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ORIGINAL ARTICLE

Mitochondrial base excision repair positively correlates with longevity in the liver and heart of mammals

Ricardo Gredilla · Inés Sánchez-Román · Alexia Gómez · Mónica López-Torres · Gustavo Barja

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Abstract Damage to DNA is especially important for aging. High DNA repair could contribute, in principle, to lower such damage in long-lived species. However, previous studies showed that repair of endogenous damage to nuclear DNA (base excision repair, BER) is negatively or not correlated with mammalian longevity. However, we hypothesize here that mitochondrial, instead of nuclear, BER is higher in long-lived than in short-lived mammals. We have thus measured activities and/or protein levels of various BER enzymes including DNA glycosylases, NTHL1 and NEIL2, and the APE endonuclease both in total and mitochondrial liver and heart fractions from up to eight mammalian species differing by 13-fold in longevity. Our results show, for the first time, a positive correlation between (mitochondrial) BER and mammalian longevity. This suggests that the low steady-state oxidative damage in mitochondrial DNA of long-lived species would be due to both their lower mitochondrial ROS generation and their higher mitochondrial BER. Long-lived mammals do not need to continuously maintain high nuclear BER levels because they release less mitROS to the cytosol. This can be the reason why they tend to show lower nuclear BER values. The higher mitochondrial BER of long-lived mammals contributes to their superior longevity, agrees with the updated version of the mitochondrial free radical theory of aging, and indicates the special relevance of mitochondria and mitROS for aging.

Keywords Mitochondria · DNA repair · AP endonuclease · DNA glycosylases · Aging

Abbreviations

APE1	Apurinic/apyrimidinic endonuclease
BER	Base excision repair
DR	Dietary restriction
mtDNA	Mitochondrial DNA
nDNA	Nuclear DNA
NTHL1	Endonuclease III homolog 1
NEIL2	Nei-like 2
5OHC	5-Hydroxycytosine

R. Gredilla
Department of Physiology, Faculty of Medicine, Complutense University of Madrid (UCM), Madrid, Spain
e-mail: gredilla@ucm.es

I. Sánchez-Román · A. Gómez · M. López-Torres · G. Barja (✉)
Department of Genetics, Physiology and Microbiology, Faculty of Biological Sciences, Complutense University of Madrid (UCM), Jose Antonio Novais s/n, 28040 Madrid, Spain
e-mail: gbarja@bio.ucm.es

I. Sánchez-Román
e-mail: Ines.sanchezroman@universidadeuropea.es

A. Gómez
e-mail: alexiagomezrguez@gmail.com

M. López-Torres
e-mail: mltorres@ucm.es

I. Sánchez-Román
Department of Basic Biomedical Science, Faculty of Biomedical and Health Sciences, Universidad Europea de Madrid, Madrid, Spain

THF	Tetrahydrofuran
ROS	Reactive oxygen species
VDAC1	Voltage-dependent anion-selective channel 1

Introduction

The prevalence of many age-related degenerative diseases has increased strongly during the last decades. Identifying the main mechanisms contributing to determine species longevity is critical to find new approaches to decrease the aging rate. Longevity varies up to 1.4-fold between individuals of the same species in dietary restricted (DR) or long-lived mutant rodents but can differ up to two orders of magnitude between different mammalian species. Investigating the causes of such huge variability is of paramount relevance to identify factors controlling the aging rate. Although correlation does not imply causation, comparative studies have been commonly used in biogerontology. A main key requisite for accepting any theory of aging is that it must be able to explain why different animals age at so different rates. The comparative approach can save a lot of research time and resources by easily discarding parameters not related to species longevity and can also suggest new longevity determinants.

There is general agreement that among causes of aging, increases in different forms of damage with age is important. Among types of macromolecular cellular damage, accumulation of DNA damage with age is especially relevant. The steady-state level of DNA damage depends both on the rate of damage generation and on its rate of repair. Thus, a low level of DNA damage could be due to a low rate of DNA damage generation, an increased DNA repair capacity, or both. There is evidence that endogenous oxidative damage generation and intrinsic susceptibility to oxidative damage are lower in long-lived than in short-lived animals. Thus, low rates of mitochondrial reactive oxygen species (ROS) production and low levels of highly unsaturated fatty acids in cellular membranes are traits of long-lived mammals and birds (Barja 2013; Hulbert et al. 2007; Naudi et al. 2013). However, there is less and more controversial information concerning repair of endogenous DNA damage in the main vital tissues.

Different repair pathways of endogenous DNA damage are known, and the activation of each pathway is dependent on the type of DNA damage. Although DNA repair mechanisms are present both in the nuclear and mitochondrial compartments, not all the DNA repair pathways described in the nucleus have been found in mitochondria. Within mitochondria the main known DNA repair pathway is the base excision repair (BER). This mechanism is the major pathway for repairing small DNA modifications caused by alkylation, deamination, or oxidation and includes four distinct sequential steps (Gredilla et al. 2010). Briefly, substrate-specific DNA glycosylases are responsible for recognition and removal of modified bases. They generate an abasic site, which is then processed by an apurinic/apyrimidinic endonuclease (APE1). Similarly to nuclear BER, mitochondrial BER (mtBER) can proceed through two different sub-pathways: short- or long-patch BER. The short-patch BER involves the incorporation of a single nucleotide into the gap by DNA polymerase γ followed by strand ligation by DNA ligase III. The long-patch BER involves the incorporation of several nucleotides, usually two to seven, by DNA polymerase γ followed by cleavage of the resulting 5′ flap by accessory proteins such as FEN-1 and ligation by DNA ligase III (Liu and Demple 2010).

