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DNA oxidative damage and life expectancy in houseflies

(aging/oxygen free radicals/DNA oxidation/oxidative stress/insects)

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ABSTRACT **The objective of this study was to explore the relationship between oxidative molecular damage and the aging process by determining whether such damage is associated with the rate of aging, using the adult housefly as the experimental organism. Because the somatic tissues in the housefly consist of long-lived postmitotic cells, it provides an excellent model system for studying cumulative age-related cellular alterations. Rate of aging in the housefly was manipulated by varying the rate of metabolism (physical activity). The concentration of 8-hydroxydeoxyguanosine (8OHdG) was used as an indicator of DNA oxidation. Exposure of live flies to x-rays and hyperoxia elevated the level of 8OHdG. The level of 8OHdG in mitochondrial as well as total DNA increased with the age of flies. Mitochondrial DNA was 3 times more susceptible to age-related oxidative damage than nuclear DNA. A decrease in the level of physical activity of the flies was found to prolong the life-span and correspondingly reduce the level of 8OHdG in both mitochondrial and total DNA. Under all conditions examined, mitochondrial DNA exhibited a higher level of oxidative damage than total DNA. The 8OHdG levels were found to be inversely associated with the life expectancy of houseflies. The pattern of age-associated accrual of 8OHdG was virtually identical to that of protein carbonyl content. Altogether, results of this study support the hypothesis that oxidative molecular damage is a causal factor in senescence.**

Recent studies in this laboratory, using transgenic *Drosophila melanogaster*, have provided direct support for the concept that oxidative stress is a causal factor in the aging process (1). It is widely postulated that oxidative stress results in the infliction of molecular damage, some of which, being irreversible, accumulates with age, causing progressive physiological attrition. Indeed, an age-related increase in the concentration of the DNA-oxidized bases (2–4) and protein carbonyl content (5–8) has been reported in a number of model systems, leading to the hypothesis that such damage, especially to key molecules such as DNA, may play a critical role in the aging process. Although this is an appealing hypothesis, the role of DNA oxidative damage in the aging process remains speculative because its association with actual life expectancy or mortality has not as yet been specifically established.

The objective of this study was to test some of the predictions of the hypothesis that oxidative stress is a causal factor in the aging process. If the hypothesis were valid, DNA oxidative damage should be accelerated by the *in vivo* increase in the level of oxidative stress and should also be inversely associated with life expectancy or physiological age of the organism. Physiological age denotes nearness to death, with 1.0 being the end point.

The present study was conducted in the adult male housefly, *Musca domestica*, using 8-hydroxydeoxyguanosine (8OHdG) concentration in DNA as an indicator of oxidative damage. It is widely believed that senescence in multicellular organisms is due to deleterious alterations in the long-lived postmitotic cells. If so, the adult housefly should be an excellent model for studying the role of age-related accumulation of oxidative damage because all of its somatic cells are postmitotic. Results indicate that 8OHdG accrual is inversely associated with the life expectancy of the flies.

MATERIALS AND METHODS

Animals. Eggs were obtained from a stock of houseflies originally procured from the Department of Zoology, University of Cambridge (U.K.), in 1980 and continuously bred in this laboratory. Eggs were placed in moist CSMA (Chemical Specialties Manufacturers' Association) fly larval medium. After their emergence from pupae, adult flies were segregated by sex and, unless specified otherwise, housed in 1-ft^3 cages, 200 flies per cage, at 25°C and 50% relative humidity. Flies were fed on sucrose and water, which promote a longer life-span than a fat- and/or protein-containing diet (9). All experiments were conducted using the male flies. Flies kept under these conditions had an average life-span of ≈20.8 ± 0.4 days.

Experimental Variation in Physical Activity and Life-Span. Earlier studies in this laboratory (10, 11) have shown that the life-span of flies is inversely related to the metabolic rate, which can be very conveniently altered by varying the level of physical activity. The level of physical activity was lowered by abolishing the flight activity. This was achieved by confining individual flies in 150-ml urine specimen bottles fitted with cardboard maze, where the flies could walk but were unable to fly due to restricted space [low activity (LA) flies]. In contrast, to achieve relatively higher levels of physical activity, flies were kept in 1-ft^3 cages where they were able to fly [high activity (HA) flies]. Under these conditions, LA flies lived more than twice as long as the HA flies (average life-span, 58 vs. 21 days, respectively).

