MITOCHONDRIAL OXIDANT GENERATION AND OXIDATIVE DAMAGE IN AMES DWARF AND GH TRANSGENIC MICE

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ABSTRACT

Aging is associated with an accumulation of oxidative damage to proteins, lipids and DNA. Cellular mechanisms designed to prevent oxidative damage decline with aging and in diseases associated with aging. A long-lived mouse, the Ames dwarf, exhibits growth hormone deficiency and heightened antioxidative defenses. In contrast, animals that over express GH have suppressed antioxidative capacity and live half as long as wild type mice. In this study, we examined the generation of H₂O₂ from liver mitochondria of Ames dwarf and wild type mice and determined the level of oxidative damage to proteins, lipids and DNA in various tissues of these animals. Dwarf liver mitochondria (24 months) produced less H₂O₂ than normal liver in the presence of succinate (p<0.03) and ADP (p<0.003). Levels of oxidative DNA damage (8OHdG) were variable and dependent on tissue and age in dwarf and normal mice. Forty-seven percent fewer protein carbonyls were detected in 24-month old dwarf liver tissue compared to controls (p<0.04). Forty percent more (p<0.04) protein carbonyls were detected in liver tissue (3-month old) of GH transgenic mice compared to wild types while 12 month old brain tissue had 53% more protein carbonyls compared to controls (p<0.005). Levels of liver malonaldehyde (lipid peroxidation) were not different at 3 and 12 months of age but were greater in Ames dwarf mice at 24 months compared to normal mice. Previous studies indicate a strong negative correla-

tion between plasma GH levels and antioxidative defense. Taken together, these studies show that altered GH-signaling may contribute to differences in the generation of reactive oxygen species, the ability to counter oxidative stress and life span.

INTRODUCTION

Aging is associated with declining physiological function. Accumulation of oxidative damage to DNA, proteins and lipids is thought to contribute to the observed decline (1). The increase in cellular damage caused by reactive oxygen species (ROS) with advancing age is supported by several studies (2-4). In addition, oxidative damage may contribute to multiple diseases including cancer, cardiovascular disease, Parkinson's disease, ischemia and chronic inflammation (5, 6). Reactive oxygen species are generated during normal cellular metabolism and although cells have various enzymatic and nonenzymatic systems to control ROS, a certain amount is able to escape these defenses and potentially cause damage to cellular components.

Elevated oxidative stress due to an increase in mitochondrial ROS generation and associated oxidative damage occur concomitant with aging (7). A significant correlation between longevity and reduced oxidative damage has been shown in several non-mammalian species (8, 9). In addition, an intervention, caloric restriction, has been shown to extend life span, modify accumulation of oxidative damage in rodents (10, 11) and may ameliorate risk factors associated with chronic human diseases (12). Ames dwarf mice represent unique genetic mutants that outlive their wild type counterparts by more than one year (13). These mutant mice are characterized by pituitary hormone deficiencies (growth hormone (GH), prolactin and thyroid stimulating hormone) that result in diminutive body size and delayed puberty. However, their antioxidant defenses, specifically catalase, are elevated in several tissues (14). The growth hormone deficiency is thought to be particularly important to the extended life span achieved by the Ames dwarf as Snell dwarf mice (phenotypically identical to Ames; 15, 16) and GH receptor knock out mice (17) also live longer than wild type siblings (18). In contrast, mice that over express GH (GH transgenics) live only half as long as normal, wild type siblings and exhibit a suppressed antioxidative capacity (14, 19).

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We were interested, therefore, in determining whether significant differences in ROS generation and oxidative damage were apparent between long-living Ames dwarf and normal wild type mice. Oxidative damage to proteins and DNA were also evaluated in short-lived GH transgenic mice.

MATERIALS AND METHODS

Animals

Ames dwarf, GH transgenic and corresponding groups of age-matched wild type male mice were maintained at the University of North Dakota (UND) vivarium facilities under controlled conditions of photoperiod (12 h light:12 h dark) and temperature (22 ± 1 °C) with ad libitum access to food and water. The Ames dwarf (df/df) mice used in this study were derived from a closed colony with heterogeneous background (over 20 years). Dwarf mice were generated by mating either homozygous (df/df) or heterozygous (df/+) dwarf males with carrier females (df/+). Ames dwarf mice were maintained under standard laboratory conditions. The GH transgenic animals were kindly provided by Dr. Andrzej Bartke (Southern Illinois University). All procedures involving animals were reviewed and approved by the UND Institutional Animal Care and Use Committee. Twenty-four month old dwarf and wild type mice from the dwarf line represent the oldest age group in this study because the average lifespan of the wild type mice in our colony is ~23 months (13). The GH transgenic animals used in these studies were derived from a single male founder (strain B6SJL) produced by microinjection of the phosphoenolpyruvate carboxykinase (PEPCK) promoter region (300 bp)/bGH hybrid gene into the male pronucleus of single-cell embryos. The creation and initial characterization of transgenic animals (transgenic males crossed to C57Bl/6J x C3H/J F1 females) were described previously (20, 21). Animals overexpressing GH typically live ~12 months of age (19, 22), therefore, the 12 month old age group represents the very oldest mice in this line. Liver, heart and brain tissues from 3, 12, and 20-24 month old animals were collected for different experiments, rapidly frozen and maintained at -80°C until analysis. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted.

ROS determination

To assess the levels of H_2O_2 production in Ames dwarf and corresponding wild type mice, mitochondria were isolated from liver tissues of 3- and 24-month old male mice using the procedure of Trounce and coworkers (23) and the H_2O_2 assay of Kwong and Sohal (24). Briefly, fresh tissue was homogenized in buffer (224 mM mannitol, 75 mM sucrose, 10 mM MOPS, 1 mM EGTA, and 0.5% BSA, pH 7.2) and centrifuged at $1500 \times g$ for 5 min at 4°C (2X). Centrifugation was repeated at $10,000 \times g$ for 15 min at 4°C to pellet mitochondria. 100 μl mitochondria, 4 μl horseradish peroxidase (1000 U/ml), 50 μl p-hydroxyphenylacetate (10mg/ml) and 2.696 ml of reaction buffer (154 mM KCl, 5 mM KH_2PO_4 , 5 mM

K_2HPO_4 , 3mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 mM EGTA, pH 7.4) were combined, placed in the fluorometer at 37°C and stirred. A volume of 100 μl of a 210 mM succinate solution in reaction buffer was added followed by 50 μl of 10.8 mM ADP in reaction buffer 2 min later. The ADP rate was obtained over the next 6 minutes and H_2O_2 production calculated based on the standard curve. The data was normalized and expressed per mg mitochondrial protein. Controls included substrate without mitochondria, mitochondria without substrate and buffer only for each tissue and substrate utilized.

