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Disparate patterns of age-related changes in lipid peroxidation in long-lived naked mole-rats and shorter-lived mice

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Summary

A key tenet of the oxidative stress theory of aging is that levels of accrued oxidative damage increase with age. Differences in damage generation and accumulation therefore may underlie the natural variation in species longevity. We compared age-related profiles of whole-organism lipid peroxidation (urinary isoprostanes) and liver lipid damage (malondialdehyde) in long living naked mole-rats [maximum lifespan (MLS) > 28.3 years] and shorter-living CB6F1 hybrid mice (MLS ~3.5 years). In addition, we compared age-associated changes in liver non-heme iron to assess how intracellular conditions, which may modulate oxidative processes, are affected by aging. Surprisingly, even at a young age, concentrations of both markers of lipid peroxidation, as well as of iron, were at least twofold ($P < 0.005$) greater in naked mole tats than in mice. This refutes the hypothesis that prolonged naked mole-rat longevity is due to superior protection against oxidative stress. The age-related profiles of all three parameters were distinctly species specific. Rates of lipid damage generation in mice were maintained throughout adulthood, while accrued damage in old animals was twice that of young mice. In naked mole-rats, urinary isoprostane excretion declined by half with age ($P < 0.001$), despite increases in tissue iron ($P < 0.05$). Contrary to the predictions of the oxidative stress theory, lipid damage levels did not change with age in mole-rats. These data suggest that the patterns of age-related changes in levels of markers of oxidative stress are species specific, and that the pronounced longevity of naked mole-rats is independent of oxidative stress parameters.

Key words: aging; isoprostanes; lipid peroxidation; longevity; malondialdehyde; naked mole-rats; non-heme iron.

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Introduction

The oxidative stress theory of aging states that the declines in organismal function that characterize the aging process result from a progressive accrual of oxidative damage to cellular constituents (Harman, 1956, 1998). Reactive oxygen species (ROS) produced as a by-product of normal aerobic metabolism continuously damage cellular nucleic acids, proteins and membrane lipids (Beckman & Ames, 1998). Many of the products of lipid peroxidation are themselves very potent ROS. Unless these are neutralized by antioxidants, they can induce considerable self-propagating oxidative damage (Halliwell & Chirico, 1993). Accumulation of damage during aging thus reflects a chronic imbalance between rates of ROS generation and the organism's capacity to neutralize ROS and/or repair any ROS-induced damage (Beckman & Ames, 1998). Organismal antioxidant defenses scavenge and neutralize ROS, while repair mechanisms remove damaged biological macromolecules preventing their accrual in tissues (Finkel & Holbrook, 2000). Within this context, natural variation in species longevity is thought to reflect disparate rates of aging caused by unequal oxidative damage accumulation (Sohal & Weindruch, 1996).

Lipids are important, ubiquitous components of biological membranes and their modification by oxidative processes can affect membrane fluidity and permeability properties with concomitant widespread aging effects (Davies, 2000). Oxidative lipid damage occurs through a sequence of self-propagating autocatalytic peroxidative reactions (Halliwell & Chirico, 1993). Phospholipids in the more metabolically active tissues (such as the liver), and in particular those lipids that comprise mitochondrial membranes that are thus close to sites of ROS generation are particularly at risk of oxidative stress. Susceptibility of membranes to lipid peroxidation also depends upon phospholipid composition; membranes higher in polyunsaturated fatty acids (PUFA) and specifically, n-3 PUFAs, such as docosahexanoic acid, are especially vulnerable to oxidative stress (Hulbert, 2005). Membrane composition reportedly is altered with caloric restriction (Faulks *et al*., 2006), and this may contribute to the observed attenuation of lipid damage accrual in response to caloric restriction (Cook & Yu 1998; Ward *et al*., 2005).

The cellular environment also can affect rates of lipid peroxidation, and thus modulate levels of damage accrual during aging. Transition metals, such as iron, initiate Fenton-type reactions leading to the formation of highly reactive hydroxyl and alkoxy radicals (Marzabadi *et al*., 1988; Spiteller, 2001). As such, age-associated changes in intracellular iron concentrations may affect rates of oxidative processes and thus damage accumulation

during an organism's life. Indeed, iron concentrations reportedly increase with age (Sohal *et al*., 1999), and these age-related changes correlate with elevated oxidative damage levels during aging in certain mouse strains (Massie *et al*., 1983) and rats (Cook & Yu, 1998). Studies on age-associated changes in accrued lipid damage have been equivocal, with results contingent on the species or tissues assayed (Palomero *et al*., 2001; Sverko *et al*., 2002; Oxenkrug & Requintina, 2003). This lack of consensus in the literature has led some to conclude that progressive accrual of oxidative lipid damage is not an integral component of organismal aging (Rikans & Hornbrook, 1997).

