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Quantification of Wild-Type Mitochondrial DNA and Its 4.8-kb Deletion in Rat Organs

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Oxidative damage to mitochondrial DNA (mtDNA) is considered a major contributor in aging. An age-dependent increase of oxidative damage and of the quantity of partially deleted mtDNA was reported for several rat and human organs. Here, a systematic investigation of ten different tissues and organs of 20-months-old rats was performed. The amount of mtDNA and age-dependent 4.8 kb deletion (ΔmtDNA^{4834}) was determined by competitive polymerase chain reaction, along with the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSHPx). The data were related to the corresponding metabolic rates. MtDNA content was highest in heart and lowest in spleen. ΔmtDNA^{4834} was detected in all ten tissues and organs, and its amount was highest in liver and lowest in intestine. In heart, lung, muscle, and bone-marrow the deletion could not be quantified because of a point mutation, an A→T transition at position 8107. Activities of SOD and GSHPx were highest in liver and lowest in intestinal mucosa. A negative correlation between mtDNA content and ΔmtDNA^{4834}, and a positive correlation between metabolic rate, GSHPx, and the deletion was found. These results suggest that the occurrence of ΔmtDNA^{4834} in rat is related to oxidative stress.

Oxidative DNA damage is considered a major cause in aging and age-associated degenerative diseases (for review, see (1)). Increase of oxidative stress during ageing inversely correlates with maximal life span potential, and increased oxidative damage and premature aging in people with Down's syndrome. Age-dependent alterations of mitochondrial functions may have serious consequences for the organism (2–5).

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Abbreviations: ΔmtDNA^{4834}, 4.8 kb deletion; GSHPx, glutathione peroxidase; mtDNA, mitochondrial DNA; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SOD, superoxide dismutase.

The level of 8-hydroxy-deoxyguanosine, a marker for oxidatively damaged DNA, is much higher in mitochondrial DNA (mtDNA)[1] than in nuclear DNA (6), and increases in mtDNA with age. Ames *et al.* (7) reported that in 24-months-old rats oxidative mtDNA damage is three-times higher than in 3-months-old animals, whereas damage of nuclear DNA is doubled. The group of Ozawa (8,9) showed age-associated accumulation of oxidatively modified bases in mtDNA of human diaphragmatic and heart muscle. The level of base oxidation in heart muscle mtDNA was highly correlated with mtDNA deletions (9). Oxidative mtDNA damage also markedly increased in an age-dependent manner in the human brain (10), where also an increase of mtDNA deletions with advancing age was found (11).

The most common and frequently examined mutation of human mtDNA is a 4.977 kb deletion. It accumulates with age in many human tissues (11–18). In liver and brain of rats an age-dependent 4.8 kb deletion (ΔmtDNA^{4834}) has been detected (19–21).

The accumulation of mtDNA damage may result from the action of free radicals produced during cellular metabolism (22), or from sunlight (23). To reduce damage caused by oxygen radicals, cells are equipped with defense systems. They comprise enzymes such as superoxide dismutases (SOD, EC 1.15.1.1), glutathione peroxidase (GSHPx, EC 1.11.1.9), catalase (EC 1.11.1.6), and oxygen radical scavengers. The extent of oxidative challenge differs from organ to organ. For example, the central nervous system is generally considered to be especially prone to free radical damage due to its high metabolic rate, high level of lipids, and the comparatively low content of protective enzymes (24).

The role of mtDNA in aging, and particularly the relationship between oxidative stress, protective enzymes, and mtDNA mutations is of great interest (25). Therefore, we systematically investigated the amount of mtDNA in total DNA, the age-dependent ΔmtDNA^{4834}, and the activities of antioxidative SOD and GSHPx in ten different tissues or organs of 20-

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months-old rats. The results indicate that in this species the occurrence of ΔmtDNA^{4834} correlates with oxidative stress.

MATERIAL AND METHODS

Material and enzymes. Chemicals of highest commercially available purity were used. Mimics (pWE-DELm and pWE-OLm) were kindly provided by Dr. C. R. Filburn, Gerontology Research Center, Baltimore, USA (19).

Animals and tissues. Four twenty-months-old male Wistar rats (*Rattus norvegicus*) were used. They were fed a normal rat chow *ad libitum*, and had a normal night-day rhythm. Rats were fasted overnight before sacrifice. After killing by cervical dislocation, the organs were immediately homogenized in potassium phosphate buffer (50mM, pH 7.4). Bone-marrow was scraped from opened femurs. For the isolation of intestinal mucosa, the middle part of the gut was taken out, rinsed with phosphate-buffered saline, opened, and the mucosa was scraped off.