Determination of oxidative DNA damage

To estimate oxidative DNA damage, 8-hydroxy-2-deoxyguanosine (8-OHdG) was quantified using high performance liquid chromatography and electrochemical detection (HPLC-EC). This guanine adduct is elevated in oxidative stress and is a major product of DNA damage. This assay was performed by ESA Laboratories (Chelmsford, MA) using procedures originally described by Floyd and coworkers (25, 26). In our lab, for preparation of samples for analysis, DNA was extracted from tissues using the Qiagen Maxi-Prep DNA Kit (Qiagen; Valencia, CA). Isolated DNA was hydrolyzed using the following procedure. Briefly, 200 μg of DNA was dissolved in 20 mM sodium acetate (pH 5.1) and incubated for 10 min at 65°C with 1 U/ μl nuclease P1. Tris-HCL (1 M) was added to each sample followed by 1 U/ μl alkaline phosphatase. Following a 1 hr incubation at 37°C , 3 M sodium acetate buffer was added and the mixture transferred to a 5 k Millipore (Bedford, MA) concentrator tube. The tubes were centrifuged at $5,000 \times g$ for 10 min and the resulting nucleosides were stored at -80°C prior to shipment to ESA. For analysis, the nucleoside mixture was injected into a reverse phase C-18 column, eluted with sodium acetate and methanol in phosphate buffer. Known amounts of characterized 8-OHdG were used as standards. An EC curve was generated, the level of sensitivity selected and unknowns were compared to standards. An 8-OHdG:2dG ratio was calculated as this value has been shown to correlate well with DNA damage and aging (27-29).

Determination of protein oxidative damage

Quantitation of protein carbonyl groups is sensitive and allows detection of differences in oxidized protein in the Ames dwarf and GH transgenic as compared to corresponding age-matched wild type animals. The carbonyl content of tissues was determined using the method of Levine and coworkers (30). Briefly, proteins were extracted from tissues using lysis buffer (10mM Tris, 0.15M NaCl, 5 mM EDTA, 1% Triton X, 1 mM phenylmethylsulfonyl fluoride, .25 ng/ μl leupeptin, .25 ng/ μl aprotinin) to prevent proteolytic degradation. Nucleic acids were eliminated by treating the samples with 1% streptomycin sulfate for 15 min, followed by a 10 min centrifugation ($11,000 \times g$). Proteins were precipitated by adding an equal volume of 20% TCA to

protein and centrifuged for 1 min. The samples were resuspended in 10 mM 2,4-dinitrophenylhydrazine (2,4-DNPH) solution and incubated at room temperature for 15-30 min. Following the addition of 20% TCA, samples were centrifuged, resulting pellets were washed in ethanol:ethyl acetate, incubated at room temperature for 10 min and centrifuged again for 3 min. The precipitate was resuspended in 6 M guanidine solution, centrifuged for 3 min and insoluble debris removed. The maximum absorbance (360 nm) of the supernatant was read against appropriate blanks (water, 2 M HCL) and the carbonyl content calculated using the molar absorption coefficient of $22,000 \text{ M}^{-1}\text{cm}^{-1}$.

Determination of lipid oxidative damage

To assess lipid peroxidation, malonaldehyde (MDA) levels were measured in liver and brain tissue homogenates and 4-hydroxyalkenal (4-HNE) in liver tissues only. A colorimetric assay (LPO-586 Kit; Oxis International; Portland, OR) based on the method of Esterbauer et al. (31) was utilized. Briefly, a 20% tissue homogenate was prepared in 20 mM Tris buffer containing 5 mM butylated hydroxytoluene (BHT) to prevent sample oxidation. Protein concentrations were determined using the Bradford method (32). Reagent 1 (N-methyl-2-phenylindole) was added to each sample, the mixture was gently vortexed and then 15.4 M methanesulfonic acid (Reagent 2) was added. Samples were incubated at 45°C for 45 min followed by centrifugation (10 min at $15,000 \times g$). The absorbance of the sample supernatants was measured at 586 nm. Sample blanks, reagent blanks and MDA and 4-HNE standards were included and the concentration of the analyte in each sample was calculated using the molar extinction coefficient at 586 nm of 110,000. The results were expressed as μM MDA/mg of protein.

Differences between means were assessed utilizing Prism (Graphpad, San Diego, CA). Analysis of variance or students t tests were used to determine significant differences among means. When needed, a Newman-Keuls post hoc test was used to test for specific differences. The accepted level of significance for Fobs was $p < 0.05$.

RESULTS

Hydrogen peroxide production was measured to determine if differences in ROS generation existed between Ames dwarf and normal, wild type mice. Young (three-month old), dwarf liver was not different from young, normal liver tissue with respect to the amount of H_2O_2 generated using either succinate or ADP as substrates (Figure 1). Mitochondria from 24-month old dwarf liver tissues consistently generated less H_2O_2 when compared to wild type mice from the same strain (Figure 1). In the presence of succinate (State 4 substrate), 42% less H_2O_2 was produced ($p < 0.03$), while in the presence of a State 3 substrate, ADP, 33% less H_2O_2 was produced by dwarf liver mitochondria ($p < 0.003$).

The determination of oxidative DNA damage was conducted in studies using dwarf, GH transgenic and corresponding age-matched normal mice. The results were variable, revealing some differences between dwarf and normal mice depending on tissue and age of the animals. No differences in liver 8OHdG:2dG were observed in three month old dwarf and normal animals. However, livers from dwarf mice tended ($p < 0.09$) to exhibit less DNA damage at 12 months of age when compared to wild type mice (Table 1). In contrast, 24-month old dwarf livers had elevated DNA damage compared to normal, wild type mice ($p < 0.02$). In this study, DNA damage appeared to accumulate with age in dwarf mice only ($p < 0.0001$; Table 1). In brain tissues of dwarf mice, no significant differences in oxidative DNA damage were noted (Table 2). Although 24 month old dwarf brains exhibited 32% lower 8OHdG:2dG ratios, when compared to wild type mice, the difference was not statistically significant ($p < 0.09$).