While data on age-related changes in oxidative lipid damage have been inconsistent, comparative studies have reported an inverse relationship between oxidative lipid damage and longevity. Specifically, long-living birds reportedly accrue less oxidative damage than similar-sized but shorter-living mammals (Pamplona *et al*., 1999), whereas among mammals, species with extended longevity generate and accumulate less lipid damage than those with shorter lifespans (Pamplona *et al*., 2000). These patterns have been ascribed to lower peroxidation indices in long-living species, reflecting relatively lower proportions of membrane n-3 PUFAs than in shorter-living animals (Pamplona *et al*., 1998; Hulbert, 2005). Similarly, the naked mole-rat, an exceptionally long-living mouse-sized rodent has membranes with a low peroxidation index (Hulbert *et al*., 2006), and we asked if a comparative study assessing age-related changes in lipid peroxidation in this rodent may provide useful insights into this controversy.

Naked mole-rats (*Heterocephalus glaber*, Bathyergidae) are subterranean hystricognath rodents that can survive to more than 28 years in captivity (Buffenstein & Jarvis, 2002). This longevity record belonged to a male caught in the wild as a subadult (~1-year-old), that then spent more than 27.5 years in captivity under standard laboratory conditions. This extraordinary lifespan is all the more impressive since laboratory atmospheric conditions are presumed to be non-physiological, and present a hyperoxic environment for subterranean rodents (Buffenstein, 2000). Naked mole-rats live approximately an order of magnitude longer than expected for a 35-g mammal, based upon the allometric equations of Prothero & Jurgens (1987). The longevity quotient (ratio of actual lifespan to that predicted based on body mass) of naked mole-rats is similar to that observed in humans and exceeded only by that reported for certain bat species (Austad & Fischer, 1991). The impressive longevity of naked mole-rats is accompanied by the absence of age-associated physiological modifications, commonly observed in other mammalian models of aging research (O'Connor *et al*., 2002), as well as the maintenance of reproductive capacity into the third decade of life (Buffenstein, 2005). Collectively, these organismal data suggest that aging is delayed, and rates of aging attenuated in naked mole-rats. As such, this long-living species may be a useful animal model with which to assess mechanisms involved in resisting aging.

Although physiological function is maintained by naked mole-rats throughout life, these long-living rodents do not possess extraordinary biochemical defenses against oxidative stress, and their antioxidant suite is not superior to shorter-living mice (Andziak *et al*., 2005). Naked mole-rats have surprisingly very low cellular glutathione peroxidase activity, without significant compensatory up-regulation of other enzymatic antioxidants (Andziak *et al*., 2005). The near absence of this important hydrogen peroxide (H_2O_2) neutralizing enzyme (Chiu *et al*., 1976) raises the possibility that mole-rats are particularly susceptible to oxidative processes, and acutely sensitive to age-related changes of factors that can modulate these processes, for example intracellular iron levels.

In the present study we compared patterns of damage generation (urinary isoprostanes) and tissue accrual (malondialdehyde) during aging in similar-sized mice (CB6F1 hybrid ~3.5 years) and mole-rats (> 28.3 years) that show an eight-fold difference in maximum longevity. In addition, we evaluated age-related changes in tissue non-heme iron levels in these two species to establish if differences in the status of the intracellular environment correlate with observed patterns of oxidative damage generation and accrual. We hypothesized that naked mole-rats would have lower levels of lipid damage generation than shorter-lived mice at physiologically equivalent ages (see Experimental procedures), and that damage would accrue at a slower rate in the longer-living species. Furthermore, we predicted that intracellular non-heme iron would be lower in naked mole-rats than in mice, reflecting a cellular environment less conducive to oxidative stress.

