Comparison of endothelial function, O$_2^*$ and H$_2$O$_2$ production, and vascular oxidative stress resistance between the longest-living rodent, the naked mole rat, and mice

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IN THE UNITED STATES, the number of persons aged $\geq$65 years is expected to increase from $\sim$35 million in 2000 to an estimated 71 million in 2030 (according to the Center for Disease Control). Epidemiological studies suggest that aging of the vascular system is directly responsible for $>50\%$ of mortality and morbidity in these age groups. Despite the growing incidence of aging-induced coronary artery disease, stroke, and diabetic vasculopathy, the factors determining successful vascular aging are still poorly understood.

Vascular aging is characterized by proatherogenic functional and phenotypic alterations, including impaired endothelial nitric oxide (NO) mediation, increased production of reactive oxygen species [ROS; such as superoxide anion radical (O$_2^*$) and H$_2$O$_2$] and enhanced endothelial apoptotic cell death (12–15, 24, 37). Few comparative studies have addressed this topic, even though species with disparate longevity may provide useful insights into the mechanisms determining successful vascular aging. We addressed this in a comparative study between four rodent species that show approximately an order of magnitude range in maximum longevity. We compared interspecies differences in endothelial function, O$_2^*$ and H$_2$O$_2$ production, and resistance to apoptotic stimuli in vessels of the three longer-living species and in arteries of shorter-living mice. Sensitivity to acetylcholine-induced, NO-mediated relaxation was smaller in carotid arteries from NMRs, GPs, and DMRs than in mouse vessels.

Measurements of production of O$_2^*$ (lucigenin chemiluminescence and ethidium bromide fluorescence) and H$_2$O$_2$ (dichlorofluorescein fluorescence) showed that free radical production in vascular endothelial and smooth muscle cells is comparable in vessels of the three longest-living species and in arteries of shorter-living mice. In mouse arteries, H$_2$O$_2$ (from $10^{-6}$ to $10^{-3}$ mol/l) and heat exposure (42°C for 15–45 min) enhanced apoptotic cell death, as indicated by an increased DNA fragmentation rate and increased caspase 3/7 activity. In NMR vessels, only the highest doses of H$_2$O$_2$ enhanced apoptotic cell death, whereas heat exposure did not increase DNA fragmentation rate. Interspecies comparison showed there is a negative correlation between H$_2$O$_2$-induced apoptotic cell death and ML. Thus endothelial vasodilator function and vascular production of reactive oxygen species do not correlate with maximal lifespan, whereas increased lifespan potential is associated with an increased vascular resistance to proapoptotic stimuli.

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The maximum lifespan of naked mole rats (NMRs; Rodentia, Bathyergidae: Heterocephalus glaber) of $>28.3$ years is greater than that of any other rodent known (8). These long-lived mouse-sized social subterranean animals have a similar longevity quotient (LQ, the ratio of actual maximum lifespan potential to that predicted by body mass) to that of humans (4). Furthermore, unlike laboratory mice, NMRs do not seem to develop age-related parenchymal tumors and lymphomas, and, in at least 250 autopsies, we have not yet found evidence for atherosclerosis in aged NMRs (Buffenstein R, unpublished observation). Because of these considerations, NMR seems to be an ideally suited model of successful aging (7). Yet there are no studies extant investigating endothelial function, ROS generation, or apoptotic cell death in this species.

Studies examining oxidative stress in NMRs did not always concur with predictions based upon the oxidative stress theory of aging and NMRs did not show lower redox states, superior antioxidant defenses or lower levels of accrued oxidative damage (2). Also surprisingly, ROS production in mitochondria isolated from NMR heart tissues reportedly is similar to that observed in mouse mitochondria (Lambert A. and Buffenstein R., unpublished observation). A by now classical study by Ku and Sohal (23) investigating long-living pigeons suggested that mitochondria isolated from species with a longer lifespan potential may exhibit a lower level of free radical generation than mitochondria isolated from shorter-living ones. A similar conclusion was reached by a recent study from Dr. Brunet-Rossini’s laboratory, that mitochondria from the heart of young little brown bats [Myotis lucifugus; maximum longevity

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Cellular apoptotic pathways and concomitant endothelial cell injury have been implicated in the initial phases of coronary artery disease (9). Recent studies revealed that advanced age promotes apoptotic cell death in various tissues, including peripheral arteries (3, 15) and the heart (22, 29, 34). Indeed increased frailty with age is often attributed to an imbalance in ROS production may, however, not necessarily reflect longevity traits but may simply be indicative of the large phylogenetic differences across phyla (birds and mammals) and mammalian orders (Chiroptera, Insectivora, and Rodentia). We redressed this issue in four phylogenetically related species, in this, the first comparative study assessing ROS generation in intact blood vessels.