Similar results were obtained from liver and brain tissues of GH transgenic mice. No significant differences were observed between GH transgenic and wild type liver and brain tissues at three and 12 months of age (data not shown). These tissue samples varied greatly from animal to animal in their 8OHdG:2dG ratios.

The amount of carbonylated protein was measured in dwarf and GH transgenic tissues as a parameter of oxidative protein damage. Three-month old dwarf and normal mice did not differ in the nmol DNPH/mg protein (Figure 2). However, at 24 months of age, livers from dwarf mice exhibited 47% fewer ($p < 0.04$) protein carbonyls when compared to age-matched normal mice. In

Table 1. Oxidative DNA damage (8OHdG:2dG) in liver tissue from 3, 12, and 24 month old Ames dwarf and normal mice.

	3 month	12 month	24 month	P value Age 3 vs 24 mon.	P value Age 12 vs 24 mon.
Dwarf	3.704 ± 0.65	1.325 ± 0.05	6.375 ± 0.48	.14	.0001
Normal	2.822 ± 0.33	1.950 ± 0.31	3.225 ± 0.93	.94	.24
P value Line	.21	.09	.02		

Values represent means $\pm$ SEM.

Table 2. Oxidative DNA damage (8OHdG:2dG) in brain tissues from 12 and 24 month old Ames dwarf and normal mice.

	12 month	24 month	P value Age
Dwarf	2.440 ± 0.38	2.025 ± 0.39	.45
Normal	2.240 ± 0.47	2.975 ± 0.26	.20
P value Line	.75	.09	

Values represent means $\pm$ SEM.

Ames dwarf mice, no significant difference was detected in oxidative protein damage to liver between three and 24 months of age. As expected, protein oxidative damage appeared to accumulate with age in normal wild type mice ($p < 0.02$). Fewer protein carbonyls ($p < 0.01$) were observed in three-month old dwarf brain tissues compared to normal mouse tissues (Figure 3). In contrast to the findings in liver, no differences were detected at 24 months, largely due to very large variances between

Fig. 1. Liver mitochondrial hydrogen peroxide generation (pmol/μg protein/min) in 3 and 24 month old Ames dwarf and normal, wild type mice. Data represent means $\pm$ SEM. *Represents differences between dwarf and normal mitochondria in the presence of succinate or ADP; * $p < 0.03$ ** $p < 0.003$.

mice. Brain tissues from dwarf mice showed accumulation of protein oxidative damage with age ($p < 0.02$), however, the variation was too great to show this in the normal wild type brains (Figure 3). No differences in protein carbonyl content were detected in heart tissues from dwarf and normal mice (data not shown).

Forty percent more ($p < 0.04$) protein carbonyls were detected in livers from three-month old GH transgenic

mice compared to wild type controls (Figure 4). No differences were observed in 12-month old animals however both groups of mice showed an increased amount of protein oxidative damage with age ($p < 0.01$). Brain protein carbonyls were 53% higher ($p < 0.005$) in GH transgenic mice at 12 months of age compared to normal wild type mice from the same strain (Figure 5). The data from animals at three months of age reflected large amounts of variation and therefore were not different.

Fig. 2. Liver protein carbonyl content (nmol DNPH/mg protein) in 3 and 24 month old Ames dwarf and normal, wild type mice. Values represent means $\pm$ SEM. *Represents differences between dwarf and normal mice at 24 months of age. #Represents differences between 3 and 24 month old normal mice. * $p < 0.04$ # $p < 0.03$.

Fig. 3. Brain protein carbonyl content (nmol DNPH/mg protein) in 3 and 24 month old Ames dwarf and normal, wild type mice. Values represent means $\pm$ SEM. *Represents differences between dwarf and normal mice at 3 months of age. #Represents differences between 3 and 24 month old dwarf mice. * $p < 0.01$ # $p < 0.02$.

Fig 4. Liver protein carbonyl content (nmol DNPH/mg protein) in 3 and 12 month old GH transgenic and normal, wild type mice. Values represent means $\pm$ SEM. *Represents differences between GH transgenic and normal mice at 3 months of age. #Represents differences between 3 and 12 month old GH transgenic mice. ##Represents differences between 3 and 12 month old normal mice. * $p < 0.04$ # $p < 0.01$ ## $p < 0.003$.

Fig 5. Brain protein carbonyl content (nmol DNPH/mg protein) in 3 and 12 month old GH transgenic and normal, wild type mice. Values represent means $\pm$ SEM. *Represents differences between GH transgenic versus normal mice at 12 months of age. * $p < 0.005$.

The level of lipid peroxidation was measured by determining the levels of malonaldehyde or malonaldehyde and 4-hydroxyalkenal. Although three-month old dwarf livers appeared to have less MDA/mg protein, this difference was not significant ($p < 0.09$). Livers from 12-month old dwarf mice did not exhibit differences in MDA content (μM MDA/mg protein; Figure 6). However, 24-month old Ames dwarf mice appeared to have more MDA/mg protein compared to normal wild type mice

Fig 6. Liver malonaldehyde levels (μM malonaldehyde/mg protein) in 3, 12 and 24 month old Ames dwarf and normal, wild type mice. Values represent means $\pm$ SEM. *Represents differences between dwarf and normal mice at 24 months of age. #Represents differences between 3 and 24 month old dwarf mice. * $p < 0.007$ # $p < 0.001$.

Fig 7. Liver malonaldehyde levels (μM malonaldehyde/mg protein) in 3, 12 and 24 month old Ames dwarf and normal, wild type mice. Values represent means $\pm$ SEM. * $p < 0.005$ dwarf versus normal mice at 12 months of age. *Represents differences between dwarf and normal mice at 24 months of age. #Represents differences between 3 and 24 month old dwarf mice. ##Represents differences between 3 and 24 month old normal mice. ** $p < 0.0001$ # $p < 0.001$ ## $p < 0.001$.

($p < 0.006$). In addition, the concentration of MDA significantly increased with aging in both dwarf ($p < 0.001$) and normal ($p < 0.001$) mice. When MDA + 4-HNE levels were determined together, 12 and 24 month old dwarf livers exhibited 47 and 77% more, respectively, compared to normal mice (Figure 7). And, as seen with MDA alone, this measure of lipid peroxidation increased with age ($p < 0.001$) in liver tissue from both dwarf and normal mice (Figure 7).

Fig. 8. Brain malonaldehyde levels (μM malonaldehyde/mg protein) in 3 and 20 month old Ames dwarf and normal, wild type mice. Values represent means $\pm$ SEM. *Represents differences between dwarf and normal mice at 3 months of age. #Represents differences between 3 and 20 month old normal mice. * $p < 0.05$ # $p < 0.001$.