Various BER activities in nuclear fractions have been compared between different mammalian species (Page and Stuart 2012). The reported results showed lack of correlation with longevity or, more frequently, lower BER enzyme activities in tissues of long-lived mammals when compared to short-lived ones. In addition, when nuclear BER activities were studied in tissues of DR rats, lower levels or no differences were observed in restricted compared to ad libitum-fed animals, despite the superior longevity of the former ones (Stuart et al. 2004). These results might indicate that nuclear BER does not play a key role in longevity extension during dietary restriction in mammals. Otherwise higher nuclear BER activities would have been expected in DR animals.

Mitochondria and mitochondrial DNA (mtDNA) seem to be important for aging (Barja 2013; Gonzalez-Freire et al. 2015; Lopez-Lluch et al. 2015; Miwa et al. 2014; Picca et al. 2018; Zsurka et al. 2018), but the repair of mtDNA has never been studied in animal species that differ in longevity. We hypothesize that BER activities are positively correlated with longevity in mitochondria, in strong contrast with the rather negative correlation with longevity observed in the nucleus. In the present investigation, we have studied for the first time different BER enzyme activities and enzyme

protein levels in liver and heart mitochondrial fractions of up to eight mammalian species showing up to 13-fold difference in (maximum) longevity. We measured two major DNA repair activities, the recognition and cleavage of the oxidative lesion 5-hydroxycytosine, and the processing of abasic sites. We have also investigated the protein levels of BER enzymes involved in those repair activities in mitochondrial and total extracts of the heart and liver from two species with low or high longevity among the selected ones. Our results suggest that mtBER likely contributes to longevity determination in mammals. This is the first study reporting higher repair activities of endogenous DNA damage in long-lived mammalian species.

Materials and methods

Animals and organ samples

Selection of species was based on the availability and reliability of maximum longevity records and on the availability of live healthy animals in order to obtain fresh tissue samples just after death. Male mice, rats, guinea pigs, gerbils, and rabbits were killed at the laboratory by decapitation. Male pigs, cows, and horses were killed at the abattoir by standard approved methods. The mean age of the animals used was 4 months (mouse), 4 months (rat), 5 months (gerbil), 1.4 years (guinea pig), 1.5 years (rabbit), 1 year (pig), and 1.5–2.0 years (cow and horse). Selection of these ages was performed to compare among adult or juvenile animals of analogous biological age. The maximum longevities of the selected species vary by 13-fold: mouse (*Mus musculus*, 3.5 years), rat (*Rattus norvegicus*, 4 years), gerbil (*Meriones unguiculatus*, 6.3 years), rabbit (*Oryctolagus cuniculus*, 9 years), guinea pig (*Cavia porcellus*, 12 years), pig (*Sus scrofa*, 27 years), cow (*Bos taurus*, 30 years), and horse (*Equus caballus*, 46 years). All the animals were in good health according to routine veterinary controls at the abattoir, and none was obese or scraggy. Heart samples were taken from ventricles and liver samples from a main hepatic lobe. The tissue samples were obtained in all species at similar time after death (around 2–5 min); they were cut into small pieces, immediately frozen in liquid nitrogen, and transferred before 2 h to a − 80 °C freezer for storage. Five different individual animals were analyzed per species unless otherwise specified.

Isolation of mitochondrial fractions

Mitochondrial fractions were isolated from the heart and liver of different species using standard procedures from the literature (Gredilla and Stevnsner 2012) that have been optimized in our laboratory (Leclere et al. 2013; Torregrosa-Munumer et al. 2016). Briefly, hearts and livers were homogenized in MSHE buffer (210 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM EDTA; pH 7.4). In order to obtain mitochondrial fractions free of nuclear contamination, homogenization was performed in the presence of the protease subtilisin A (2 U/ml). After homogenization, the protease was removed by centrifugation at 8500 g for 8 min. The supernatant was discarded, and the pellet was resuspended in MSHE buffer. All subsequent steps were performed in the presence of protease inhibitors (protease inhibitor cocktail, Sigma). After centrifugation at 500 g for 10 min, the pellet was discarded, and the mitochondria were spun down by centrifugation of the supernatant at 10,000 g for 9 min. The mitochondrial pellet was resuspended in MSHE buffer, and the final mitochondrial fraction was obtained after centrifugation at 8500 g for 9 min. All steps were carried out at 4 °C. The final mitochondrial fractions were resuspended in Mitobuffer (20 mM HEPES, 1 mM EDTA, 2 mM DTT, 5% glycerol; pH 7.4), aliquoted, and stored at − 80 °C until use.

Oligonucleotides

Oligonucleotides containing a tetrahydrofuran (THF) residue, an abasic analog [5′- ATA TAC CGC GC(THF) CGG CCGATC AAG CTT ATT- 3′], or a 5-hydroxycytosine (5OHC) residue [5′- ATA TAC CGC G(5OHC)G CGG CCG ATC AAG CTT ATT- 3′] were purchased from DNA technology (Aarhus, Denmark) and were 5′-end-labeled using T4 polynucleotide kinase (PNK) and γ-^{32}P-ATP (PerkinElmer). Mixtures of 100 μg oligonucleotide containing a specific DNA lesion or no damage, 20 units of T4 PNK (Fermentas), PNK forward buffer A (Fermentas), and 333 μCi γ-^{32}P-ATP were incubated for 90 min at 37 °C and then stopped with 2 μl 0.5 M EDTA. The unincorporated γ-^{32}P-ATP was removed by binding to G25 Microspin columns (GE Healthcare, Amersham). Duplexing of the 5′-end-labeled oligonucleotide with the corresponding complementary strand was carried out by heating at 90 °C followed by gradual cooling to room temperature.