Enzyme activity measurements. Tissue homogenates were centrifuged for 5 min at 4°C at 10,000 g. The supernatants were used for SOD and GSHPx activity measurements, the pellets for total DNA isolation. Total SOD (Cu/Zn SOD and MnSOD) was assayed according to Marklund and Marklund (26), GSHPx according to Paglia and Valentine (27) modified by Lawrence and Burk (28).

Total DNA isolation. Pellets (see above) were suspended in 10mM Tris-HCl, 1mM EDTA, pH 8.0. DNA was isolated according to Maniatis *et al.* (29), and quantified fluorimetrically with the dye Hoechst 33258.

Competitive polymerase chain reaction (PCR). Prior to PCR amplification total DNA was digested with *SAU* 3A to improve PCR specificity and efficiency, and to avoid amplification of the normal 5.3 kb product. PCR was carried out with a constant amount of total DNA and increasing amounts of the appropriate mimic as described by Edris *et al.* (19). Cycling conditions for PCR were as follows: 1) 2 min 95°C; 2) 35 cycles: 30 sec 94°C, 50 sec 60°C, 50 sec 72°C; 3) 10 min 72°C. For the detection and quantification of wildtype mtDNA the following primers were used: L4395 (5′-AGGACTTAACCAGACGCCAAACACG-3′, nt 4395-4418) and H5164 (5′-CCTCTTTTCTGATAGGCGGG-3′, nt 5164–5145). Ten ng of total DNA and 10^{-12} to 10^{-13} g of the mimic pWE-OLm were used. To quantify the age-dependent ΔmtDNA^{4834} 100 to 2000 ng of *SAU* 3A-digested total DNA, 10^{-18} g to 10^{-15} g of mimic pWE-DELm, additional 1U Taq-Polymerase, and the primers L7825 (5′-TTTCTTCCCAAACCTTTCCT-3′, nt 7825-7944) and H13117 (5′-AAGCCTGCTAGGATGCTTC-3′, nt 13117-12997) were used. After amplification, the 459 bps fragment (deletion product) and the 770 bps product (wildtype mtDNA) were subjected to restriction digest with *Ban* II and *Rsa* I, respectively. The fragments were analysed on a 5% polyacrylamide gel, stained with 0.5μg/ml ethidium bromide, and the bands were densitometrically quantified (Molecular Dynamics Scanner).

Calculation. For mimics, relative units of the bands' density were plotted against the number of molecules initially added to the PCR, and a logarithmic regression was calculated. For the fragments generated from the total DNA a polynominal regression of the second degree was done. By numeric approximation the initial amount of molecules derived from mtDNA (deleted and wildtype, respectively) was determined. The amount of ΔmtDNA^{4834} is expressed as the ratio of the 459 bps and 770 bps molecules.

Sequencing. Sequence analysis was performed on both mtDNA strands by cycle sequencing. The thermostable polymerase dCODE Enzyme (Genesys Ltd. UK) and infrared-fluorescent primers (IR-41) were used. Sequencing gels were analysed with a LICOR 4000L in a semi-automatic modus, using the software BaseImagIR V2.30. Sequencing data were compared and analysed by the pileup and bestfit operation of GCG Wisconsin using the GeneEMBL data bank.

Statistics. All values are expressed as mean ± S.E.M.. ANOVA for all measured parameters revealed $p<0.05$. For each parameter organ pair differences were analysed by both the Tukey HSD test (honest significant difference) and the Fisher LSD (least significant difference). Statistical data in the Results section were obtained by the more conservative Tukey HSD test. Both tests comprise a multivariant analysis. The Fisher LSD test showed significant differences ($p<0.05$) for nearly all cases. Correlations were calculated by the Spearman's rank correlation.

RESULTS

To quantify mtDNA and ΔmtDNA^{4834}, a competitive PCR method with an internal standard (mimic) (19) was employed (see Materials and Methods). The amplified and subsequently restricted DNA was analyzed by PAGE and quantified densitometrically.