The level of MDA in brain protein from three-month old normal mice was elevated 30% ($p < 0.05$) over that of dwarf mice while levels at 20 months of age were not found to differ significantly (Figure 8). Interestingly, less MDA was noted in older normal mouse brain tissue ($p < 0.001$) although no differences in MDA with age were revealed for dwarf mice.

DISCUSSION

Ames dwarf mice live longer than wild type mice of the same strain. Heightened antioxidative defenses may play a role in the observed life span extension (14, 33). In the current studies, we have shown that Ames dwarf mice also generate fewer ROS and in many cases, likely results in reduced oxidative damage when compared to their normal counterparts. In addition, a premature aging mouse, the GH transgenic, tend to exhibit greater protein oxidative damage. This finding in transgenic mice, along with evidence of suppressed antioxidative defenses (14), suggest that oxidative stress may play a role in premature aging as well.

Determining the level of ROS production in Ames dwarf mice was critical in relating the heightened antioxidative defenses to the dramatic extension of lifespan observed previously (13). According to the free radical theory of aging, less oxidative damage accumulates leading to longer life if either fewer free radicals are generated and/or elevated antioxidative defenses are in place. In this study, reduced production of H_2O_2 was observed in 24 month old Ames dwarf liver mitochondria utilizing succinate, a State 4 substrate, and ADP, a State 3 substrate. These results suggest that at least in liver tissue, dwarf mice are subjected to less oxidative stress and may need less O_2 to control membrane leakiness when compared to normal mice (7). In addition,

while chronic exposure to ROS can result in oxidative damage to protein, lipids and DNA, acute exposure can inactivate electron transport chain complexes (I, II, III) resulting in reduced mitochondrial energy production (34, 35). We have preliminary evidence indicating that in fact, protein levels of Complexes I, II and V are elevated in dwarf mice (36). Calorically restricted animals also exhibit lower ROS production rates compared to ad libitum fed animals (37). In the cell, H_2O_2 is detoxified by both catalase and glutathione, both of which are significantly elevated in dwarf liver tissue (14, 38). GH-deficient dwarf mice are also hypothyroid and exhibit a lower core body temperature when compared to age-matched wild type mice (39). Presumably, metabolic activity is lower leading to fewer ROS generated in these mice. However, GH receptor KO mice are only slightly hypothermic and still live 30-50% longer suggesting that the hypothyroidism may not be the key player in the life span extension of the Ames dwarf mouse. Rather, a deficiency in GH signaling may be responsible for the dramatic extension in life span observed in these two types of mice.

Hydroxyl free radicals attack DNA producing 8-OHdG and other oxidation products. DNA damage plays a role in mutagenesis, carcinogenesis, cell death and is highly correlated with aging as less damage is observed in longer living species. High performance liquid chromatography is a sensitive method used to measure guanine adducts. With this method, we found no differences in young (3 month) liver, less damage in dwarfs at 12 months and in old dwarfs (24 month) higher 8-OHdG:2dG ratios compared to controls. A recent report supported earlier studies demonstrating significant age-related increases in 8OHdG in both nuclear and mitochondrial DNA from various tissues of mice and rats (40). Early reports showed that normal oxidative damage to both nuclear and mitochondrial DNA was extensive (41). Even though antioxidative defenses are elevated and less H_2O_2 is produced in dwarf liver, some enzymes may not be as effective in repairing damaged (base excision repair) DNA. In addition, both the amount and significance of cumulative oxidative damage in aging is unclear (42). Another recent report showed a linear increase in mitochondrial 8OHdG glycosylase with age in mouse liver (43). In brain tissues, less oxidative DNA damage was observed in dwarf mice suggesting better protection against oxidative stress or repair of altered structures. It has been shown that brain is one organ most vulnerable to oxidative stress due to high O_2 consumption, high concentrations of unsaturated fatty acids, a large store of iron and low antioxidant capacity. Our previous studies did not find major differences in levels of catalase and SOD in whole brains of Ames dwarf mice (14; unpublished data). However, others have evaluated antioxidative enzymes in specific brain regions and demonstrated differences in the Ames dwarf mouse (44). Therefore, there may be region specific oxidative damage (45) in these animals that

was not detected in our whole brain preparation. In addition, we have found that dwarf mice appear vulnerable to oxidative stress induced by MPP⁺ administration as Parkinson's-like tremors develop at the same rate as normal wild type mice (Brown-Borg, unpublished data). In contrast, Ames dwarf mice exhibit greater survival than normal mice following paraquat-induced oxidative stress suggesting better systemic oxidative defenses (46).

Oxidative protein damage occurs when free radicals interact with neighboring proteins. This free radical attack results in the formation of carbonyls on afflicted proteins, among other types of specific damage due to oxidant exposure. Dwarf mice exhibited less oxidative damage to protein in both liver and brain tissues compared to normal mice. This is not surprising in light of the reduced H₂O₂ production and heightened antioxidative defense capacity. Although we measured whole tissue protein oxidative damage, recent studies have shown no evidence for increased protein oxidative damage in mitochondria relative to cytosol (47). Interestingly, protein damage did not accumulate with age in dwarf mice suggesting possible differences in protein degradation processes resulting in fewer residual damaged proteins. The proteasome is located in the cytosol and nuclei and in general, is involved in selective degradation of regulatory intracellular proteins and of malfunctioning protein molecules (48, 49). Proteasome activity has been shown to decline with aging (50-53) possibly resulting in the accumulation of modified proteins. Preventing protein oxidation or removing damaged proteins more efficiently (increased turnover) may be one mechanism that dwarf mice utilize that results in the increased longevity of these mice (54). For example, if the PARP protein (polyadenosine diphosphate ribose polymerase) became oxidized, DNA repair would be inhibited, allowing DNA damage to accumulate and result in a shorter life span (55). An additional mechanism that plays a role in resistant to oxidative stress is glutathione (GSH; 56). Several tissues from dwarf mice exhibit significantly elevated GSH levels (38).

