Navarro A, Gomez C, Lopez-Cepero J, Boveris A. Beneficial effects of moderate exercise on mice aging: survival, behavior, oxidative stress, and mitochondrial electron transfer. *Am J Physiol Regul Integr Comp Physiol.* 2004; 286:R505–R511. [PubMed: 14615275]

- Yakes MF, Van Houten B. Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. *PNAS*. 1997; 94:514–519. [PubMed: 9012815]
- Acevedo-Torres K, Berrios L, Rosario N, Dufault V, Skatchkov S, Eaton MJ, Torres-Ramos CA, Ayala-Torres S. Mitochondrial DNA damage is a hallmark of chemically induced and the R6/2 transgenic model of Huntington's disease. *DNA Repair (Amst)*. 2009a; 8:126–36. [PubMed: 18935984]
- Mandavilli BS, Ali SF, Van Houten B. DNA damage in brain mitochondria caused by aging and MPTP treatment. *Brain Res*. 2000; 885:45–52. [PubMed: 11121528]
- Vogel K, Perez M, Momand J, Acevedo-Torres K, Hildreth K, Garcia R, Torres-Ramos C, Ayala-Torres S, Prihoda T, McMahan CA, Walter C. Age-Related Instability in Spermatogenic Cell Nuclear and Mitochondrial DNA Obtained from Apex1 Heterozygous Mice. *Molecular Reproduction and Development*. In Press.
- Martin R, Fitzl G, Mozet C, Martin H, Welt K, Wieland E. Effect of age and hypoxia.reoxygenation on mRNA expression of antioxidative enzymes in rat liver and kidneys. *Exp. Gerontol*. 2002; 37:1481–1487. [PubMed: 12559417]
- Muradian K, utko N, Fraifeld V, Mozzhukhina T, Pishel I, Litoshenko A. Superoxide dismutase, catalase, and glutathione peroxidase activities in the liver of young and old mice: linear regression and correlation. *Arch. Gerontol. Geriatr*. 2002; 35:205–214. [PubMed: 14764359]
- Muller-Hocker J, Aust D, Rohrbach H, Napiwotzky J, Reith A, Link T, Seibel P, Holzel D, Kadenbach B. Defects of the respiratory chain in the normal human liver and in cirrhosis during aging. *Hepatology*. 1997; 26:709–719. [PubMed: 9303502]
- Aleman J, de la Cruz M, Roncero I, Miquel J. Effects of aging on respiration, ATP levels and calcium transport in rat liver mitochondria. Response to theophylline. *Exp. Gerontology*. 1988; 23:25–34.
- Ferreira F, Moreno A, Seica R, Santos M, Palmeira C. Mitochondrial bioenergetics, diabetes and aging: Top-down analysis using the diabetic goto-kakizaki (GK) rat as a model. *Toxicol. Mech. Methods*. 2006; 16:323–330. [PubMed: 20021031]
- Hagen T, Yowe D, Bartholomew J, Wehr C, Do K, Park J-Y, Ames B. Mitochondrial decay in hepatocytes from old rats: Membrane potential declines, heterogeneity and oxidants increase. *Proc. Natl. Acad. Sci. USA*. 1997; 94
- Modi H, Katyare S, Patel M. Ageing-induced alterations in lipid/phospholipid profiles of rat brain and liver mitochondria: implications for energy-linked functions. *J. Membr. Biol*. 2008; 221:51–60. [PubMed: 18097631]
- Serviddio G, Bellanti F, Romano A, Tamborra R, Rollo T, Altomare E, Vendemiale G. Bioenergetics in aging: mitochondrial proton leak in aging rat liver, kidney and heart. *Redox Rep*. 2007; 12:91–95. [PubMed: 17263918]
- Sabaretnam T, Kritharides L, O'Reilly J, Le Couteur D. The effect of aging on the response of isolated hepatocytes to hydrogen peroxide and tert-butyl hydroperoxide. *Toxicology in Vitro*. 2010; 24:123–128. [PubMed: 19720132]
- Sastre J, Pallardo F, Pla R, Pellin A, Juan G, O'Connor J, Estrela J, Miquel J, Vina J. Aging of the liver: age-associated mitochondrial damage in intact hepatocytes. *Hepatology*. 1996; 24:1199–1205.
- Gredilla R, Bohr VA, Stevnsner T. Mitochondrial DNA repair and association with aging - An Update. *Experimental Gerontology*. 2010; 45:478–488. [PubMed: 20096766]
- Hiona A, Sanz A, Kujoth G, Pamplona R, Seo A, Hofer T, Someya S, Miyakawa T, Nakayama C, Samhan-Arias A, Servais S, Barger J, Portero-Otin M, Tanokura M, Prolla T, Leeuwenburgh C. Mitochondrial DNA mutations induce mitochondrial dysfunction, apoptosis and sarcopenia in skeletal muscle of mitochondrial DNA mutator mice. *PLoS ONE*. 2010; 5:e11468. [PubMed: 20628647]
- Roth G, Mattison J, Ottinger M, Chachich M, Lane M, Ingram D. Aging in rhesus monkeys: Relevance to human health interventions. *Science*. 2004; 305:1423–1426. [PubMed: 15353793]
- Ayala-Torres, S.; Chen, Y.; Svoboda, T.; Rosenblatt, J.; Van Houten, B. Analysis of gene-specific DNA damage and repair using quantitative PCR. In: Doetchst, P., editor. *Methods: A Companion to Methods in Enzymology*. Academic Press; 2000.