Results

Urinary isoprostanes

We compared isoprostane concentrations in urine samples from known age mice and mole-rats. Urinary isoprostanes of mice and naked mole-rats showed neither a significant effect of age, nor an interaction effect of age and species (Fig. 1). However, levels of this marker of *in vivo* oxidative stress exhibited a

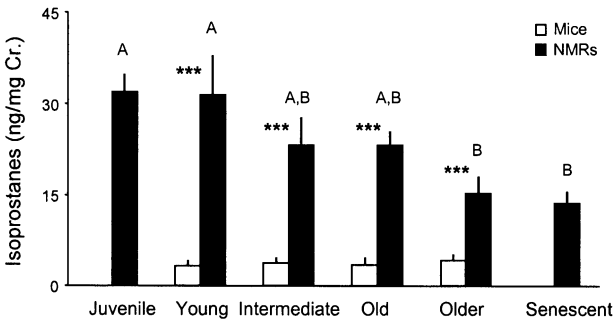

Fig. 1 Urinary isoprostane levels in naked mole-rats (NMR) and mice at different ages. While urinary isoprostane excretion remained constant with age in the four mouse cohorts (4–24 months), there was a pronounced age-related decline across the six NMRs age groups (6–290 months). NMRs had higher isoprostane levels than mice for all four comparisons of physiologically equivalent age groups. Results are means ± SEM. Significant differences between age-matched mice and NMRs: ***$P < 0.001$. A and B: NMR age-cohort means that do not share a common superscript are significantly different.

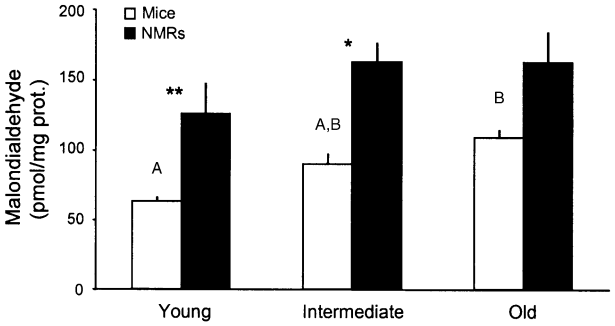

Fig. 2 Levels of accrued malondialdehyde (MDA) in livers of mice and naked mole-rats (NMR) at different ages. MDA increased with age in mice, but remained constant in NMRs. NMRs had higher MDA levels than mice in the two younger age cohorts. Results are means ± SEM. Significant differences between age-matched mice and NMRs: $^{**}P < 0.01$, $^{*}P < 0.05$. A and B: mouse cohort means that do not share a common superscript are significantly different.

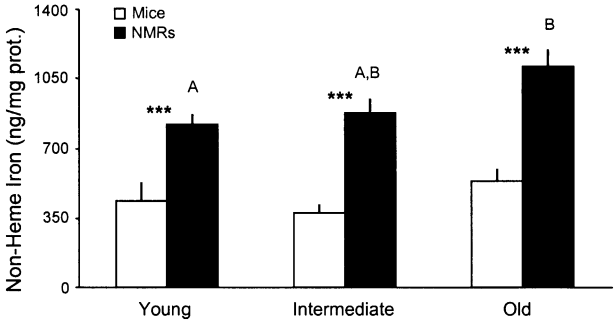

Fig. 3 Liver non-heme iron levels in mice and naked mole-rats (NMR) at different ages. Iron levels did not change with age in mice, but increased in NMRs. NMRs had higher liver levels of non-heme iron than mice for all three age-cohort comparisons. Results are means ± SEM. Significant differences between age-matched mice and NMRs: $^{***}P < 0.001$. A and B: NMR age cohort means that do not share a common superscript are significantly different.

significant species effect ($P < 0.001$). Naked mole-rats had five- to tenfold greater isoprostane levels (23.30 ± 2.16 ng mg^{-1} creatinine, $n = 28$) than did mice (3.64 ± 0.51 ng mg^{-1} creatinine, $n = 24$), for all four age cohort comparisons ($P < 0.005$). Urinary isoprostane values for our young mice were higher than those previously reported (Pratico *et al*., 2001). However, in both that and our study, no age-related changes in urinary isoprostane excretion were evident. This was not the case for naked mole-rats.

Between the ages of 6 months and 290 months naked mole-rat urinary excretion of isoprostanes declined by half ($P < 0.001$; Fig. 1). The two youngest mole-rat cohorts (6 months, 31.98 ± 2.87 ng mg^{-1} creatinine; 30 months, 31.47 ± 6.39 ng mg^{-1} creatinine) had two-fold greater concentrations of isoprostanes than both 180 months (15.29 ± 2.81 ng mg^{-1} creatinine; $P < 0.05$) and 290 months (13.67 ± 1.95 ng mg^{-1} creatinine; $P < 0.01$) animals.