Close proximity to increased oxidant generative processes leaves membranes susceptible to oxidative insults resulting in lipid peroxidation. Accordingly, the effects of hydroxyl radical attack on lipids initiate a chain reaction that produces hundreds or thousands of lipid hydroperoxide molecules from a single initial event (57). In contrast to these toxic effects of lipid hydroperoxides and 4HNE, lower levels have regulatory and messenger functions, particularly in stress signaling (58, 59). Malonaldehyde and 4-hydroxyalkenal are commonly used indicators of lipid peroxidation. Aged dwarf mice had more MDA/mg protein when compared to wild type mice. Initially, this observation was surprising although there is at least one report indicating that elevated catalase levels increased both MDA and conjugated diene levels in liver tissue (60). This suggests that although dwarf mice have elevated hepatic catalase activity, it may not play an important role in the

development of hepatic lipid peroxidation or may not be present in amounts necessary to prevent lipid peroxidation. The dwarf may exhibit greater protection against oxidative protein and DNA damage while mechanisms protecting from oxidative lipid damage may operate at less than optimal levels [i.e. glutathione peroxidases (GPX), glutathione-S-transferase-catalyzed conjugation]. Correspondingly, dwarf mice have lower liver GPX activity (33) and we have preliminary evidence indicating that GPX protein levels are also significantly lower in dwarf liver tissue when compared to wild type liver (Brown-Borg, unpublished data). In addition, we have previously demonstrated that 12 month old liver inorganic peroxide levels (using procedure of Meitattini, 1985; 61) were lower in dwarf versus normal, wild type mice (62). This previous observation was confirmed (63) in 26 month-old dwarf mice – again showing lower inorganic peroxide levels in liver and kidney compared to normal mice. In this study (63), levels of lipid peroxides found were variable. Male dwarf mice exhibited less liver lipid peroxides while female dwarfs did not differ from age-matched controls (63). Dwarf kidney levels of lipid peroxides were similar to normal animals. Different results could be attributed to assay type and sensitivity and the fact that lipid peroxidation is more transient in nature when compared to other forms of oxidative damage. There are reports showing that lipid peroxidation is not altered in hypothyroid mice (64) or rats (65) compared to euthyroid animals.

Superoxide generation has been shown to be significantly elevated in rodents overexpressing GH (66). In the current study, we found that GH transgenic mice exhibited elevated levels of oxidative protein damage to both liver and brain at different ages compared to their normal counterparts. Recently, we showed that catalase is significantly suppressed in tissues from GH transgenic mice (14) while others have found that GH excess is associated with early declines in SOD and GPX (67). No differences in the amount of oxidized DNA (8OHdG) were detected between GH transgenic and normal mice. Lipid peroxidation was not measured in this study as previous reports indicated that indices of lipid peroxidation were significantly higher in animals with high plasma GH compared to controls (66).

The specific role of GH in aging, and antioxidative processes remains to be elucidated. Body size has been shown to play a role in aging as smaller breeds of animals (dogs and horses) live longer than larger breeds (68, 69) and short humans tend to live longer than tall individuals (70, 71). The interactions between GH and the insulin-like growth factors in determination of both final body size and metabolism may contribute to possible alterations in antioxidative defenses resulting in differences in oxidative damage and overall life span.

Other mammalian models of GH insufficiency also live longer. The extended life span of Ames and Snell dwarf mice (15, 16) that are homozygous for unrelated

mutations on different chromosomes (72, 73) strongly suggests that this effect is due to phenotypic characteristics common to the two mutants. Mice unable to respond to GH, GH receptor knockouts, have also been shown to outlive their normal counterparts (17, 18). Importantly, evidence presented by Krzisnik and co-workers (74) shows increased longevity in hypopituitary patients with mutations at the Prop-1 locus, the same gene that is mutated in the Ames dwarf. In sharp contrast, mice overexpressing GH live significantly shortened lives, exhibit increased indices of free radical processes (66), decreased antioxidative defenses (14, 67), signs of accelerated aging including reduced replicative potential of cells in vitro (75), and signs of premature central nervous system aging including reduced catecholamine turnover, increased astrogliosis, and impaired learning and memory (19, 76, 77). In addition, these animals typically reach sexual maturation earlier and cessation of reproduction sooner than wild type siblings (21, 78). According to Kajiura and Rollo (79), the increased rate of physiological decline in mice overexpressing GH maybe due to a disproportional allocation of energy towards growth versus repair mechanisms, reproduction and behavioral activities.

Although GH deficiency in elderly humans is characterized by increased adiposity, muscle atrophy and reduced cardiac function, and can be reversed by GH administration (80, 81), the long term effects of GH replacement on lifespan are unknown. The dwarf gene may selectively modify various components of the antioxidant defense system leading to less oxidative damage and an overall extension in lifespan. Notably, the delayed aging observed in Ames dwarf mice is not limited to phenotypic and biochemical parameters but also behavioral parameters. Growth hormone deficient Ames dwarf mice do not experience age-related declines in cognitive function and locomotor activity found in wild type mice (82).

Our laboratory has significant evidence showing that GH and IGF-1 (main mediator of growth hormone's actions on growth) suppress the activities of both catalase and glutathione peroxidase in vitro (83). In addition, the relationship between extended life span and GH/IGF hormones is not limited to studies conducted in mammals. There are reports in yeast (*PKA* and *Sch9*—both involved in nutrient utilization/Akt/PKB; 84), worms (*c. elegans*; *daf-2-85*; *age-1-86*; both components of insulin/IGF pathway), and flies (insulin receptor mutant-87; *chico*-insulin receptor substrate gene- 88) that reduced activity of the GH/IGF/insulin signaling pathway results in significant extensions in life span. In addition, disrupting the *p66^{shc}* gene, which is involved in intermediary metabolism, also extends life span in rodents (89). Therefore, the control of metabolic activity by the GH/IGF/insulin signaling pathway may be a universal regulator of aging across species. Another notable point is that there are numerous types of oxidative damage other than those measured in the present study (8OHdG,

protein carbonyls and malonaldehyde). The altered metabolic state of the Ames dwarf and GH transgenic animals may shift the balance towards these other types of damage (i.e. nitrotyrosine, 3-methylguanine, 5-hydroxycytidine). Finally, although aging is associated with an overall decline in antioxidative enzyme activities, a significant increase in H₂O₂ production, and an accumulation of oxidative damage, the decline in GH that occurs with aging may benefit overall antioxidant status.