DNA glycosylase and abasic site processing activities in heart and liver mitochondrial fractions

The mitochondrial recognition and cleavage of 5OHC and abasic site processing are mainly, but not exclusively, related to NTHL1 and NEIL activities and APE1 activity, respectively. These activities were determined in vitro in heart and liver mitochondria by incision assays of 5OHC and THF essentially as previously described (Gredilla and Stevnsner 2012). After permeabilization in the presence of 0.05% Triton X-100 and 0.3 M KCl, mitochondria were incubated for 1 h or 30 min at 37 °C with 180 fmol of a duplexed ^{32}P-labeled 30-mer oligonucleotide containing 5OHC or a THF residue, respectively. Reactions (20 μl) contained 20 mM HEPES, 5 mM EDTA, 75 mM KCl, 1 mM $MgCl_2$, 5% glycerol, 5 mM DTT, and 0.1 mg/ml BSA. The amount of mitochondrial protein added in the reaction varied depending on the tissue and oligonucleotide used. Thus, 200 ng (heart) or 75 ng (liver) of mitochondrial extracts were added to investigate APE1 activity, while 2 μg of both heart and liver mitochondrial fractions were used for DNA glycosylase activity. Reactions were stopped by addition of 0.4% SDS and 0.2 μg/μl proteinase K, followed by 30 min of incubation at 55 °C. Samples were mixed with 20 μl of formamide loading buffer (80% formamide, 10 mM EDTA, 1 mg/ml xylene cyanol FF, and 1 mg/ml bromophenol blue) heated to 90 °C for 5 min and loaded on a denaturing 20% polyacrylamide gel. The radioactively labeled DNA was visualized and quantified using Gene Snap software (Syngene). Mitochondrial DNA glycosylase and APE1 activities were calculated as the amount of radioactivity in the product lane relative to the total radioactivity in the lane as described elsewhere (Gredilla and Stevnsner 2012).

Western blotting

Absence of nuclear contamination in the mitochondrial samples was confirmed by western blot analysis. Mitochondrial and total samples (60 μg protein) of the different species were separated on 4–15% Mini-PROTEAN® TGXTM gels (Bio-Rad) and transferred to PVDF membranes (Millipore). The absence of the cytosolic protein tubulin was assayed using a polyclonal mouse antibody (1:10000 dilution; Sigma), and the mitochondrial presence was assayed by detection of VDAC-1 (polyclonal rabbit antibody, 1:200 dilution; Abcam). The total and mitochondrial amounts of specific proteins involved in BER were compared in short-lived and long-lived species. NTHL1 was detected by using a polyclonal rabbit antibody (1:250 dilution; Santa Cruz Biotechnology), and NEIL2 was detected by using a monoclonal rabbit antibody (1:5000 dilution; Abcam). On the other hand, APE1 was detected by using a monoclonal mouse antibody (1:2000 dilution; Novus biologicals). Secondary anti-rabbit or anti-mouse antibodies were applied at different dilutions depending on the primary antibody. Proteins on the membranes were visualized using ECL plus® (GE Healthcare, Amersham) and quantified using Gene Snap software (Syngene).

Statistics

All results are described as mean ± standard error of the mean (SEM) using samples from at least five independent individual animals unless otherwise indicated. The association between maximum life-span potential and BER activities was analyzed by linear regression using GraphPad Software Inc. Comparisons of parameters between paired species were statistically analyzed by Student's t test. The predetermined minimum significance level was $p < 0.05$.

Results

When measuring parameters in tissue fractions like mitochondria, the purity of the mitochondrial fractions must be confirmed to validate the results. We observed that VDAC1, the most abundant mitochondrial marker protein on the outer mitochondrial membrane, was present in mitochondrial samples of all the different species used at levels much higher than in total samples (Fig. 1a and b). Conversely, α-tubulin, the main protein of microtubules, a major component of cytoskeleton, was present in total but not in mitochondrial samples from all the animal species (Fig. 1a and b) confirming that the mitochondrial fractions were free of extramitochondrial protein contamination.

To our knowledge, comparative studies on mtBER in mammalian species with different longevities have not been reported. We first investigated the dynamic of both 5OHC and THF incision activities in mitochondrial fractions from all the species, determining optimal extract concentration for each assay and tissue (Fig. 1c–f).