Exposure to X-Rays. Live houseflies were placed in clear plastic tubes and exposed to 6-krad (1 rad = 0.01 Gy) x-rays using a Philips x-ray generator RT-305, operating at 100 kV, 10 mA, at a delivery rate of 200 rad/min.

Exposure to Hyperoxia. Flies were exposed to hyperoxia by placing the housing cage in a sealed Plexiglas container connected to a cylinder containing 100% oxygen. The gas was bubbled through water prior to its passage through the chamber at a steady rate.

Isolation and Hydrolysis of DNA. Nuclear DNA (total DNA) was isolated by extraction of the whole body homogenate of ≈25 flies with phenol, followed by precipitation with cold ethanol, and RNase A and RNase T1 treatment (12). To obtain mitochondrial DNA, mitochondria were isolated from ≈200 flies by differential centrifugation, as described (13), and the DNA was extracted. The mitochondrial DNA was

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Abbreviations: 8OHdG, 8-hydroxydeoxyguanosine; HA, high levels of activity; LA, low levels of activity.

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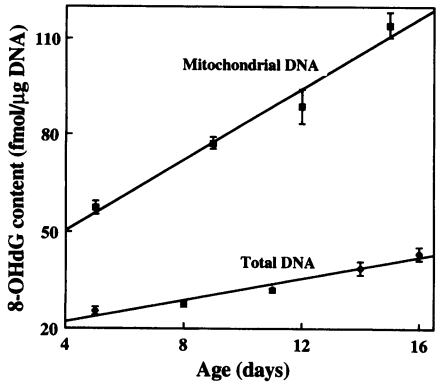

FIG. 1. Age-related changes in 8OHdG concentration in total and mitochondrial DNA of male houseflies. Flies were housed in 1-ft³ cages, 200 flies per cage. Total DNA was extracted from whole body homogenate and mitochondrial DNA was extracted from isolated mitochondria. DNA was enzymatically hydrolyzed to nucleosides and the amount of 8OHdG was determined by HPLC electrochemical detector. Values are averages ± SEM of three to six determinations.

found to contain no detectable nuclear DNA after *Bam*HI digestion, agarose gel electrophoresis, and ethidium bromide staining, as described by Richter *et al.* (14). DNA concentration was determined spectrophotometrically by measuring absorbance at 260 nm. DNA yield was 4 μg per fly (for total DNA) and 0.2 μg per fly (for mitochondrial DNA). About 50–100 μg of DNA was enzymatically digested to nucleosides using nuclease P1 (ICN) and *Escherichia coli* alkaline phosphatase (Sigma), as described by Fraga *et al.* (15).

Determination of 8OHdG in DNA. The concentration of 8OHdG in the DNA hydrolysate was determined by HPLC following the method of Shigenaga *et al.* (16). The separation was made on a 3-μm Supelcosil LC18DB (15 cm × 4.6 mm) analytical column with 50 mM phosphate buffer, containing 7.5% methanol (pH 5.5) as the mobile phase at a flow rate of 0.8 ml/min and an ESA Coulochem detector (Coulochem II) equipped with a model 5010 analytical cell operating at 300 mV. Peaks were identified by using authentic 8OHdG (gift from M. Shigenaga, University of California, Berkeley) as an internal standard and were quantified by comparing the height of the peak from DNA hydrolysate with that obtained from the standard sample.

RESULTS

Effect of Age on 8OHdG Content. The average life-span of flies kept in cages was 20.8 ± 0.4 days; however, the rapid dying phase, indicated by the sharp downward slope of the survivorship curve, started around 16 days of age (indicated in Fig. 3*A*). Previous studies in this laboratory (17) have shown that in cross-sectional age-related studies, to avoid potential sampling errors, investigation should be restricted to a period prior to the beginning of the rapid dying phase, because the survivors progressively represent subsets undergoing relatively slower rates of aging. Therefore, the concentration of 8OHdG in DNA was measured in flies ranging from 5 to 16 days of age (Fig. 1). The relative amount of 8OHdG in total DNA increased steadily with age, exhibiting an almost 70% higher level in 16-day-old flies as compared to the 5-day-old flies. Mitochondrial DNA showed a 2- to 3-fold higher 8OHdG concentration than the total DNA. The 8OHdG content in mitochondrial DNA also increased progressively with age, showing an almost 100% increase between 5 and 16 days of age. However, the amount of age-related augmentation of 8OHdG was ≈3.3-fold greater in mitochondrial than in total DNA ($P = 0.001$).