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REFERENCES

1. Harman, D: The aging process. *Proc. Natl. Acad. Sci. (USA)* 78, 7124-7128, 1981.
2. Pryor, WA: The free radical theory of aging revisited: A critique and a suggested disease-specific theory, in *Modern Biological Theories of Aging*, edited by Warner, HR, Butler, RN, Sprott, RL and Schneider, EL, Raven Press, New York, 1987, pp. 89-112.
3. Sohal, RS, and Dubey, A: Mitochondrial oxidative damage, hydrogen peroxide release, and aging. *Free Rad. Biol. Med.* 16:621-626, 1994.
4. Orr, W, and Sohal, RS: Extension of life-span by overexpression of superoxide dismutase and catalase in *Drosophila melanogaster*. *Science* 257:1220-1224, 1994.
5. Ames, BN, Shigenaga, MK, and Hagen, TM: Oxidants, antioxidants and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. (USA)* 90, 7915-7922, 1993.
6. Halliwell, B and Gutteridge, JMC: Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* 219:1-14, 1984.
7. Wei, YH: Oxidative stress and mitochondrial DNA mutations in human aging. *Proc. Soc. Exp. Biol. Med.* 217:53-63, 1998.
8. Sohal, RS, Svensson I, and Brunk, UT: Hydrogen peroxide production by liver mitochondria in different species. *Mech. Age. Dev.* 53:209-215, 1990.
9. Ku, HH, and Sohal, RS: Comparison of mitochondrial prooxidant generation and antioxidant defenses between rat and pigeon: Possible basis of variation in longevity and metabolic potential. *Mech. Age. Dev.* 72:67-76, 1993.

10. Sohal, RS, and Weindruch, R: Oxidative stress, caloric restriction, and aging. *Science* 273:59-63, 1996.
11. Forster, MJ, Sohal, BH, and Sohal, RS: Reversible effects of long-term caloric restriction on protein oxidative damage. *J. Gerontol.* 55A:B522-B529, 2000.
12. Weindruch, R: The retardation of aging by caloric restriction: studies in rodents and primates. *Toxicol Pathol.* 24:742-5, 1996.
13. Brown-Borg, HM, Borg, KE, Meliska, CJ, and Bartke A: Dwarf mice and the aging process. *Nature* 384:33, 1996.
14. Brown-Borg, HM, and Rakoczy, SG: Catalase expression in delayed and premature aging mouse models. *Exp Gerontol* 35:199-212, 2000.
15. Miller, RA: Are there genes for aging? *J Gerontol. Biol Sci* 54A:B297-B307, 1999.
16. Flurkey, K, Papconstantinou, J, Miller, RA, and Harrison, DA: Lifespan extension and delayed immune and collagen aging in mutant mice with defects in growth hormone production. *Proc. Natl. Acad. Sci.* 98:6736-6741, 2001.
17. Kopchick, JJ, and Laron, Z: Is the Laron mouse an accurate model of Laron Syndrome? *Mol. Genet. Metab.* 68, 232-236, 1999.
18. Coschigano, KT, Clemmons, D, Bellush, LL, and Kopchick JJ: Assessment of growth parameters and life span of GHR/BP gene-disrupted mice. *Endocrinology.* 141:2608-13, 2000.
19. Steger, RW, Bartke, A, and Cecim, M: Premature aging in transgenic mice expressing growth hormone genes. *J Repro Fertil Suppl* 46:61-75, 1993.
20. McGrane, MM, DeVente, J, Yun, J, Bloom, J, Park, E, Wynshaw-Boris, A, Wagner, T, Rottman, FM, and Hanson, FW: Tissue-specific expression and dietary regulation of a chimeric phosphoenolpyruvate carboxykinase/bovine growth hormone gene in transgenic mice. *J Biol Chem* 263:11443-11451, 1988.
21. Steger, RW, Bartke, A, Parkening, TA, Collins, T, Cerven, R, Jun, JS, and Wagner, TE: Effects of chronic exposure to bovine growth hormone (bGH) on the hypothalamic-pituitary axis in transgenic mice: relationship to the degree of expression of the PEPCK.bGH hybrid gene. *Transgenics* 1:245-253, 1994.
22. Cecim, M, Bartke, A, Yun, JS and Wagner, TE: Expression of human, but not bovine, growth hormone genes promotes development of mammary tumors in transgenic mice. *Transgenics* 1:431-437, 1994.
23. Trounce, IA, Kim, YL, Jun, AS, and Wallace, DC: Assessment of mitochondrial oxidative phosphorylation in patient muscle biopsies, lymphoblasts, and transmittochondrial cell lines. *Methods Enzymol.* 264:484-509 (1996).
24. Kwong, LK, and Sohal, RS: Substrate and site specificity of hydrogen peroxide generation in mouse mitochondria. *Arch. Biochem. Biophys.* 350:118-126, 1998.
25. Floyd, RA, Watson, JJ, Wong, PK, Altmiller, DH, and Rickard, RC: *Free Rad. Res. Comm.* 1:163-172, 1986.
26. Park, JW, and Floyd, RA: Lipid peroxidation products mediate the formation of 8-hydroxydeoxyguanosine in DNA. *Free Rad. Biol. Med.* 122:245, 1989.
27. de Zwart, LL, Meerman, JHN, Commandeur, JNM, and Vermeulen, NPE: Biomarkers of free radical damage applications in experimental animals and in humans. *Free Rad. Biol. Med.* 26:202-226, 1999.
28. Shigenaga, MK, Aboujaoude, EN, Chen, Q, and Ames, BN: Assays of oxidative DNA damage biomarkers 8-oxo-2'-deoxyguanosine and 8-oxoguanine in nuclear DNA and biological fluids by HPLC with electrochemical detection. *Methods Enzymol.* 334:16-59, 1994a.
29. Shigenaga, MK, Hagen, TM, and Ames, BN: Oxidative damage and mitochondrial decay in aging. *Proc. Natl. Acad. Sci.* 91:10771-10778, 1994b.
30. Levine, RL, Garland, D, Oliver, CN, Amici, A, Climent, I, Lenz, AG, Ahn, BW, Shmuel, S, and Stadtman, ER: *Methods Enzymology* 186:464-478, 1990.
31. Esterbauer, H, Schaur, RJ, and Zollner, H: Chemistry and biochemistry of 4-hydroxynoneal, malonaldehyde and related aldehydes. *Free Rad. Biol. Med.* 11:81, 1991.
32. Bradford, MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248-254, 1976.
33. Brown-Borg, HM, Bode, AM, and Bartke A: Antioxidative mechanisms and plasma growth hormone levels: Potential relationship in the aging process. *Endocrine* 11:41-48, 1999.
34. Wallace, DC: Mitochondrial diseases in man and mouse. *Science.* 283:1482-8, 1999.
35. Melov S, Coskun, P, Patel, M, Tuinstra, R, Cottrell, B, Jun, AS, Zastawny, TH, Dizdaroglu, M, Goodman, SI, Huang, TT, Miziorko, VH, Epstein, CJ, and Wallace, DC: Mitochondrial disease in superoxide dismutase 2 mutant mice. *Proc. Natl. Acad. Sci.* 96:846-51, 1999.