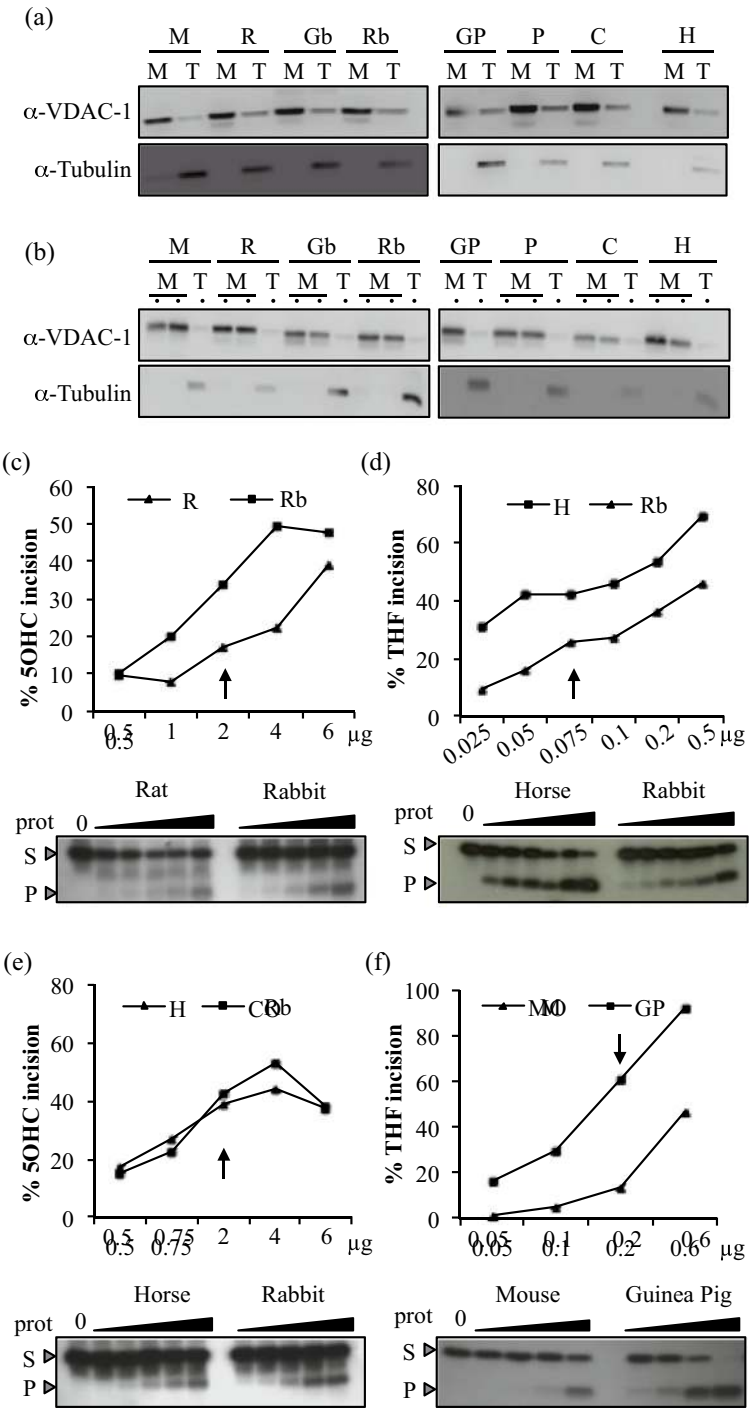

Fig. 1 Confirmation of mitochondrial purity and setting of incision assay conditions. Presence of VDAC-1 and tubulin was assayed by western blot in liver (**a**) and heart (**b**) mitochondrial and total tissue fractions of the mammalian species used in this investigation; representative blot images are shown. 5-Hydroxycytosine (5OHC) and THF incision assays in liver (**c** and **d**) and heart (**e** and **f**) mitochondrial fractions were performed with increasing amounts of mitochondrial protein in all species in order to determine the precise amount to be used in the final incision assays; bottom panels show representative gels in two illustrative species; top graphics show the quantification of the representative gels. The arrow indicates the amount of extract chosen. *M* mouse, *R* rat, *Gb* gerbil, *Rb* rabbit, *GP* guinea pig, *P* pig, *C* cow, *H* horse, *Mt* mitochondrial fraction, *T* total fraction, *S* substrate, *P* product