- Santos JH, Meyer JN, Mandavilli BS, Van Houten B. Quantitative PCR-based measurement of nuclear and mitochondrial DNA damage and repair in mammalian cells. *Methods Mol Biol.* 2006; 314:183–99. [PubMed: 16673882]
- Ohkawa H, Oshishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbuturic acid reaction. *Anal. Biochem.* 1979; 95:351–358. [PubMed: 36810]
- Acevedo-Torres K, Fonseca-Williams S, Ayala-Torres S, Torres-Ramos CA. Requirement of the *Saccharomyces cerevisiae* APN1 gene for the repair of mitochondrial DNA alkylation damage. *Environ Mol Mutagen.* 2009b; 50:317–27. [PubMed: 19197988]
- Brown W, George MJ, Wilson A. Rapid evolution of animal mitochondrial DNA. *Proc Natl Acad Sci USA.* 1979; 76:1967–1971. [PubMed: 109836]
- Gokey N, Caio Z, Pak J, Lee D, McKiernan S, McKenzie D, Weindruch R, Aiken J. Molecular analyses of mtDNA deletion mutations in microdissected skeletal muscle fibers from aged rhesus monkeys. *Aging Cell.* 2004; 3:319–326. [PubMed: 15379855]
- Colman R, Anderson R, Johnson S, Kastman E, Kosmatka K, Beasley T, Allison D, Cruzen C, Simmons H, Kemnitz J, Weindruch R. Caloric restriction delays disease onset and mortality in rhesus monkeys. *Science.* 2009; 325:201–204. [PubMed: 19590001]
- Gredilla R, Barja G. Minireview: The role of oxidative stress in relation to caloric restriction and longevity. *Endocrinology.* 2005; 146:3713–3717. [PubMed: 15919745]
- Masoro E. Dietary restriction and aging. *J Am Geriatr Soc.* 1993; 41:994–999. [PubMed: 8409187]
- Gredilla R, Barja G, Lopez-Torres M. Effect of short-term caloric restriction on H₂O₂ production and oxidative DNA damage in rat liver mitochondria and location of the free radical source. *J Bioenerg Biomembr.* 2001; 33:279–287. [PubMed: 11710804]
- Lopez-Torres M, Gredilla R, Sanz A, Barja G. Influence on aging and long-term caloric restriction on oxygen radical generation and oxidative DNA damage in rat liver mitochondria. *Free Radic Biol Med.* 2002; 32:882–889. [PubMed: 11978489]
- Forster M, Sohal B, Sohal R. Reversible effects of long-term caloric restriction on protein oxidative damage. *J Gerontol A Biol Sci Med Sci.* 2000; 55:B522–B529. [PubMed: 11078084]
- Lass A, Sohal BH, Weindruch R, Fotster MJ, Sohal RS. Caloric restriction prevents age-associated accrual of oxidative damage to mouse skeletal muscle mitochondria. *Free Rad. Biol. Med.* 1998; 25:1089–1097. [PubMed: 9870563]
- Zainal T, Oberley T, Allison D, Szweda L, Weindruch R. Caloric restriction of rhesus monkeys lowers oxidative damage in skeletal muscle. *FASEB J.* 2000; 14:1825–1836. [PubMed: 10973932]
- Chance B, Sies H, Boveris A. Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.* 1979; 59:527–605. [PubMed: 37532]
- Iantomasi S, Favilli T, Marraccini P, Lunghi B, Vincencini M, Treves C. Glutathione metabolism in heart and liver of the aging rat. *Biochem. Cell Biol.* 1994; 72:58–61. [PubMed: 7915119]
- Esposito L, Kokoszka J, Waymire K, Cottrell B, MacGregor G, Wallace D. Mitochondrial oxidative stress in mice lacking the glutathione peroxidase-1 gene. *Free Radic Biol Med.* 2000; 28:754–766. [PubMed: 10754271]
- Brunk U, Terman A. Lipofuscin: mechanisms of age-related accumulation and influence on cell function. *Free Radic. Biol. Med.* 2002; 33:611–619. [PubMed: 12208347]
- Ikeda H, Tauchi H, Sato T. Fine structural analysis of lipofuscin in various tissues of rats of different ages. *Mech Aging Dev.* 1985; 33:77–93. [PubMed: 4079478]
- Bradbury M. Lipid metabolism and liver inflammation. I. Hepatic fatty acid uptake: possible role in steatosis. *Am J Physiol Gastrointest Liver Physiol.* 2006; 290:G194–G198. [PubMed: 16407588]
- Pessayre D, Mansouri A, Fromentry B. Mitochondrial dysfunction in steatohepatitis. *Am J Physiol Gastrointest Liver Physiol.* 2002; 282:G193–G199. [PubMed: 11804839]
- Humphreys V, Martin R, Ratcliffe B, Duthie S, Wood S, Gunnell D, Collins A. Age-related increases in DNA repair and antioxidant protection: A comparison of the Boyd Orr Cohort of elderly subjects with a younger population sample. *Age and Ageing.* 2007; 36:521–526. [PubMed: 17913757]
- Grosch S, Fritz G, Kaina B. Apurinic endonuclease (ref-1) is induced in mammalian cells by oxidative stress and involved in clastogenic adaptation. *Cancer Res.* 1998; 58:4410–4416. [PubMed: 9766671]