Thus the present study was designed to compare endothelial function, O$_2^•$ and H$_2$O$_2$ production, and vascular oxidative stress resistance between three hystricognath rodents (NMRs; ML $\sim$ 30 yr, LQ 5.0), Damara mole rats (DMRs, Cryptomys damarensis; ML $\sim$ 16 yr, LQ 2.1), guinea pigs (Cavia porcellus; ML $\sim$ 6 yr, LQ 0.6) that show divergent MLs and LQs and shorter-living laboratory C57 mice (Mus musculus; ML $\sim$ 3.5 yr, LQ 0.7). Specifically, we tested the hypothesis that endothelial ROS production and/or vascular resistance to apoptotic stimuli correlate with ML.

**METHODS**

**Animals.** All animal use protocols were approved by the Institutional Animal Care and Use Committee of the City College of New York and the New York Medical College. Young NMRs (n = 10; age <2 yr old, weighing 25–38 g), DMRs (n = 5; approximate age <4 yr old, weighing ~160 g), and guinea pigs (4–5 mo old, weighing ~700 g) were from the well-characterized colonies maintained in Dr. Buffenstein’s laboratory (2, 7, 30). Young (6–8 wk old) C57 mice (weight 17–20 g) were purchased from Charles River (Austin, TX) and maintained under standard conditions. The chronological age of the animals in each strain was less then one-fifth of their predicted maximum lifespan potential [based on the allometric equation of Austad and Fischer (4) for nonflying eutherian mammals (predicted longevity = 10.67 $\times$ M$^{0.189}$, M = body mass in kg). Longevity quotients (LQs) were calculated from the ratio of maximum longevity (ML) to the predicted MLSP.

Endothelial function was assessed as previously described (10, 17). In brief, carotid arteries of each animal were cut into ring segments 2 mm in length and mounted on 40-$\mu$m stainless steel wires in the myographs chambers (Danish Myo Technology) for measurement of isometric tension. The vessels were superfused with Krebs buffer solution (118 mM NaCl, 4.7 mM KCl, 1.5 mM CaCl$_2$, 25 mM NaHCO$_3$, 1.1 mM MgSO$_4$, 1.2 mM KH$_2$PO$_4$, and 5.6 mM glucose, at 37°C; gassed with 95% air and 5% CO$_2$). After an equilibration period of 1 h, during which an optimal passive tension was applied to the rings (as determined from the vascular length-tension relationship), relaxation of precontracted (by 10$^{-6}$ mol/l phenylephrine) vessels to acetylcholine (ACh; from $10^{-9}$ to $10^{-4}$ mol/l) and the NO donor S-nitroso-penicillamine (SNAP; from $10^{-9}$ to $3 \times 10^{-5}$ mol/l) were obtained. The effects of the NO synthase inhibitor N$^\text{-}$nitro-L-arginine methyl ester (L-NAME $3 \times 10^{-4}$ mol/l) or the free radical scavengers superoxide dismutase (SOD; 200 U/ml) plus catalase (200 U/ml) on ACh-induced responses of NMR vessels were also tested.

**Ethidium bromide fluorescence.** Production of O$_2^•$ in segments of the same carotid arteries that were used for functional studies was determined. Hydroethidine, an oxidative fluorescent dye, was used to localize superoxide production in situ as we previously reported (13, 36, 38). In brief, vessels were incubated with hydroethidine (10$^{-6}$ mol/l, at 37°C for 60 min). Then the arteries were washed three times, embedded in OCT medium, and cryosectioned. Vascular sections were imaged using a Zeiss AxiosCam Mrm camera mounted on a Zeiss Axiovert 200 fluorescence microscope (Carl Zeiss, Germany). Images were captured at $\times 20$ magnification and analyzed using the Zeiss Axiovision imaging software. Ten to fifteen entire fields per group were analyzed with one image per field. The mean fluorescence intensities of ethidium bromide (EB)-stained nuclei in the endothelium and medial layer were measured in each view field.

Measurement of vascular O$_2^•$ levels by lucigenin chemiluminescence. O$_2^•$ production was assessed from aortic segments by the lucigenin chemiluminescence (5 mmol/l) method as we previously described (13, 35, 36, 38). In separate experiments, O$_2^•$ production was assessed in vessels preincubated (for 1 h) with of diphenylideneiodonium [DPI, 10$^{-5}$ mol/l, an inhibitor of flavoprotein-containing oxidases, including NAD(P)H oxidases] or SOD (200 U/ml).