Quantification of mtDNA

To determine the amount of mtDNA in total DNA, an infrequently deleted region necessary for replication of mtDNA - the light strand origin of replication - was amplified using the primers L4395 and H5164. This resulted in a 770 bps product. Increasing levels of the mimic pWE-OLm were added to a fixed amount of total DNA derived from the various tissues and organs. After restriction with *Rsa* I and polyacrylamide gel electrophoresis (PAGE), the 770 bps product and the 495 and 275 restricted bps products appeared, which originate from the mimic pWE-OLm and the wildtype mtDNA, respectively (Fig. 1). This method allowed reliable quantification of mtDNA in total DNA. Fig. 2 and Table 1 show the percentage of mtDNA in total DNA isolated from the ten different tissues and organs. Heart, brain, and muscle had a relatively high mtDNA content (>0.10% of total DNA). In the other tissues and organs the content was lower than 0.055%.

Detection and Quantification of the 4.8 kb Deletion

ΔmtDNA^{4834} was undetectable in three-months-old rats (data not shown) but was detectable in all tissues and organs of 20-months-old rats investigated. For precise quantitation a competitive PCR assay with the primers L7825 and H13117 was employed. DNA cleavage by *Ban* II and subsequent PAGE revealed three bands (Fig. 3): the undigested 459 bps product of the mimic pWE-DELm, and two bands corresponding to the expected 281 and 178 bps restriction fragments of the 459 bps deletion product. Fig. 2 and Table 1 show that liver contained the highest amount of ΔmtDNA^{4834}, followed by spleen and pancreas. The ΔmtDNA^{4834} in bone-marrow and muscle of only one rat could be quantified, whereas for heart and lung the quantification failed. The 459 bps bands derived from bone-marrow and muscle of three of the four

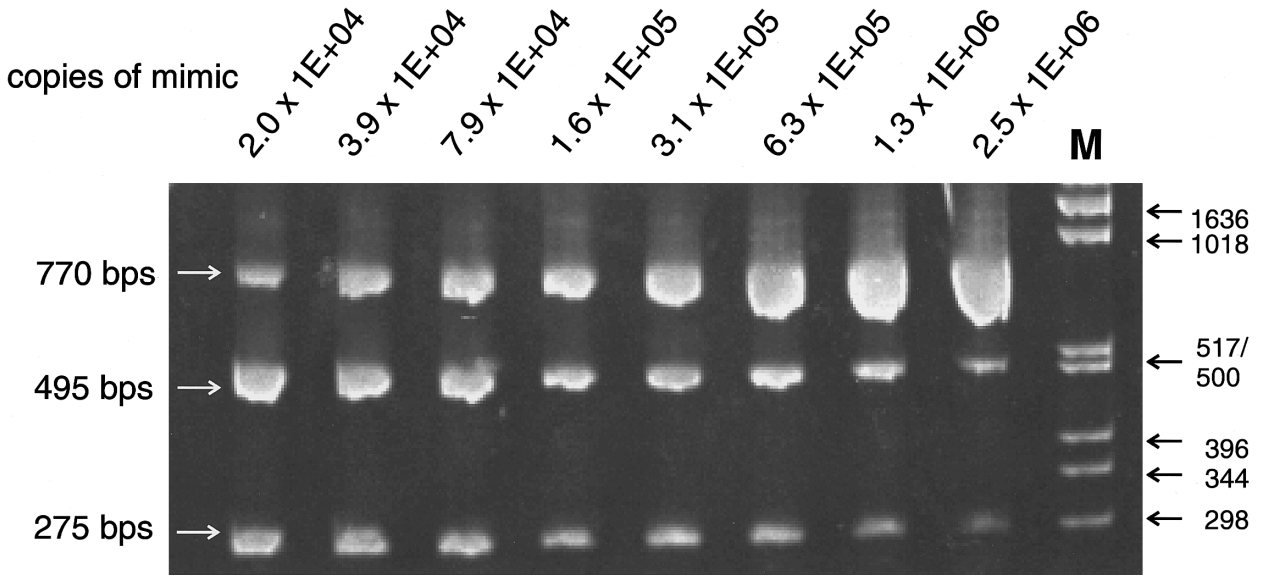

FIG. 1. Quantification of wildtype mtDNA. Competition between the mimic pWE-OLm and the mtDNA in 10 ng of lung total DNA. Following amplification a *Rsa* I digest was performed and DNA electrophoresed on a 5% polyacrylamide gel. The upper band (770 bps) corresponds to the mimic, the two lower bands originate from lung mtDNA.

rats, and from lung and heart of all rats were only partially digestible with *Ban* II (see below).