Malondialdehyde (MDA)

We compared age-related changes in lipid damage accumulation in naked mole-rats and mice by measuring MDA levels in liver samples, Fig. 2. Both 30 month old (125.8 ± 21.9 pmol mg^{-1} protein) and 75 month old (161.2 ± 13.9 pmol mg^{-1} protein) mole-rats had significantly more MDA than physiologically age-matched mouse cohorts (4 months, 63.0 ± 3.3 pmol mg^{-1} protein, $P < 0.01$; and 12 months, 90.3 ± 7.4 pmol mg^{-1} protein, $P < 0.05$), leading to a significant species effect ($P < 0.001$). Malondialdehyde values obtained in this study for mice were similar to those previously reported using the same technique in CBA and AKR mouse strains (Sverko *et al*., 2002).

While MDA concentrations were similar in the three mole-rat age groups, lipid damage levels in 18 month old mice (108.9 ± 5.8 pmol mg^{-1} protein) were nearly twice that of the 4 month old cohort ($P < 0.05$), and contributed to a significant age effect on accrued MDA content ($P < 0.01$). There was no significant interaction effect of age and species for this marker.

Rates of accrued damage differed markedly between species. Oxidative damage in mice increased in a linear manner (3.3 pmol mg^{-1} protein per month) from 4 to 18 months of age. In mole-rats, however, accrued damage increased with age from 30 months to 75 months (0.8 pmol mg^{-1} protein per month). Thereafter, between 75 months and 130 months MDA was unchanged.

Non-heme iron

We measured levels of non-heme iron in naked mole-rat and mouse liver samples, in order to compare age-associated changes in cellular conditions that may affect oxidative reactions. While the interaction effect of age and species was not significant in these two species, naked mole-rats had greater non-heme iron levels than mice ($P < 0.001$), for all comparisons among physiologically age-matched cohorts (mole-rats: 30 months, 821 ± 64 ng mg^{-1} protein; 75 months, 877 ± 72 ng mg^{-1} protein; 180 months, 1109 ± 86 ng mg^{-1} protein vs. mice: 4 months, 435 ± 98 ng mg^{-1} protein, $P < 0.001$; 12 months, 378 ± 40 ng mg^{-1} protein, $P < 0.001$; 18 months, 536 ± 64 ng mg^{-1} protein, $P < 0.001$, Fig. 3). Mouse values (175 μg g^{-1} liver) obtained in this study were similar to those previously published for AKR, 129 and C57 mouse strains (Gerhard *et al*., 2002), but were higher than three (C57Bl/6; BALB and DBA) of the data published for four mouse strains used in the Clothier *et al*. (2000) study. There was a significant effect of age ($P < 0.05$) on iron levels, which was primarily due to age-related changes in naked mole-rats; 180 months old mole-rats had higher iron levels than 30-month animals ($P < 0.05$).

Discussion

This study assessed if the extraordinary longevity of naked mole-rats was due to enhanced intracellular protection against oxidative damage and attenuated oxidative damage accrual. Specifically, we asked if mole-rats have similar amounts of lipid damage to mice at physiologically equivalent ages, and if rates

of damage accrual vary between species. Furthermore, we asked if amounts of lipid peroxidation can be attributed to non-heme iron levels, and if these results may explain interspecific differences in longevity. Our comparative data did not correspond to differences in species longevity; young naked mole-rats (i) had higher iron levels contributing to a more stressful intracellular environment, (ii) generated more lipid damage (as indicated by higher levels of urinary isoprostanes), and (iii) accrued more oxidative damage in liver tissue than did shorter-lived mice. Very different species responses were evident during aging. While there was an age-related increase in non-heme iron in naked mole-rats, urinary isoprostane levels of our oldest cohort (290 months) were 50% lower than those of juveniles (6 months), and liver MDA concentrations remained constant between 30 and 130 months. In mice, no age-associated changes in non-heme iron and urinary isoprostane concentrations were apparent. Despite this, however, accrued liver damage doubled with age (4 to18 months) in mice. Oxidative lipid damage generation and accumulation thus follow different species patterns that appear unrelated to both intracellular environmental conditions and interspecific differences in longevity.