Measurement of vascular H$_2$O$_2$ production. The cell-permeant oxidative fluorescent indicator dye 5- (and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate-acetyl ester (C-H$_2$DCFDA; Invitrogen, Carlsbad, CA) was used to assess H$_2$O$_2$ production in isolated carotid arteries according to the modified protocols of Miura et al. (27). C-H$_2$DCFDA is a 2'-7'-dichlorofluorescein (DCF) derivative that has longer retention within the cells. In brief, vessel segments were treated with C-H$_2$DCFDA ($10^{-5}$ mol/l, at 37°C for 60 min). Untreated arteries were used as controls. Then the arteries were washed three times. Fluorescent images of the endothelial layer of en
face preparations were captured and analyzed using the Axiosvision software (Carl Zeiss). Each experiment was performed in quadruplicate. Ten to fifteen entire fields per group were analyzed with one image per field. The background-corrected mean fluorescent intensities of each image were averaged. In some experiments, vessels co-incubated with catalase were used as positive controls.

**Vessel culture.** Aortic segments of the mice and NMRs were isolated and maintained in organoid culture under sterile conditions in F12 medium (GIBCO-BRL) containing antibiotics (100 UI/l penicillin, 100 mg/l streptomycin, and 10 μg/l fungizone) and supplemented with 5% FCS (Boehringer-Mannheim), as previously described (15, 35, 36, 38). Vessel segments were treated with H2O2 (10−8 to 10−4 mol/l) for 6 h. After washout, the vessels were maintained in H2O2-free medium for 18 h. In separate experiments, carotid arteries were exposed to heat (42°C) for 15, 30, or 45 min and then cultured under standard conditions (at 37°C) for 24 h. After the culture period, vessels were snap-frozen in liquid nitrogen.

**Detection of apoptotic cell death by ELISA.** H2O2-pretreated cultured arteries were lysed, and cytoplasmic histone-associated DNA fragments, which indicate apoptotic cell death, were quantified by the Cell Death Detection ELISAPLUS kit (Roche Diagnostics, Indianapolis, IN) as described previously (15). Results are reported as normalized arbitrary optical density (OD) units.

**Caspase activity assay.** Cultured arteries were homogenized in lysis buffer, and caspase activities were measured using the Caspase-Glo 3/7 assay kit according to the manufacturer’s instruction (Promega, Madison, WI). In 96-well plates, a 50-μl sample was mixed gently for 30 s with 50 μl of Caspase-Glo 3/7 reagent and incubated for 2 h at room temperature. The lysis buffer with the reagent served as blank. Luminescence of the samples was measured using an Infinite M200 plate reader (Tecan, Research Triangle Park, NC). Luminescent intensity values were normalized to the sample protein concentration.

**Data analysis.** Data were normalized to the respective control mean values and are expressed as means ± SD or SE. Statistical analyses of data were performed by Student’s t-test or by two-way ANOVA, followed by Tukey’s post hoc test, as appropriate. *P < 0.05 was considered statistically significant.

**RESULTS**

**NO-mediated vascular relaxation.** ACh elicited relaxation of NMR carotid arteries that was significantly inhibited by L-NAME (Fig. 2A). ACh-induced relaxations of NMR vessels were unaffected by SOD plus catalase (Fig. 2A). NMR arteries showed only weak relaxation to bradykinin (10−6 mol/l, 18 ± 7%; 10−5 mol/l, 24 ± 3%). ACh elicited relaxations of mouse carotid arteries in a lower concentration range than for NMR vessels (Fig. 2B). Sensitivity of guinea pig carotid arteries to ACh was similar to that of NMR arteries, whereas DMR arteries exhibited poor ACh-induced relaxations (Fig. 2B). ACh-induced relaxations of mouse, guinea pig, and DMR arteries were abolished by L-NAME (not shown). The NO donor SNAP elicted relaxations in arteries from mice, NMRs, and guinea pigs. Data are means ± SE (n = 4–8 vessels for each group). *P < 0.05.

**Vascular superoxide production.** In carotid arteries of NMRs, mice, DMRs, and guinea pigs, the relative number of EB-positive nuclei was similar (Fig. 3, A–C). EB staining was present in both the endothelium (arrows) and media (double arrows) of the vessels (Fig. 3, A–C). Analysis of nuclear EB fluorescence intensities showed that endothelial cells and smooth muscle cells in vessels from each species tended to produce comparable amounts of O2− (Fig. 3D). Lucigenin chemiluminescence measurements showed that O2− generation in both NMR and mouse vessels can be inhibited by DPI (Fig. 3E) and SOD (not shown), consistent with the view that NAD(P)H oxidase activity (13, 16) is responsible for a significant portion of vascular O2− production under basal conditions in each species.