Sequencing

As the 459 bps deletion product of heart, muscle, bone-marrow and lung could not totally be cleaved by *Ban* II, we suspected the presence of a point mutation within its restriction site and therefore sequenced the heart, muscle, lung, and liver mtDNA fragments. The latter served as a control. We identified a point mutation inside the *Ban* II restriction site at position 8107, which is located in the gene of ATPase subunit 6 (Fig. 4).

Activities of GSHPx and SOD

Fig. 5 and Table 1 report the activities of the two antioxidative enzymes GSHPx and SOD, measured in all organs and tissues under identical conditions at 37°C. Liver has the highest, and intestinal mucosa the lowest activity of these enzymes.

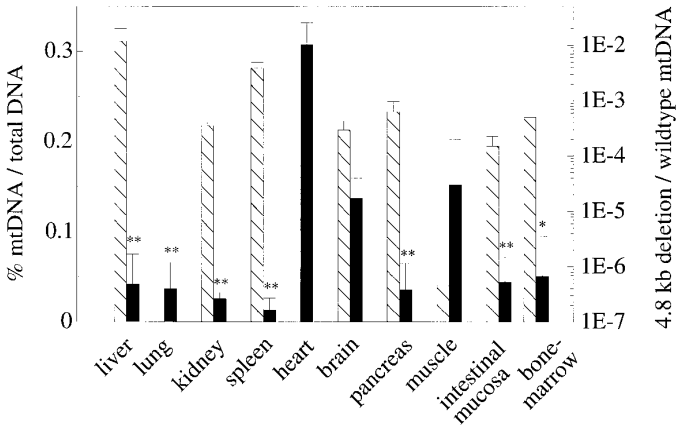

FIG. 2. Amount of mtDNA and ΔmtDNA^{4834} in ten different organs and tissues. Values represent average ± S.E.M. of total DNA of the ten tissues and organs of 20-months-old rats (n = 4) assessed in triplicate for each assay as described in Materials and Methods. Solid bars represent amount [%] mtDNA in total DNA, slanted bars represent the ratio of ΔmtDNA^{4834} to wildtype mtDNA. (*: $p<0.01$; **: $p<0.005$).

DISCUSSION

The reported amount of mtDNA in total DNA of mammals usually ranges from 0.1–2%, and can be as large as 20–30% in yeast cells and in the single large mitochondrion or kinetoplast of kinetoplastid flagellates (30). In rats, Gadaleta *et al.* (20) detected 0.23, 0.59, and 0.48% of mtDNA in liver, heart and the cerebral hemisphere, respectively, of seven-months-old animals. However, no systematic studies concerning the amount of mtDNA molecules and mtDNA deletions in different tissues of mammals have been published.

The levels of mtDNA molecules carrying the common 4.977 kb deletion in human mitochondrial myopathies can be as high as 95% of total mtDNA (30). These levels can be readily detected and quantified by restriction enzyme digestion and Southern hybridization. However, the levels of the common deletion in normal aging are generally much lower than 1%, which is beyond the sensitivity of those techniques (31–33). Various laboratories have developed differ-