Effect of Experimental *in Vivo* Oxidative Stress on 8OHdG Content. To ascertain whether 8OHdG in DNA was associated with the *in vivo* oxidative stress, live houseflies were subjected to experimentally induced oxidative stress by exposure to x-rays or hyperoxia, both of which are known sources of reactive oxygen species generation (18, 19). As shown in Fig. 2*A*, exposure of live flies to 6 krad of x-rays caused an increase in 8OHdG content in total DNA. Furthermore, older flies showed a relatively higher increase in 8OHdG content in response to x-irradiation than the younger ones (e.g., 62% in 12-day-old vs. 31% in 5-day-old flies). Similarly, exposure of 5-day-old flies to 100% oxygen for 3 days was found to increase 8OHdG content of total DNA by 37% and by 100% in mitochondrial DNA, as compared to the unexposed control group (Fig. 2*B*).

Effect of Physical Activity on Life-Span and 8OHdG Content. Flies kept under low levels of physical activity—i.e., those prevented from flying—exhibited a >2-fold extension of the average as well as the maximum life-span as compared to flies kept under HA conditions (Fig. 3*A*). The average and maximum life-spans of LA flies were 58 days and 81 days, respectively, whereas they were 20.8 days and 42 days in HA flies. The concentration of 8OHdG content in both the total DNA (Fig. 3*B*) as well as the mitochondrial DNA (Fig. 3*C*) increased with age at a significantly faster rate in the HA group as compared to the LA group flies ($P = 0.001$ for total DNA; $P = 0.006$ for mitochondrial DNA).

8OHdG Content and Physiological Age. To determine whether DNA oxidative damage was related to the physiological or the chronological age, the physiological age of the flies, kept under HA and LA conditions, was calculated by dividing chronological age by the average life-span, with 1.0 representing the end point. As shown in Fig. 4, at similar

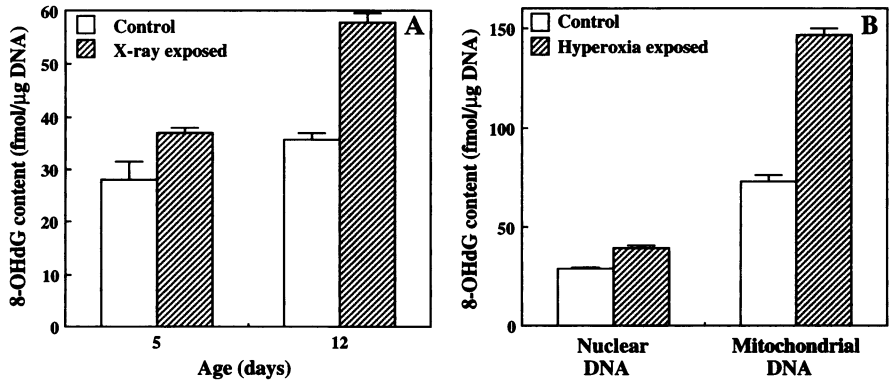

FIG. 2. (*A*) 8OHdG content in total DNA after 6 krad of x-irradiation of 5- and 12-day-old flies. Live flies were placed in clear plastic tubes and exposed to x-rays delivered at 200 rad/min. (*B*) Effect of hyperoxia on 8OHdG content in total and mitochondrial DNA. Five-day-old flies, kept in 1-ft³ cages, were placed in 100% oxygen for 3 days. Values are averages ± SEM of three to six determinations.