In vivo oxidative stress

Urinary isoprostanes are key markers for arachidonic acid peroxidation and are indicative of *in vivo* oxidative stress and organismal oxidative damage generation (Roberts & Morrow, 2000). Higher isoprostane levels in naked mole-rats may reflect a greater proportion of arachidonic acid and other n-6 PUFAs, and lower amounts of n-3 PUFAs in their membranes when compared to those of mice (Hulbert *et al*., 2006). It is plausible that markers representative of n-3 PUFA peroxidation (e.g. neuroprostanes) may reveal divergent species differences to those reported here. Interspecific differences in membrane composition may also contribute to the higher MDA content in mole-rat tissues, since MDA is a by-product of arachidonic acid peroxidation (Esterbauer *et al*., 1991). However, in addition to having greater lipid damage than mice, naked mole-rats also show higher levels of oxidative damage to DNA and proteins in all tissues studied to date (heart, liver, muscle and kidney; Andziak *et al*., 2006). The robustness of these findings, across a broad range of biological molecules implies that the ten-fold interspecies difference in isoprostane levels (as well as the two-fold greater MDA concentrations) in young mole-rats than in mice, are not consistent with disparate availability of arachidonic acid for peroxidative reactions. Instead, our findings more likely point toward higher rates of oxidative damage generation by this long living species.

Higher urinary isoprostane levels in naked mole-rats may reflect the hyperoxic gaseous environment encountered by animals reared in captivity (21%), relative to that found (< 15%) in their natural subterranean sealed burrow milieu (Buffenstein, 2000). Indeed, isoprostanes are reportedly elevated following laboratory-induced hyperoxia in both clinical (Delanty *et al*., 1997) and experimental (Jaensch *et al*., 2001) settings. Newborn and young naked mole-rats, like prenatal, neonatal, and juvenile mice, most likely do not have a fully developed antioxidant defense system (Utsumi *et al*., 1977; Salminen *et al*., 1988; Fantel & Person, 2002) and would be particularly vulnerable to oxidative stress induced by comparatively high oxygen atmospheres. Greater damage accrual during early stages of life also could reflect augmented ROS generation due to high cell proliferation and concomitant elevated metabolic rates of young animals (Fraga *et al*., 1990; Fantel & Person, 2002).

It is possible that the observed higher isoprostane levels of naked mole-rats reflect detection by the assay antibody of substances other than 15-isoprostane F2t. Indeed, isoprostane immunoassays have been criticized for a lack of specificity due to the potential cross reactivity with other isoeicosanoids (Proudfoot *et al*., 1999; Meagher & FitzGerald, 2000). We do not believe this to be the case in this study: (i) Both markers of lipid peroxidation (isoprostane and MDA) show identical trends; (ii) In addition, in a previous study (Andziak *et al*., 2006), in which we examine liver and muscle samples, we obtained similar findings using both the technique employed here, and the 'gold standard' method of gas chromatography/negative-ion chemical ionization/mass spectrometry (GC-NICI-MS). The consistency of these results, regardless of technique, corroborates our urinary isoprostane data and leads us to conclude that *in vivo* lipid peroxidation is elevated in the long-living rodents.

Interstrain mouse comparisons reveal that liver non-heme iron levels of our CB6F1 strain are elevated even at a young age (Clothier *et al*., 2000) and remain constant throughout adult life. Naked mole-rat non-heme iron concentrations are substantially higher than those of the mice used in this study and also considerably higher than most previously published mouse values (Clothier *et al*., 2000; Gerhard *et al*., 2002). High non-heme iron concentrations in naked mole-rats suggest that their cellular milieu is more susceptible to peroxidative reactions and damage accrual. Indeed, in certain mouse strains hepatic non-heme iron levels are reportedly positively correlated with lipid peroxidation (Gerhard *et al*., 2002), as well as with an increased susceptibility to oxidative stress (Clothier *et al*., 2000). In addition, feeding treatments that induce dietary iron overload can lead to significant increases in oxidative lipid damage (Stimson & Fischer, 1997; Valerio & Petersen, 1998), whereas energy restriction results in both attenuated iron levels and oxidative damage generation in mice (Sohal *et al*., 1999) and rats (Cook & Yu, 1998).