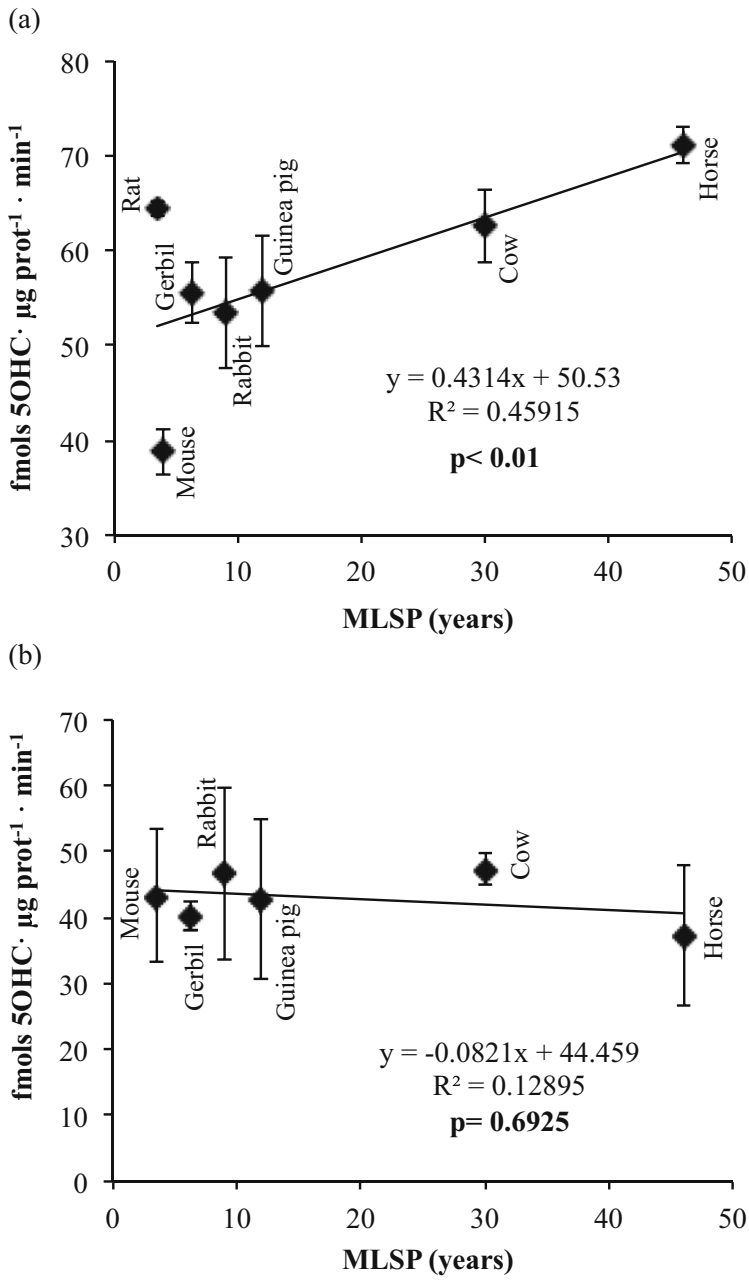

Fig. 2 Incision of 5-hydroxycytosine containing oligo in liver (**a**) and heart (**b**) mitochondrial fractions of mammalian species with different longevities. Graphics represent mean ± SEM of duplicate measurements from five independent fractions except horse liver mitochondria and guinea pig and pig heart mitochondria ($n = 4$)

Hence, we ensured that repair activities were not saturated under those experimental conditions.

Recognition and cleavage of 5OHC in liver mitochondria was positively correlated with longevity in the mammalian species studied ($p < 0.002$; Fig. 2a). In the case of heart mitochondria, no correlation was detected ($p = 0.692$), and a high variability of data around the mean values was observed in four of the six species studied (Fig. 2b).

The incision of a THF-containing oligonucleotide was positively correlated with longevity across mammalian species with different longevities both in liver ($p < 0.0001$; Fig. 3a) and heart ($p < 0.0001$; Fig. 3b) mitochondrial fractions.

We then compared the protein levels of selected BER components between two species, one showing low (rat) and one showing high (cow or horse) longevities. Cow or horse was selected as most long-lived species in

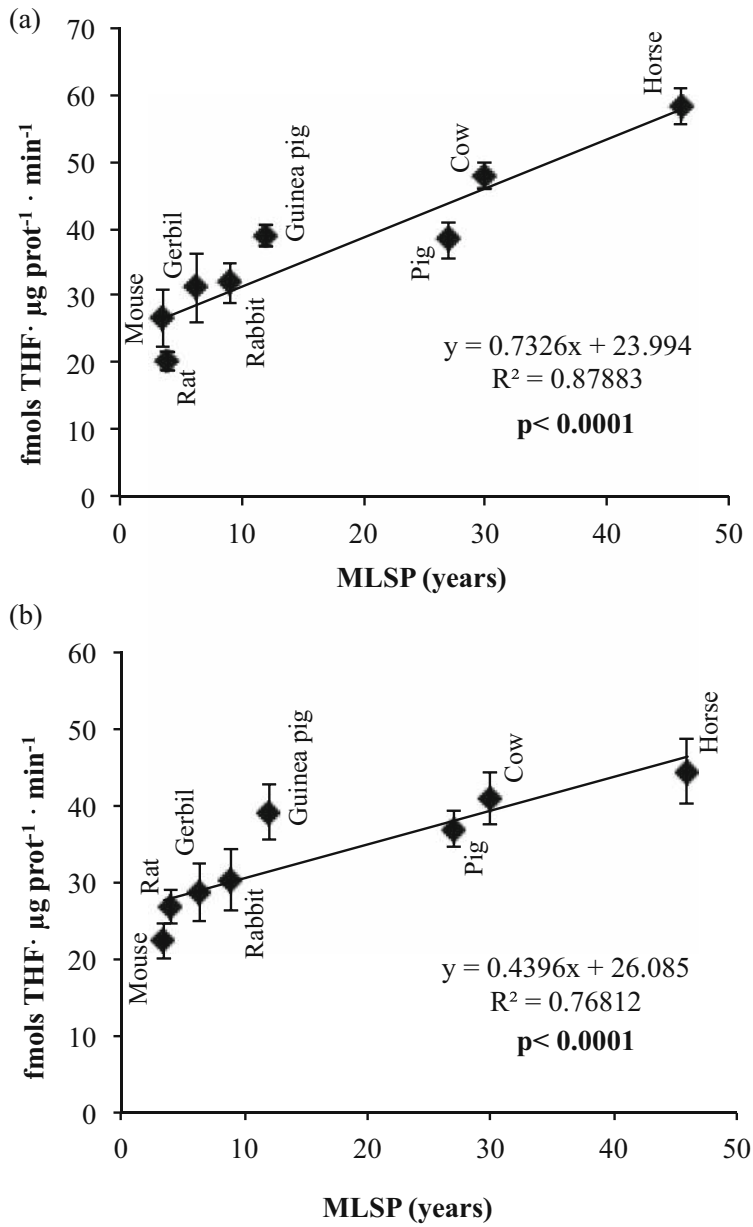

Fig. 3 Incision of THF-containing oligo in liver (**a**) and heart (**b**) mitochondrial fractions of mammalian species with different longevities. Graphics represent mean ± SEM of duplicate measurements from five independent fractions except rat and mouse liver mitochondria and guinea pig and gerbil heart mitochondria ($n = 4$)