36. Brown-Borg, HM, and Harman, SM: Hormones and oxidative stress, in *Oxidative Stress and Aging: Advances in Basic Science, Diagnostics and Intervention*, edited by Cutler, RG, and Rodriguez, H, World Scientific Pub., New Jersey, 2002 (in press).
37. Kim, JD, McCarter, RJM, and Yu, BP: Influence of age, exercise, and dietary restriction on oxidative stress in rats. *Age. Clin. Exp. Res.* 8:123-129, 1996.
38. Brown-Borg, HM, Rakoczy, SG, Kennedy, MA, and Romanick, MA: Relationship between plasma growth hormone, antioxidants and oxidative damage in premature and delayed aging mice. *Endocrine Society Abstract* p. 237-238, 2001.
39. Hunter, WS, Croson, WB, Bartke, A, Gentry, MV, and Meliska, CJ: Low body temperature in long-lived Ames dwarf mice during rest and during stress. *Physiol. Behav.* 67:433-437, 1999.
40. Hamilton, ML, Van Remmen, H, Drake, JA, Yand, H, Guo, ZM, Kewitt, Walter, CA and Richardson, A: Does oxidative damage to DNA increase with age? *Proc. Natl. Aca. Sci.* 2001.
41. Richter, C, Park, JW, and Ames, BN: Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proc. Natl. Acad. Sci.* 85:6465-6467, 1988.
42. Forman, HJ, and Azzi, A: On the virtual existence of superoxide anions in mitochondria: thoughts regarding its role in pathophysiology *FASEB J.* 11:374-5, 1997.
43. de Souza-Pinto, NC, Hogue, BA, and Bohr, VA: DNA repair and aging in mouse liver: 8-oxoDG glycosylase activity increase in mitochondrial but not in nuclear extracts. *Free Rad. Biol. Med.* 30:916-923, 2001.
44. Hauck, S, and Bartke, A: Effects of growth hormone on hypothalamic catalase and CuZn superoxide dismutase. *Free Rad Biol Med* 28:970-978, 2000.
45. Cardoza-Pelaez, S, Song, S, Parthasarathy, A, Epstein, CJ, and Sanchez-Ramos, J: Attenuation of age-dependent oxidative damage to DNA and protein in brainstem of Tg Cu/Zn SOD mice. *Neurobiol. Aging* 19:311-316, 1998.
46. Bartke, A: Delayed aging in Ames dwarf mice. Relationships to endocrine function and body size, in *Results and Problems in Cell Differentiation: The Molecular Genetics of Aging*, Vol, 29, edited by Hekimi, Springer-Verlag, Verlin, 2000, pp. 181-202.
47. Davies, SMK, Poljak, A, Duncan, MW, Smythe, GA, and Murphy, MP: Measurements of protein carbonyls, ortho- and meta-tyrosine and oxidative phosphorylation complex activity in mitochondria from young and old rats. *Free Rad. Biol. Med.* 31:181-190, 2001.
48. Coux, O, Tanaka, K, and Goldberg, AL: Structure and functions of the 20S and 26S proteasomes. *Annu. Rev. Biochem.* 65:801-847, 1996.
49. Rivett, AJ: Intracellular distribution of proteasomes. *Curr Opin Immunol.* 10:110-4, 1998.
50. Stadtman, ER: Protein oxidation and aging. *Science* 257, 1220-1224, 1992.
51. Starke-Reed, PE, and Oliver, CN: Protein oxidation and proteolysis during aging and oxidative stress. *Arch. Biochem. Biophys.* 275:559, 1989.
52. Conconi, M, and Friguet, B: Proteasome inactivation upon aging and on oxidation-effect of HSP 90. *Mol Biol Rep.* 24:45-50, 1997.
53. Petropoulos, I, Conconi, M, Wang, X, Hoenele, B, Bregegere, F, Milner, Y, and Friguet, B: Increase of oxidatively modified protein is associated with a decrease of proteasome activity and content in aging epidermal cells. *J. Gerontol.* 55A:B220-B227, 2000.
54. Davies, KJA: Protein damage and degradation by oxygen radicals. I. General aspects. *J. Biol. Chem.* 262:9895-9901, 1987.
55. Pero, RW, Hoppe, C, and Sheng, Y: Serum thiols as a surrogate estimate of DNA repair correlates with human life span. *J. Anti-Aging Med.* 3:241-249, 2000.
56. Ehrhart, J, and Zeevalk, GD: Hydrogen peroxide removal and glutathione mixed disulfide formation during metabolic inhibition in mesencephalic cultures. *J. Neurochem.* 77:1496-1507, 2001.
57. Gutteridge, JMC, and Halliwell, B: The measurement and mechanism of lipid peroxidation in biological systems. *Trends Biochem. Sci.* 15:12-135, 1990.
58. Dianzani, MU: 4-hydroxynoneal and cell signaling. *Free Rad. Res.* 28:553-560, 1998.
59. Uchida, K, Shiraishi, M, Naito, Y, Torii, Y, Nakamura, Y, and Osawa, T: Activation of stress signaling pathways by the end product of lipid peroxidation. 4-hydroxy-2-nonenal is potential inducer of intracellular peroxide production. *J. Biol. Chem.* 274:2234-2242, 1999.
60. Nilakantan, V, Spear, BT, and Glauert, HP: Effect of the peroxisome proliferator ciprofibrate on lipid peroxidation and 8-hydroxydeoxyguanosine formation in transgenic mice with elevated hepatic catalase activity. *Free Rad. Biol. Med.* 24(9):1430-1436, 1998.
61. Meattini, F: Inorganic peroxides, in *Methods of Enzymatic Analysis*, 2nd edition, edited by Bernt, E, Bergmeyer, H, Academic Press, New York, 1985, pp. 566-571.