The high iron content of naked mole-rat tissues may be an evolved trait associated with underground life in the lateritic soils of Northeast Africa. Naked mole-rats dig through these iron-rich soils (Brett, 1991) using their chisel-like incisors. This may very likely result in the ingestion of considerable amounts of iron. Fossil evidence reveals that naked mole-rats have led a strictly subterranean existence since the Miocene (Lavocat, 1978). This species, thus, may have evolved great tolerance for a high oral intake of iron-rich substrates and naturally high non-heme iron levels. This trait appears to still persist in our captive population, even though their diet has a six-fold lower iron content than that of mice (see Experimental procedures). Therefore,

although the internal environment naked mole-rats may be more conducive to lipid peroxidation than it is in mice, it is unlikely that interspecific differences in non-heme iron levels are related to mechanisms involved in longevity determination.

Regardless of the causes for the observed differences in *in vivo* oxidative stress between mice and naked mole-rats, our findings of significantly higher lipid peroxidation in the longer-living rodent are in conflict with the widely accepted oxidative stress theory of aging. Our findings are divergent from previously published studies showing inverse correlations between susceptibility to peroxidation (Cutler, 1985), and/or lipid damage accrual (Pamplona *et al*., 1999, 2000), and species longevity. Naked mole-rats generate more oxidative lipid damage than mice and accumulate it in greater quantities early in life, yet they are able to live with this damage burden. They appear extremely tolerant of damage, with no adverse affects evident (O'Connor *et al*., 2002; Andziak *et al*., 2006), and routinely survive and continue to breed for an additional 26 years while 2 year-old mice have less than 2 more years of potential lifespan.

Age-related changes in oxidative stress

Changes in cellular iron during aging may affect species-specific patterns of oxidative damage generation and accrual (Massie *et al*., 1983). Increases in liver iron reportedly accompany age-associated elevated damage accumulation in both rats (Cook & Yu, 1998) and C57BL/6J mice (Massie *et al*., 1983). Our CB6F1 mice, however, maintained constant high non-heme iron levels over the range of ages assayed (4–24 months). Maintenance of an unchanging pro-oxidative cellular environment in our mice was reflected in steady levels of lipid peroxidation (urinary isoprostanes) between 4 and 24 months and a constant rate (linear) of MDA accrual between 4 and 18 months. Given that a nine-fold variation in hepatic non-heme iron levels has been reported between different mouse strains (Clothier *et al*., 2000), and that the association between liver non-heme iron levels and lipid peroxidation only holds true for some mouse strains (Gerhard *et al*., 2002), our unchanged iron levels with age may thus reflect strain-specific differences that exist in this species.

Liver concentrations of non-heme iron were high in young naked mole-rats, and further increased with age. However, elevated iron levels in these rodents were not associated with greater oxidative damage. Contrary to expectations, declines in liver non-heme iron were accompanied by reductions in both rates of lipid damage generation and MDA accrual. Our findings parallel previous reports in which both energetically restricted mice and rats, despite age-related increases in non-heme iron, showed attenuated oxidative damage accrual during aging (Lass *et al*., 1998; Sohal *et al*., 1999). Collectively, our naked mole-rat results suggest that age-associated changes in levels of intracellular non-heme iron are not linked to patterns of oxidative lipid damage generation and accrual during aging in this long-living species.

Isoprostane and MDA concentrations in biological samples generally correlate well with each other (Roberts & Morrow, 2000), and age-related changes in tissue MDA levels commonly mirror the profiles of urinary isoprostane excretion. This was indeed the case for our mice and naked mole-rats, even though each species displayed their own distinct patterns for isoprostane excretion and MDA accrual (Figs 1 and 2). In mice, urinary isoprostane excretion remained constant during adulthood (4–24 months), suggesting a maintenance of steady rates of damage generation. MDA levels in liver increased at a net rate of 3.3 pmol mg^{-1} protein per month, and doubled between 4 and 18 months cohorts. Our data thus parallel previous mouse studies, which reported both constant urinary isoprostane excretion during aging (Pratico *et al*., 2001), as well as a progressive increase in liver MDA content (Sverko *et al*., 2002).