different comparisons depending on the antibody sensitivity observed. We analyzed the levels of two DNA glycosylases involved in 5OHC processing, NEIL2 and NTHL1, as well as APE1, which is involved in the processing of abasic sites. Mitochondrial NEIL2 was higher in horse than in rat liver (Fig. 4a) and heart (Fig. 4c), although it only reached statistically significance in hepatic mitochondria ($p < 0.05$). On the other hand, mitochondrial APE1 levels were significantly higher in cow than in rat samples both in liver (Fig. 4b $p < 0.05$) and heart (Fig. 4d; $p < 0.001$) mitochondria. Similar changes were also observed when we investigated the levels of NTHL1 and APE1 in total hepatic and cardiac fractions (Table 1).

Discussion

Previous studies have found no correlation or negative correlation of different nuclear BER activities with

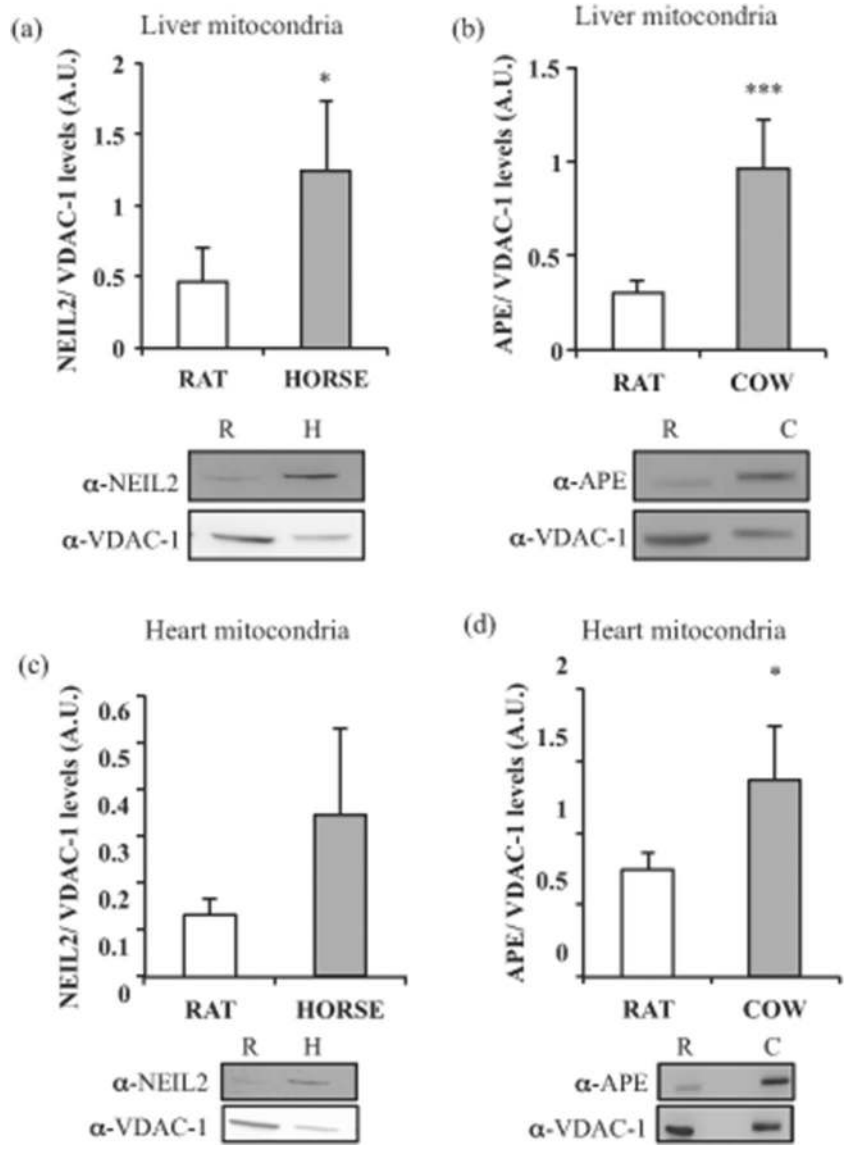

Fig. 4 Protein levels of mitochondrial NEIL2 (NEIL2/VDAC-1) and mitochondrial APE1 (APE/VDAC-1) in liver (**a**, **b**) and heart (**c**, **d**) of short- and long-lived species. Bottom panels show representative blots. Bars represent mean ± SEM of duplicate measurements from 4 independent samples. *R* rat, *C* cow, *H* horse. Asterisks denotes significant differences; $*p \leq 0.05$, $***p \leq 0.001$

Table 1 Protein levels of NTHL1 and APE1 in total fractions of the liver and heart from rat and cow

		Liver	Heart
NTHL1	Rat	0.29 ± 0.07	0.80 ± 0.08
	Cow	2.12 ± 0.49***	0.49 ± 0.07*
APE1	Rat	0.61 ± 0.03	0.91 ± 0.10
	Cow	1.92 ± 0.45***	1.72 ± 0.18**