- Grosch S, Kaina B. Transcriptional activation of apurinic/apyrimidinic endonuclease (ape, Ref-1) by oxidative stress requires CREB. *Biochem Biophys Res Commun.* 1999; 261:859–863. [PubMed: 10441516]
- Pines A, Bivi N, Romanello M, Damante G, Kelley M, Adamson E, D'Andrea P, F Q, Moro L, Tell G. Cross-regulation between Erg-1 and APE/Ref-1 during early response to oxidative stress in the human osteoblastic HOBIT cell line: evidence for an autoregulatory loop. *Free Radic Biol Med.* 2005; 39:269–281.
- Ramana CV, Boldogh I, Izumi T, Mitra S. Activation of apurinic/apyrimidinic endonuclease in human cells by reactive oxygen species and its correlation with their adaptive response to genotoxicity of free radicals. *PNAS.* 1998; 95:5061–5066. [PubMed: 9560228]
- Izumi T, Brown D, Naidu C, Bhakat K, Macinnes M, Saito H, Chen D, Mitra S. Two essential but distinct functions of the mammalian abasic endonuclease. *PNAS.* 2005; 102

HIGHLIGHTS

- Aged rhesus show increased levels of mitochondrial DNA (mtDNA) damage in the liver and blood cells.
- Aged rhesus show increased oxidative stress as a potential cause for increases in mtDNA damage.
- MtDNA damage may be contributing to liver age-associated mitochondrial dysfunction.
- Peripheral blood cells may represent a surrogate tissue to measure aging in rhesus monkeys.

Fig. 1. Mitochondrial DNA damage increases with age in liver from rhesus monkeys

Total DNA was isolated from liver obtained from 0.6–2.0 year old (infant), 3–8 year old (young), 9–17 year old (middle age), and 19–24 years of age (aged) monkeys and analyzed by QPCR. A) Upper panel, representative gel showing an age-dependent decrease in the amplification of a 10 kb mtDNA amplicon from two monkeys per age group. Lower panel, bars indicate relative levels of amplification of a 10 kb mtDNA fragment after normalization for changes in mtDNA abundance. * $p < 0.0001$ and ** $p < 0.0001$ versus young and ‡ $p < 0.05$ versus middle age. B) Upper panel, representative gel showing the amplification of a 100 bp mtDNA fragment. Lower panel, relative abundance of mtDNA molecules during aging. ** $p < 0.0001$ versus young. C) Frequency of mtDNA lesions per 10 kb per strand. * $p < 0.0001$ and ** $p < 0.0001$ versus young and ‡ $p < 0.05$ versus middle age. $n = 5$ infant, $n = 7$ young; $n = 6$ middle age, and $n = 3$ old; $n = 4$ QPCR analyses.