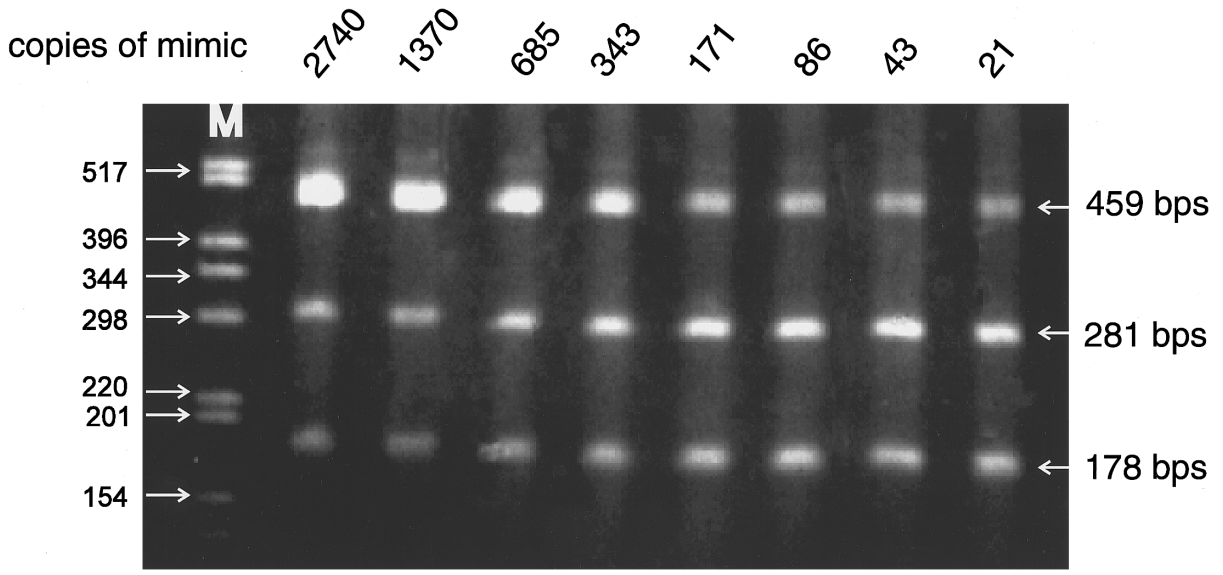

FIG. 3. Quantification of the ΔmtDNA^{4834}. Competition between the mimic pWE-DELm and the ΔmtDNA^{4834} product of 500 ng of brain total DNA. Amplification products were digested with *Ban* II and analysed on a 5% polyacrylamide gel. The undigested 459 bps band derived from the mimic, the 281 and 178 bps bands originated from ΔmtDNA^{4834} of brain mtDNA.

ent quantitative PCR strategies to detect and quantify these and other deletions. Zhang *et al.* (31) quantified a particular mtDNA deletion by serial dilution, kinetic and two internal standard PCR methods. Comparable values were obtained using the serial dilution procedure and the two internal standard methods, whereas kinetic PCR gave a value two orders of magnitude higher. On the basis of the considerations presented here, internal standard (competitive) PCR using a standard with a modified restriction site seems most appropriate. In our study a competitive PCR method with an internal standard (mimic) (19) was employed to quantify mtDNA and ΔmtDNA^{4834}.

Here, we found values for mtDNA content ranging from 0.01% (spleen) to 0.3% (heart). These values differ from previously published data (20,30). It must be pointed out that different methodologies and animals of different ages were chosen in the present and previous studies. Gadaleta *et al.* (20) detected total DNA with diphenylamine staining and mtDNA by Southern blot hybridization. We used Hoechst 33258 to quantify total DNA, a competitive PCR assay to quantify the amount of mtDNA in total DNA and a *SAU* 3A digest of total

TABLE 1

Amounts of mtDNA in Total DNA, ΔmtDNA^{4834}, GSHPx and Total SOD Activities in Ten Different Organs and Tissues

	mtDNA	ΔmtDNA^{4834}	SOD	GSHPx	Metabolic rate from (37)
Tissue	% mtDNA in total DNA	Ratio of ΔmtDNA^{4834} to wildtype mtDNA	U SOD per mg protein	nmol NADPH per min per mg protein	ml O_2 per min per g tissue
Liver	0.039 ± 0.03	1.17E-02 ± 8E-03	15.89 ± 2.03	172.37 ± 6.7	2.01
Lung	0.037 ± 0.03	n.d.	7.44 ± 0.68	46.98 ± 4.75	1.25
Kidney	0.026 ± 0.01	3.53E-04 ± 4E-05	7.03 ± 1.02	100.56 ± 15.72	4.12
Spleen	0.014 ± 0.01	3.85E-03 ± 1E-03	2.88 ± 0.33	42.09 ± 2.05	1.33
Heart	0.308 ± 0.02	n.d.	11.35 ± 1.73	37.24 ± 7.77	1.935
Brain	0.137 ± 0.02	2.95E ± 1E-04	6.72 ± 1.1	20.94 ± 2.28	1.84
Pancreas	0.036 ± 0.03	6.39E-04 ± 3E-04	3.30 ± 0.29	22.04 ± 3.83	n.d.
Muscle	0.152 ± 0.05	4.59E-07	3.41 ± 0.6	23.50 ± 5.01	0.875
Intestine	0.045 ± 0.03	1.52E-04 ± 7E-05	2.97 ± 0.23	8.33 ± 0.96	1.01
Bone-marrow	0.051 ± 0.04	4.70E-04	1.95 ± 0.48	23.77 ± 2.0	n.d.

Values are expressed as means ± S.E.M. of four 20-months-old rats. n.d. = no data.

DNA prior to PCR to enlarge its sensitivity and efficiency. It should also be noted that the mtDNA content decreases with age (34). The quantification of mtDNA by competitive PCR has not yet been published; a comparison is therefore not possible.