**Vascular H2O2 production.** Vascular H2O2 generation was measured by the DCF fluorescence method. We found that NMR arteries tend to produce more H2O2 than mouse vessels, whereas H2O2 production in DMR and guinea pig vessels was comparable (Fig. 4). DCF fluorescence could be inhibited by catalase, showing the specificity of the signal (not shown).

**Vascular apoptotic cell death.** Previously, we have demonstrated that DNA fragmentation and caspase activities are good measures of endothelial apoptotic cell death in blood vessels (15). Pretreatment with H2O2 (for 6 h) significantly increased
DNA fragmentation (Fig. 5A) and caspase 3/7 activity (Fig. 5B) in cultured arteries of mice, even at the lowest concentrations. In contrast, in NMR vessels, H$_2$O$_2$ elicited only minimal increases in DNA fragmentation rate or caspase activity only at the highest concentrations studied (Fig. 5A), indicating that NMR vessels are resistant to the proapoptotic effects of H$_2$O$_2$. Preexposure of mouse arteries to heat for 15, 30, or 45 min elicited substantial increases in DNA fragmentation (Fig. 5C), indicating that NMR vessels are resistant to apoptotic cell death in longer-living species. Comparison of the basal DNA fragmentation rates (Fig. 5D) and basal caspase activities (not shown) in mouse, guinea pig, NMR, and DMR vessels showed no correlation between basal apoptotic cell death and maximal lifespan potential. In contrast, $10^{-6}$ mol/l H$_2$O$_2$-induced increases in DNA fragmentation rate (Fig. 4E) and caspase activities (Fig. 5F) showed a negative correlation ($R^2$, 0.61 and 0.71, respectively) with maximal lifespan potential.

**DISCUSSION**

There are four salient findings in this study, namely, 1) interspecies differences in vascular relaxation in response to acetylcholine stimulation do not correlate with maximum lifespan potential, 2) arteries of the longest living rodent are more tolerant of oxidative stress than vessels of shorter living mice, 3) cellular ROS production does not seem to correlate with longevity, and 4) resistance to apoptosis is a better predictor of longevity than is ROS production.

We have demonstrated that NO plays a central role in mediating ACh-induced vascular relaxations in NMR arteries (Fig. 2A), extending previous findings in other mammals. Under basal conditions, ACh-induced dilations were not significantly affected by scavenging of the free radicals O$_2^-$ and H$_2$O$_2$. Preexposure of mouse arteries to heat for 15, 30, or 45 min elicited substantial increases in DNA fragmentation (Fig. 5C), indicating that NMR vessels are resistant to apoptotic cell death in longer-living species. Comparison of the basal DNA fragmentation rates (Fig. 5D) and basal caspase activities (not shown) in mouse, guinea pig, NMR, and DMR vessels showed no correlation between basal apoptotic cell death and maximal lifespan potential. In contrast, $10^{-6}$ mol/l H$_2$O$_2$-induced increases in DNA fragmentation rate (Fig. 4E) and caspase activities (Fig. 5F) showed a negative correlation ($R^2$, 0.61 and 0.71, respectively) with maximal lifespan potential.

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H$_2$O$_2$ (Fig. 2B). Comparison of vasodilator responses between carotid arteries of mice and NMRs showed that mouse vessels exhibit an ~10-fold greater sensitivity toward ACh (Fig. 2B). Carotid arteries of the guinea pig, which is like NMRs, is a member of hystricognath sub-order of rodents, showed ACh-induced relaxations with similar EC$_{50}$ values (Fig. 2B). Interestingly, ACh elicits relaxation in vessels of DMRs (Fig. 2B) and shorter-living rats (10) with a significantly higher EC$_{50}$.

One can speculate that these differences in ACh-induced vasodilator responses are due to interspecies differences in the expression of ACh receptors and/or endothelial NO synthase, which do not correlate with maximum lifespan potential. Because relaxations of mouse, NMR, and guinea pig carotid arteries to an exogenous NO donor were comparable (Fig. 2C), which may explain the poor endothelial responses (Fig. 2B) in this species.