62. Bartke, A, Brown-Borg, HM, Bode, AM, Carlson, J, Hunter, WS, and Bronson, RT: Does growth hormone prevent or accelerate aging? *Exp. Geron.* 33:675-687, 1998.
63. Carlson, JC, Bharadwaj, R and Bartke, A: Oxidative stress in hypopituitary dwarf mice and in transgenic mice overexpressing human and bovine GH. *Age* 22:181-186, 1999.
64. Guerrero, A, Pamplona, R, Portero-Otin, M, Barja, G, and Lopez-Torres, M: Effect of thyroid status on lipid composition and peroxidation in the mouse liver. *Free Rad. Biol. Med.* 26:73-80, 1999.
65. Asayama, K, Dobashi, K, Hayashibe, H, Megata, Y, and Kato, K: Lipid peroxidation and free radical scavengers in thyroid dysfunction in the rat: a possible mechanism of injury to heart and skeletal muscle in hyperthyroidism. *Endocrinology* 121:2112-2118, 1987.
66. Rollo, CD, Carlson, J, and Sawada, M: Accelerated aging of giant transgenic mice is associated with elevated free radical processes. *Can. J. Zool.* 74:606-620, 1996.
67. Hauck, S, and Bartke, A: Free radical defenses in the liver and kidney of human growth hormone transgenic mice: possible mechanisms of early mortality. *J Gerontol Bio Sci* 56A:B153-162, 2001.
68. Promislow, DE: On size and survival: progress and pitfalls in the allometry of life span. *J. Gerontol.* 48, B115-B123, 1993.
69. Patronek GJ, Waters, DJ, and Glickman, LT: Comparative longevity of pet dogs and humans: Implications for gerontology research. *J. Gerontol.* 52A:B171-178, 1997.
70. Samaras, T: How body height and weight affect our performance, longevity, and survival. *J. Wash. Aca. Sci.* 84:131-156, 1996.
71. Samaras, TT, Elrick, H, and Storms, LH: Height, health and growth hormone. *Acta Paediatr* 88:602-609, 1999.
72. Somson, MW, Wu, W, Dasen, JS, Flynn, SE, Norman, DJ, O'Connell, SM, Gukovsky, I, Carriere, C, Ryan, AK, and Miller, AP: Pituitary lineage determination by the prophet of pit-1 homeodomain factor defective in Ames dwarfism. *Nature* 384:327-333, 1996.
73. Li, S, Crenshaw, EB, 3rd, Rawson, EJ, Simmons, DM, Swanson, LW, and Rosenfeld, MG: Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene pit-1. *Nature* 347:528-533, 1990.
74. Krzisnik, C, Kolacio, Z, Battelino, T, Brown, M, Parks, JS, and Laron, Z: The 'little people' of the Island of Krk – revisited. Etiology of hypopituitarism revealed. *J Endo Genet* 1:9-19, 1999.
75. Pendergrass, WR, Li, Y, Jiang, D, and Wolf NS: Decrease in cellular replicative potential in giant mice transfected with the bovine growth hormone gene correlates to shortened life span. *J. Cell. Physiol.* 156, 96-103, 1993.
76. Miller, DB, Bartke, A, and O'Callaghan, JP: Increased glial fibrillary acidic protein (GFAP) levels in the brains of transgenic mice expressing the bovine growth hormone (bGH) gene. *Exp. Gerontol.* 30, 383-400, 1995.
77. Meliska, CJ, Burke, PA, Bartke, A, and Jensen, RA: Inhibitory avoidance and appetitive learning in aged normal mice: comparison with transgenic mice having elevated plasma growth hormone levels. *Neurobiol. Learn. Mem.* 68:1-12, 1997.
78. Naar EM, Bartke A, Majumdar SS, Buonomo FC, Yun JS, and Wagner TE: Fertility of transgenic female mice expressing bovine growth hormone or human growth hormone variant genes. *Biol Reprod.* 45:178-87, 1991.
79. Kajiura, LJ and Rollo, CD: A mass budget for transgenic 'supermice' engineered with extra growth hormone genes: evidence for energetic limitation. *Can. J. Zool.* 72:1010-1017, 1994.
80. Rudman, D, Feller, AG, Nagraj, HS, Gergans, GA, Lalitha, PY, Goldberg, AF, Schenkler, RA, Cohn, L, Rudman, IW, and Mattson, DE: Effects of human growth hormone in men over 60 years old. *New. Engl. J. Med.* 323:1-6, 1990.
81. Sonksen, PH, Cuneo, RC, Salomon, F, McGauley, G, Wiles, CM, Wilmschurst, P, Byrne, C, Hesp, R, Lowy, C, and Weissberger, A: Growth hormone therapy in adults with growth hormone deficiency. *Acta Paediatr Scand Suppl.* 379:139146, 1991.
82. Kinney, BA, Meliska, CJ, Steger, RW, and Bartke, A: Evidence that Ames dwarf mice age differently from their normal siblings in behavioral and learning and memory parameters. *Horm Behav* 39:277-285, 2001.
83. Brown-Borg, HM, Rakoczy, SG, Romanick, MA, and Kennedy, MA: Effects of growth hormone and insulin like growth factor-1 on hepatocyte antioxidative enzymes. *Exp. Biol. Med.* (in press), 2002.
84. Fabrizio P, Pozza, F, Pletcher, SD, Gendron, CM, and Longo, VD: Regulation of longevity and stress resistance by Sch9 in yeast. *Science* 292:288-290, 2001.
85. Kenyon, C, Chang J, Gensch, E, Rudner, A, and Tabtiang, RA: *Caenorhabditis elegans* mutant that lives twice as long as wild type. *Nature* 266:461, 1993.

86. Johnson, TE, and Hutchinson, EW: Aging in *Caenorhabditis elegans*: update 1988, in Review of Biological Research in Aging, Vol. 4, edited by Rothstein, M, New York, Alan R Liss, 1990.
87. Tatar, M, Kopelman, A, Epstein, D, Tu, MP, Yin, CM, and Garofalo, RS: A mutant *drosophila* insulin receptor homolog that extends life-span and impairs neuroendocrine function. *Science* 292:107-110, 2001.
88. Clancy, DJ, Gems, D, Harshman, LG, Oldham, S, Stocker, H, Hafen, E, Leivers, SJ, and Partridge, L: Extension of life-span by loss of CHICO, a *Drosophila* insulin receptor substrate protein. *Science* 292:104-106, 2001.
89. Migliaccio, E, Giorgio, M, Mele, S, Pelicci, G, Reboldi, P, Pandolfi, PP, Lanfranccone, L, and Pelicci, PG: The p66^{shc} adaptor protein controls oxidative stress and life span in mammals. *Nature* 402:309-313, 1999.