We found that the ratio of partially deleted mtDNA to wild-type mtDNA ranges between 1.2×10^{-2} (liver) and 2×10^{-4} (intestine), values which are higher than those previously published (19,20). Edris *et al.* (19) detected and quantified ΔmtDNA^{4834} in liver of 24-months-old rats by competitive PCR and obtained a ratio in the range of 10^{-3}. With the same method, Filburn *et al.* (21) found a ratio of about 7.5×10^{-5} in distinct brain areas. These values are 10-fold lower than those detected by us. Gadaleta *et al.* (20) quantified the deletion by serial dilution PCR, a method which does not use an internal standard. They found a ratio of 2×10^{-4} in liver of 31-months-old rats. The overall differences may also reflect genetic differences between rat strains, differences in diets used, or uncertainty in methods other than competitive PCR. It should be noted that with our very sensitive PCR technique the ΔmtDNA^{4834} was not detectable in three-months-old rats.

The activities of SOD and GSHPx differ substantially from organ to organ. The ranking order of the organs reported here is consistent with published data (35,36). Thus, activities of SOD and GSHPx are highest in liver. Brain and intestinal mucosa have very low activities of these antioxidative enzymes, and lung has an intermediate activity. Separate analysis of cytosolic and mitochondrial antioxidative enzymes could, unfortunately, not be performed in the present study. The scarcity of the biological material and the need for isolation of mitochondria for such a study precluded this informative comparison.

Is there a relationship between the metabolic rate, the amount of mtDNA in total DNA, the occurrence of ΔmtDNA^{4834}, and enzymatic antioxidants? To address this question we relied on published values of metabolic rates of rat tissues and organs (37). Table 1 summarizes the relevant parameters. No correlation was found between the metabolic rate and mtDNA content

FIG. 4. Analysis of the point mutation. The ΔmtDNA^{4834} of heart, muscle and liver was partially sequenced. Nucleotide numbering corresponds to the GeneEMBL rat mtDNA (X14848) (mtDNA*) sequence of the L-strand reading 5′ to 3′ (38). The bold letter indicate the nucleotide position 8107.

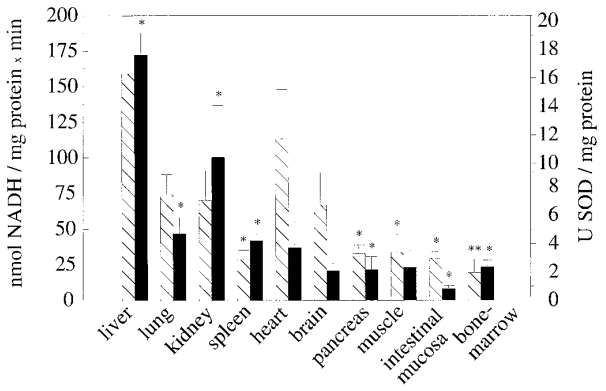

FIG. 5. GSHPx and SOD activity ten different organs and tissues. Values represent average ± S.E.M. from homogenates of the ten tissues and organs of 20-months-old rats (n = 4) assessed in duplicate for each assay as described in Materials and Methods. Solid bars represent units SOD per mg of protein, slanted bars represent activity of GSHPx expressed as nmol NADPH per min and per mg protein. (*: $p<0.005$; **: $p<0.001$).

($r = -0.24$). The more deletions were present, the less mtDNA was detected ($r = -0.67$, $p<0.025$). GSHPx correlates positively with the metabolic rate ($r = 0.64$, $p<0.025$) and the amount of ΔmtDNA^{4834} ($r = 0.64$, $p<0.025$). There is also a positive correlation between metabolic rate and the deletion ($r = 0.71$, $p<0.025$). No correlation was found between the tissues' replicative state (postmitotic or replicative) and any parameter measured here.

In summary, our systematic study presents the detection and quantitation of ΔmtDNA^{4834}, the quantitation of mtDNA content in total DNA, SOD and GSHPx activities in ten different rat organs and tissues, as well their correlation among each other. Since oxidative stress parallels the metabolic rate our findings suggest that oxidative stress favours the presence of the age-dependent ΔmtDNA^{4834}. Whether oxidative stress acts directly on mtDNA, *e.g.*, by inducing strand breaks followed by faulty ligation, or indirectly by damaging the replication machinery requires further studies.

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