Rates of lipid damage generation declined with age in naked mole-rats, such that isoprostane excretion by older (180 and 290 months) animals was half that observed in younger (6 and 30 months) cohorts. Liver MDA showed a concomitant attenuation in age-related accrual. MDA levels were moderately higher (~28%) in 75 month old mole-rats than they were in 30 month old individuals with a concomitant 0.8 pmol mg^{-1} protein per month rate of damage accrual. Despite a decade interval between the two oldest mole-rat cohorts, levels of accrued damage were similar. The absence of MDA accrual over a 10-year period implies that rates of oxidative damage generation were matched by either antioxidant defense, and/or damage repair. Indeed this may play a pivotal role in their extraordinary longevity.

Elevated isoprostane excretion is associated with certain pathological conditions including Alzheimer's disease (Pratico *et al*., 1998; Montine *et al*., 2002) and atherosclerosis (Pratico *et al*., 1997, 2001), and is also evident in transgenic mouse models of these diseases. Interestingly, significant age-related increases in this marker of *in vivo* oxidative stress are reportedly found only in individuals afflicted with one of these diseases, and not in healthy age-matched controls (Pratico *et al*., 2001; Montine *et al*., 2002). These findings suggest that isoprostane excretion during aging reflects distinct pathological states and not the aging process itself. The absence of age-related increases in urinary isoprostane levels of either mice or naked mole-rats used in our study supports this premise, since these animals were healthy and devoid of any apparent pathology. Indeed, in the case of naked mole-rats urinary isoprostanes declined with age, as presumably these animals acclimated to the relatively high oxygen content in the laboratory and induced a highly effective response to oxidative stressors.

Conclusions

Our findings of high levels of oxidative damage generation and accrual, as well as an intracellular environment that favors peroxidative reactions in the longest-living rodent do not support the key tenet of the oxidative stress theory of aging, namely that sustained low levels of oxidative damage are an important component of longevity. While age-associated increases in non-heme iron were evident in naked mole-rats, there was no concomitant age-related increase in markers of lipid peroxidation at least over a ~10-year period. This infers that naked mole-rats

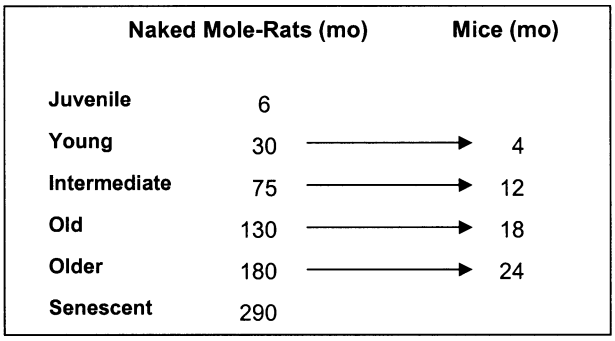

Fig. 4 Ages of cohorts of physiologically age-matched naked mole-rats (NMR) and mice used in this study. For comparisons between mice and NMRs, age groups were specifically chosen as proportions of their respective maximum species lifespans. In addition, 'Juvenile' and 'Senescent' NMRs cohorts were used when comparing urinary isoprostane excretion at different stages of life in this species.

are able to maintain steady states of oxidative damage, albeit at a higher level than mice, with no concomitant adverse effects. Other factors, such as a larger stem cell reservoir, and enhanced stability of key biological macromolecules, are more likely to be the determinants of the extraordinary longevity of this species.

Experimental procedures

Animals

We assessed and compared levels of lipid damage and non-heme iron in naked mole-rats and hybrid CB6F1 mice (*Mus musculus*) at different ages. Age cohorts were specifically selected to facilitate comparisons between these two species at physiologically equivalent ages (Fig. 4).

With the exception of the 'Senescent' cohort (290 months), all naked mole-rats were born in captivity. Senescent animals were captured in Kenya in 1980, as juveniles (~6 to 18 months), and comprised the parental stock from which all the other experimental naked mole-rats originated. Mice were obtained from Charles River Laboratories (Wilmington, MA, USA) and from the National Institute of Aging (Bethesda, MD, USA). Animals were maintained at the City College of New York following standard housing protocols (O'Connor *et al*., 2002; Andziak *et al*., 2005). The average dietary iron content (dry weight per 100 g of dry diet) of naked mole-rats was approximately 5 mg (Sizer & Whitney, 2006), whereas that of mice was 27.0 mg (PMI International, Brentwood, MO, USA). Similarly the fruit diet supplemented with a protein-rich cereal (Pronutro, Bokomo, South Africa) provided a lower lipid content (< 3 g lipid per 100 g) than the commercial mouse chow (> 4.5 g lipid per 100 g; PMI International).