Total fractions of the liver and heart from rat and cow were analyzed by western blot in order to investigate total levels of NTHL1 and APE1. Results are means ± SEM from 4 independent samples (arbitrary units; normalized by tubulin levels). Asterisks denotes significant differences between species in the same tissue and enzyme. $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$

longevity in mammalian tissues. That kind of result is contradictory with a potential role for nuclear DNA repair in determination of mammalian longevity. In our study, both the activities and protein levels of BER enzymes were studied for the first time in mitochondria from tissues of mammals with widely different longevities. In strong contrast with nuclear BER, mitochondrial BER activities and protein levels generally showed a highly significant positive correlation with mammalian longevity both in the liver and heart. Together with the known negative correlation of both mitochondrial ROS production and oxidative damage to mtDNA with mammalian longevity (Barja and Herrero 2000), these results

agree with predictions from an updated mitochondrial free radical theory of aging (Barja 2013) and point to a high relevance of mitochondria and ROS in the determination of species longevity (Barja 2013; Lopez-Lluch et al. 2015; Miwa et al. 2014; Picca et al. 2018; Zsurka et al. 2018).

The mainstream view is that aging has as multifactorial origin. Accumulation of somatic DNA damage and increased genetic instability with age occurs in rodents, humans, and other mammals (Macedo et al. 2018; Moskalev et al. 2013), likely contributing to aging of tissue cells and organisms. DNA damage is diverse, including oxidation, alkylation, and glycation, DNA adducts, cross-linkages, and single- and double-strand breaks. Different reports suggest that the increase in DNA damage with age could be partly due to decreases in many forms of DNA repair during aging (Gorbunova et al. 2007; Gredilla et al. 2010; Moskalev et al. 2013). However, the contribution of DNA repair mechanisms to the determination of species aging rate and longevity is far from clear. Three major approaches have attempted to analyze the relation between longevity and DNA repair: transgenic animals for DNA repair genes, DR animals – which show superior longevity – and interspecies comparisons. Up to now these investigations, which have been mainly conducted on nDNA, have yielded results inconsistent with a role for DNA repair in longevity.

Nuclear BER deficiency caused by polymerase ß haploinsufficiency increases nDNA damage including oxidative damage, DNA single-strand breaks, and chromosomal aberrations and raises dimethyl sulfate mutagenicity in mouse tissues (Cabelof et al. 2003a). However, overexpression of nuclear genes involved in recognition of DNA damage, BER and nuclear excision repair (NER) genes, and genes involved in repair of strand breaks in *Drosophila melanogaster* increases or decreases longevity depending on sex, developmental stage, the conditional or constitutive, and the ubiquitous or limited to the nervous system character of the overexpression (Shaposhnikov et al. 2015). Notably, overexpression limited to adults or constitutive in the brain decreased longevity instead of increasing it. Also, heterozygous nuclear APE1 ± mice expressing half APE1 activity had higher mutation frequencies (Huamani et al. 2004) and were more susceptible to ROS-induced damage, but longevity was unaffected (Meira et al. 2001). Similarly, heterozygous nuclear polymerase ß ± mice had around 50% less polymerase ß protein levels and activity in various tissues but unchanged longevity (Cabelof et al. 2006).

During the last decades, comparative studies have been important to investigate the potential determinants of aging rate and longevity, including DNA repair mechanisms. Various studies performed in the 1970s and 1980s of the last century analyzed the NER capacity in relation with longevity in mammals. These studies found positive correlation with longevity for global genome NER in mammalian fibroblasts (Cortopassi and Wang 1996; Francis et al. 1981; Hall et al. 1984; Hart and Setlow 1974; Treton and Courtois 1982), suggesting that DNA repair substantially contributes to species longevity. Since those investigations explored the rate of DNA synthesis in skin fibroblasts after subjecting the cells to UV radiation, they analyzed the capacity for repair of DNA damage from *exogenous* origin. Double-strand break recognition positively correlates with species longevity in skin fibroblasts as well (Lorenzini et al. 2009). Considering that long-lived species are more exposed to exogenous sources of DNA damage, such as UV radiation, a higher capacity for repairing those sorts of DNA lesions would be expected from evolutionary adaptation. It would not make sense to create a species that can live, e.g., 50 years, if due to a lack of enough defenses against *exogenous* UV radiation it could not live more than a decade or less. Therefore, although long-lived species do have more repair of DNA damage induced by exogenous radiation in skin fibroblast ("unscheduled DNA synthesis", reviewed in Cortopassi and Wang 1996), this does not mean that their slower *endogenous* aging rate is due to that kind of higher DNA repair capacity. In order to have a more clear picture of the role DNA repair plays in the determination of the aging rate, it is most important to investigate the DNA repair capacity of the *endogenously* generated DNA damage.

Poly(ADP-ribose) polymerase activity in leukocytes is positively correlated with species longevity in mammals (Grube and Burkle 1992). Among repair forms *of endogenous* DNA damage in tissues, BER is the primary pathway that eliminates small DNA modifications caused by alkylation, deamination, or oxidation. The role of BER in aging as well as in age-related diseases has been extensively investigated (reviewed in Gredilla et al. (2012); Hou et al. (2017); Jeppesen et al. (2011); Zarate et al. (2017)). Different studies have reported a decline in BER with aging in different tissues, although such decline is especially significant in the brain

(reviewed in Gredilla et al. (2017)). Interestingly, age-related neurodegenerative disorders have been associated with a decline in BER capacity (Canugovi et al. 2014; Iida et al. 2002; Soltys et al. 2019; Sykora et al. 2015; Weissman et al. 2007). The role that BER plays in lifespan extension when animals are subjected to DR or in the lower aging rate of long-lived species has also been investigated. Regarding DR studies, mixed results have been reported.