In blood vessels, the cell membrane-associated NAD(P)H oxidase is a significant source of ROS production, and an increased vascular NAD(P)H oxidase has been shown to contribute importantly to the symptoms of vascular aging, including increased O$_2^*$ production, decreased bioavailability of the vasodilator and anti-apoptotic NO (1, 12, 13, 15, 19, 24, 37), increased cardiac oxygen demand (1), and vascular inflammation (Ungvari Z, unpublished observation). EBD fluorescence measurements revealed that both the endothelial and smooth muscle cells of NMR vessels produce O$_2^*$ (Fig. 3, A and D). We found no correlation between maximal lifespan potential and O$_2^*$ production in vessels of NMRs, mice, DMRs, and guinea pigs (Fig. 3D). Lucigenin chemiluminescence measurements also showed that NAD(P)H oxidase activity contributes significantly to vascular O$_2^*$ production in both NMR and mice

![Graph A](image1.png)

**A** DNA fragmentation in cultured arteries of NMRs and mice. Vessels were pretreated (for 6 h) with H$_2$O$_2$ (from 0 to 10$^{-3}$ mol/l) and then maintained in organoid culture for 18 h. Data ± SE are normalized to the mean values of untreated controls ($n = 5–6$ for each data point). *P < 0.05 vs. untreated control. **B**: caspase 3/7 activity (see METHODS) in H$_2$O$_2$-treated NMR and mouse vessels. Data ± SE are normalized to the mean value of untreated mouse control ($n = 5–6$ for each data point). *P < 0.05 vs. untreated control. **C**: DNA fragmentation in cultured arteries of NMRs and mice. Vessels were exposed to heat (42°C for 15, 30, or 45 min) and then maintained in organoid culture for 24 h. Data ± SE are normalized to the mean values of untreated controls ($n = 5–6$ for each data point). *P < 0.05 vs. untreated control. **D**: lack of correlation between maximal lifespan potential and baseline DNA fragmentation rate in arteries of mice, NMRs, DMRs, and guinea pigs. **E** and **F**: negative correlation between maximal lifespan potential and H$_2$O$_2$ (10$^{-4}$ mol/l)-induced DNA fragmentation (**E**) and caspase activity (**F**) in vessels of mice, NMRs, DMRs, and guinea pigs ($n = 5–6$ for each data point).
studies revealed that, in the liver of NMRs, glutathione perox-
ide activity is 70 times lower than in mice (2). It is significant that ML and vascular H$_2$O$_2$ production did not show any correlation in the species studied (Fig. 4). Importantly, other studies also failed to demonstrate a relationship between ML and the rate of mitochondrial H$_2$O$_2$ production (33) and between maximum species lifespan potential and H$_2$O$_2$ scavenging enzymes (catalase and glutathione peroxidase) in various long-living animal models (21).

We would like to emphasize that our data do not challenge the view that oxidative stress is a major factor in aging-related vascular disease. Further studies are definitely needed to compare aging-induced ROS production and rats of accrued damage in response to oxidative stressors in short-lived and long-lived animals. It also might be informative to conduct a comparative study of ROS-producing systems (such as NADPH oxidases and mitochondrial sources) and antioxidant defense systems (including SOD isoforms, catalase, glutathione peroxidase) in each of the species to determine whether there is a correlation among such variables with age.

The third and potentially the most important finding of our study is that arteries of NMRs exhibit a marked resistance against the proapoptotic effects of H$_2$O$_2$ (Fig. 5, A and B). It is significant that, in mouse arteries, the same concentrations of H$_2$O$_2$ substantially enhanced apoptotic cell death (as shown by an increased DNA fragmentation rate and caspase activation; Fig. 5, A and B). Although apoptosis may reduce the replication of mutated or damaged cells and concomitant risk of cancers in nonvascular tissues, limiting apoptotic responses to oxidative stressors in blood vessels is likely beneficial and facilitates sufficient time for repair to occur. Interspecies comparison revealed a negative correlation between maximum lifespan potential and H$_2$O$_2$-induced apoptotic cell death (but not with baseline rate of apoptosis) in vessels of mice, NMRs, DMRs, and guinea pigs (Fig. 5, D–F). These findings support that increased resistance to the proapoptotic effects of oxidative stress (rather than an attenuated basal production of ROS) may be associated with exceptional longevity and/or a slower rate of cardiovascular aging. This view is also supported by studies showing an increased resistance to oxidative challenge in cells of long-lived mouse models (such as the Ames and Snell dwarf mice; Refs. 26, 28, 32) and avian species (budgerigar, Melopsittacus undulatus, ML ~ 20 yr; Ref. 31) as well as NMRs (Salmon A, Miller R, Buffenstein R, personal communication). The mechanisms underlying the increased oxidative stress resistance in long-living animals are not completely understood. One would expect that long-lived animals have superior antioxidant defenses; however, recent studies revealed that, in the liver of NMRs, glutathione perox-

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