Urine and liver collection

We collected a 6-h urine (12:00–18:00) sample from mice and naked mole-rats that were housed individually in plastic collection chambers (Sterilite, Townsend, MA, USA). Chambers were lined with parafilm (American National Can, Menasha, WI, USA), on top of which a wire platform was mounted preventing animals from physically dispersing or contaminating excreted urine. Animals were constantly monitored and upon detection of micturition, all urine voided was collected and transferred on ice for storage at –80 °C. Prior to the isoprostane assays, we centrifuged the urine samples at 500 ***g*** for 3 min (4 °C) to remove any debris (e.g. skin or fur) that may have been accidentally collected.

Animals were euthanized with an intraperitoneal injection of sodium pentobarbital (60 mg kg^{-1}). Liver was dissected out within 5 min of injection, rinsed in ice-cold Ringer solution and flash frozen in liquid nitrogen and stored at –80 °C until later analyses.

Lipid peroxidation

We compared age-related changes in *in vivo* organismic oxidative stress in mice (n = 6 per age cohort) and naked mole-rats (n = 6–8 per age cohort) through measurement of isoprostane content in urine. This marker of lipid damage generation was assayed using EA 85 ELISA kits (Oxford Biomedical Research, Oxford, MI, USA), as described in the manufacturer's protocol. Urinary isoprostanes are reported per milligram of creatinine.

We determined MDA content using BIOXYTECH MDA-586 kits (OxisResearch, Portland, OR, USA), as described by the manufacturer's protocol. We quantified levels of this marker of accrued lipid damage in liver samples (mice, n = 6 per age group; naked mole-rats, n = 8 per age group). Liver samples were initially washed with 0.9% NaCl containing 0.16 mg mL^{-1} of heparin and 2.5 mM butylated hydroxytoluene (BHT) in acetonitrile, and then were homogenized in 20 mM potassium phosphate containing 2.5 mM BHT (pH 7.4 at 4 °C). We centrifuged the homogenates at 3000 ***g*** for 10 min at 4 °C and stored the resulting supernatants at –80 °C until time of assay.

Absorbance readings were made across a range of wavelengths (650 nm–450 nm, DU530, Beckman-Coulter, Fullerton, CA, USA), spanning the expected peak absorbance (586 nm) of the resulting assay reaction product (carbocyanine dye). We isolated distinct MDA peaks using third derivative spectroscopy. MDA data are expressed per milligram of protein.

Non-heme iron

Liver non-heme iron content was determined using the modified method of Rebouche *et al*. (2004). Briefly, we homogenized liver samples (n = 6 for each species' age cohort) in high purity water using a mechanical homogenizer. Homogenates were combined with an equal volume of protein precipitation solution (1 N HCl and 10% trichloroacetic acid), mixed and incubated at 95 °C for 120 min. Following the incubation, the solutions were mixed and centrifuged at 8200 ***g*** for 10 min (20 °C). The resulting supernatants were stored on ice until assay later that same day.

Equal volumes of sample and chromogen [0.508 mM ferrozine, 1.5 M sodium acetate and 0.1% (v/v) thioglycolic acid] were reacted in microplates. The mixtures were incubated in the dark for 30 min (20 °C), after which absorbance readings were taken at 562 nm. Sample concentrations were determined using a calibration curve based on a set of iron standards (High Purity Standards, Charleston, SC, USA). Non-heme iron data are reported per milligram of protein.

Creatinine and protein content

We measured both creatinine and protein content in our samples using commercially available assays (creatinine in urine: CR 01 kits; Oxford Biomedical Research and protein in liver homogenates: Pierce BCA Protein Assay, Pierce, Rockford, IL, USA).

Statistical analyses

Statistical analyses were performed using NCSS Version 97 (Kaysville, UT, USA). Results are presented as untransformed means ± SEM. The effects of age and species differences on urinary isoprostanes, malondialdehyde and non-heme iron in liver were tested for significance by a two-way analysis of variance (ANOVA) using a general linear model. In addition, we tested the effect of age of isoprostanes in the six NMR age cohorts using a one-way ANOVA. All age-cohort contrasts were made using the Bonferroni multiple-comparison test. Prior to analysis data were transformed to their natural logarithm to meet the assumptions of the statistical tests that data be normally distributed. Statistical significance for all analyses was set at $P < 0.05$.

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