Fig. 2. Mitochondrial DNA in peripheral blood mononuclear cells (PBMCs) from rhesus monkeys increases with age

A) Relative amplification of a 10 kb mtDNA fragment that was normalized for changes in mtDNA abundance. B) Frequency of mtDNA lesions per 10 kb per strand based on the relative levels of amplification of a 10 kb mtDNA fragment. n=11 young (6–7 year-old); n=10 middle age (middle age) and n=4 old (>22 years) monkeys. *p<0.002; **p = 0.05 versus young.

Fig. 3. Protein damage in liver and peripheral blood mononuclear cells (PBMCs) from rhesus monkeys increases with age

A) Lipid peroxidation as malondialdehyde (MDA) levels. MDA levels were measured as nM of MDA per mg of Protein. n=5 young, n=4 middle age, and n=4 old monkeys; Old versus young **p<0.01 and middle aged versus young *p<0.001. B) Detection of carbonylated proteins in liver from rhesus monkeys. Densitometry of modified proteins using anti-DNP antibody. Protein loading was normalized using α -tubulin expression. n=7 young; n=6 middle age, and n=2 old monkeys. **p<0.005 ***p=0.0005 †p=0.01. C) Detection of carbonylated proteins in PBMCs from rhesus monkeys. Densitometry of modified proteins using anti-DNP antibody. Protein loading was normalized using α -tubulin expression. n=5 young, n=2 middle age, and n=4 old monkeys. *p<0.0001 versus young.

Fig. 4. Age-associated activity of hepatic antioxidant enzymes and protein expression of APE1 and MnSOD in liver of rhesus monkeys

A) Age-dependent decreases in the activity of glutathione peroxidase (GHSPx). One unit of GHSPx activity is equal to mM of NADPH oxidized/min/mg of protein. n=5 young, n=4 middle age, and n=3 old monkeys; *p<0.05 versus young. B) Age-dependant catalase activity. Catalase activity was determined as units/mg protein. One unit of activity is equal to the moles of H₂O₂ degraded min⁻¹ mg protein⁻¹; n=5 young, n=3 middle age, and n=3 old monkeys; *p<0.05 versus young. C) Total SOD activity. Activity was expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, which is equal to 1 unit. n=5 young, n=2 middle age, n=4 old monkeys. *p<0.05 versus middle age. D) Expression levels of APE1 and MnSOD in PBMCs from rhesus monkeys. Protein loading was normalized using α -tubulin expression. n=5 young, n=2 middle age, and n=4 old monkeys. *p<0.0001 and **p<0.0001 versus young; ***p<0.05 versus young.

Fig. 5. Age-related pathology in liver from rhesus monkeys

Liver sections from young, middle age, and old rhesus were stained with hematoxylin and eosin. Panels A and C are representative liver sections from 10-year-old rhesus (n=3; middle age). Panels B, D, E, F are representative sections from 20–25 year-old rhesus monkeys (n=3; old). The old individuals used for this experiment were healthy monkeys. 200X magnification. Arrow in panel D shows fibrosis. Arrow in panel E shows steatosis. Arrow in panel F shows area of inflammation.

Fig. 6. Proposed model of aging liver and pathology

Our results using rhesus monkeys are consistent with a model of mitochondrial aging involving the increased generation of ROS, increased mtDNA damage/lesions, decreased antioxidant activity and increased liver pathology, culminating in mitochondrial dysfunction. Dysfunctional mitochondria may enhance inflammatory responses and fibrogenesis induced by drugs and xenobiotics and negatively affect detoxification reactions. The response of hepatocytes to substrate metabolism (glycolysis, gluconeogenesis, tricarboxylic acid cycle, oxidative phosphorylation, ATP synthesis) may lead to insulin resistance and type 2 diabetes mellitus.

Table 1

Number of animals used for the different experiments

Analyses	Young (0.6–8 yrs)	Middle age (9–17 yrs)	Old (>19 yrs)
Liver mtDNA lesions and mtDNA abundance	12	6	3
Liver glutathione peroxidase activity	5	4	3
Liver total SOD activity	5	2	4
Liver catalase activity	5	3	3
Liver lipid peroxidation	5	4	4
Liver protein carbonylations	7	6	2
Liver APE1 and MnSOD expression	7	6	2
Liver histology	5	3	3
PBMCs mtDNA lesions	11	10	4
PBMCs APE1 and MnSOD expression	5	2	4
PBMCs protein carbonylations	5	2	4