It has been described that DR increases G/U mismatch repair and both activity and protein levels of polymerase ß in tissues of Fisher 344 rats (Cabelof et al. 2003b). However, DR in C57BL6 mice tissues (kidney, heart, brain, and liver) led in most cases to decreases or lack of significant changes in total BER capacity as well as in individual steps of the pathway including NTHL1, APE1, and polymerase γ in the brain and kidney, while 8-oxodG and uracil incision were increased by DR in liver and kidney mitochondria, respectively (Stuart et al. 2004). In another study, total APE1 activity increased after DR in five brain rat regions, while its protein levels or those of polymerase ß and DNA ligase III did not change (Kisby et al. 2010). In contrast, DR-induced increases in polymerase ß activity and protein amount but not on T4 DNA ligase or primer extension activity in rat cortical neurons have been described (Swain et al. 2016).

The studies analyzing the role of BER in the low aging rate of long-lived animals are scarce. In an extensive investigation, APE1 and polymerase ß activities were measured in liver and brain nuclear fractions of 13 mammals and 2 birds (Page and Stuart 2012), and none of these activities positively correlated with species longevity. Rather, these activities generally showed negative correlation with longevity although statistical significance was not reached in various cases. It is important to stress that all those studies were performed in *nuclear* fractions and no equivalent studies in mitochondrial fractions are available.

Mitochondria have been related to aging and longevity in a multitude of studies using many different approaches. Therefore, we considered important to test whether BER activities in mitochondria, in contrast to nuclear fractions, could be correlated with species longevity. The results of the present investigation generally support this hypothesis. In our study we investigated different BER components in liver and heart mitochondrial fractions of up to 8 mammals with 13-fold different longevities, measuring both activities and protein levels. Liver and heart mitochondrial APE1 activity and liver mitochondrial 5OHC incision showed highly significant positive correlation with mammalian longevity. The lack of significant correlation for heart mitochondrial 5OHC incision could be due to the high variability of the data observed.

In order to investigate whether the changes observed in mtBER activities among species could be related to differences in BER protein levels, we measured the amount of various BER components in total and mitochondrial extracts in species with extremely low (rat) or high (cow or horse) longevities. We analyzed two of the DNA glycosylases involved in the removal of 5OHC, NTHL1, and NEIL2 as well as the protein levels of APE1. Globally, the results observed at protein level matched those obtained for mitochondrial BER activities, suggesting that the higher mitochondrial 5OHC and THF incision activity in long-lived species is likely due to their higher levels of mtBER proteins. Interestingly, the fact that other BER proteins like polymerase γ also tended to show higher levels in horse than in rat in heart mitochondria (data not shown) suggests that such an increase in mitochondrial BER protein levels in long-lived animals might be general.

Our study has limitations concerning the selection of the species studied. Although mammalian species are of utmost relevance for extrapolation to human aging, knowledge on what is the situation concerning mtBER in other animal groups is also relevant, especially to more definitely test the proposed hypothesis from an evolutionary biology perspective. Concerning mammals, the inclusion in our study of rodents like rats and mice that have undergone decades of laboratory evolution may or may not have affected the results obtained. Future studies should optimally include other mammals like carnivores and mammalian species with exceptional longevity like squirrels, naked mole rats, or primates including human tissues.

Similarly to BER activities, various studies have shown that antioxidant enzyme activities follow the same tendency. Thus, total tissue antioxidant enzyme activities (CuZn SOD, catalase, glutathione peroxidase, and reductase) generally *negatively* correlate (or sometimes do not correlate) with longevity in mammals and vertebrates in general (Page et al. 2010; Pamplona and Costantini 2011; Perez-Campo et al. 1998). However, both the activity (Page et al. 2010) and the amount (Brown and Stuart 2007) of the mitochondrial form of superoxide dismutase,

MnSOD, *positively* correlated with mammalian longevity, similarly to what we report in the current study for mtBER. Other studies also point to an especially important role of mitochondrial compared to total cell tissue antioxidants concerning longevity (Schriner et al. 2005; Munro and Baldy 2019; Munro and Pamenter 2019).

The higher mitochondrial BER of long-lived mammals can contribute to their superior longevity. In mitochondria, the combination of low rates of mitROS generation, high rates of BER, and perhaps high antioxidant levels described at least for MnSOD would all contribute to produce a low level of mtDNA steady-state damage and a low mitochondrial damage in general in long-lived species. These mitochondrial parameters, taken together, can help to explain the large quantitative differences in aging rate and longevity among different species. Other aging effectors, such as autophagy, membrane fatty acid unsaturation, telomere shortening rate, apoptosis, or inflammaging, might contribute as well. Our results are consistent with both the updated mitochondrial free radical theory of aging (Barja 2013), and with the increasingly held view that aging is regulated at a different level in each species by a cellular aging program most likely lying on the cell nucleus which acts through different effector mechanisms (Barja 2019). The finding of a higher repair of endogenous DNA damage observed in mitochondria but not in the cell in general in long-lived animals supports the updated version of the mitochondrial free radical theory of aging and highlights again the importance of mitochondria in the determination of aging rate and species longevity.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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