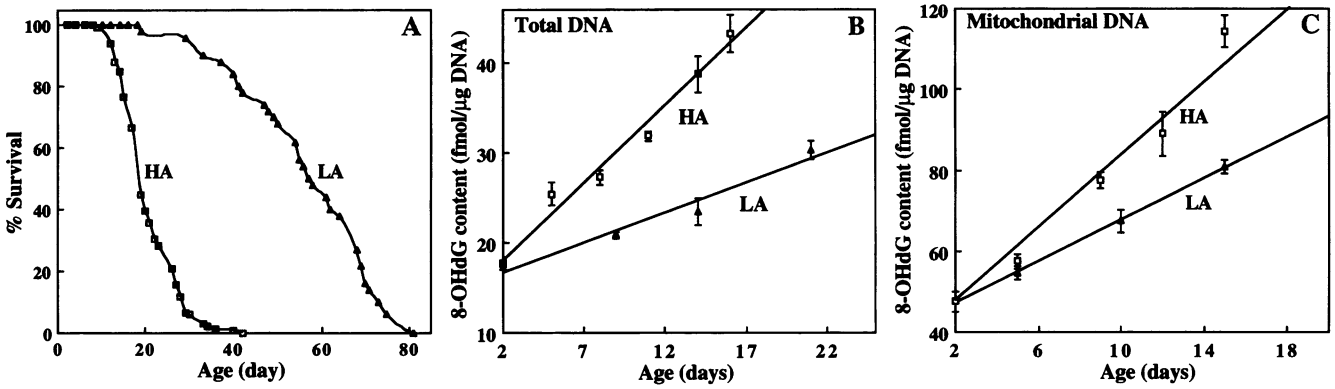

FIG. 3. (*A*) Survivorship curves of houseflies kept under different levels of physical activity. HA flies were housed in 1-ft^3 cages (200 flies per cage), where they were able to fly, whereas LA flies were individually confined in 150-ml urine specimen bottles (50 flies), fitted with cardboard maze, where flies were able to walk but could not fly because of lack of space. (*B*) Age-related accumulation of 8OHdG in total DNA of HA and LA flies. (*C*) Age-related accumulation of 8OHdG in mitochondrial DNA of HA and LA flies. Values in *B* and *C* are averages ± SEM of three to six determinations.

physiological ages—i.e., relative nearness to death—there is statistically no significant difference in 8OHdG content between the HA and LA groups (P = 0.733), indicating that 8OHdG content is associated with the life expectancy rather than the chronological age of the flies.

Age-Associated Pattern of 8OHdG and Protein Carbonyl Accumulation. Previously we conducted a similar study on the relationship between aging and protein oxidative damage, as indicated by the protein carbonyl content (11). It was therefore of interest to determine whether protein oxidative damage and DNA oxidative damage follow a similar age-associated pattern. As shown in Fig. 5, the age-related increase in 8OHdG concentration (in total DNA) virtually parallels that in the protein carbonyl content.

DISCUSSION

Results of this study suggest the existence of an inverse relationship between DNA oxidative damage and the life expectancy of flies. The concentration of 8OHdG has been widely regarded as an indicator of DNA oxidation (3, 4, 20, 21); however, skepticism about the formation of 8OHdG *in vivo* has recently been expressed (22). The evidence presented in this study supports the view that 8OHdG is indeed a product of DNA oxidative damage *in vivo*. For example, the exposure of live flies to x-rays, a well recognized source of reactive oxygen species, caused an elevation in the level of 8OHdG. Second, exposure of flies to 100% oxygen, which has been demonstrated to increase the rate of mitochondrial $O_2^{\cdot-}$ and H_2O_2 generation, was also found to enhance the 8OHdG concentration. Since the tissues of the experimental and control flies were handled identically, it is highly improbable that the elevated levels of 8OHdG, in response to hyperoxia or x-ray exposure, are procedural artifacts.

Our results support the finding by Richter *et al.* (14) that mitochondrial DNA is much more susceptible to oxidative damage than nuclear DNA because of a multiplicity of factors including the proximity of mitochondrial genome to the sites of $O_2^{\cdot-}$ and H_2O_2 generation within mitochondria. Results of this study tend to support this explanation. Hyperoxia is known to cause an elevation in mitochondrial $O_2^{\cdot-}$ and H_2O_2 generation as well as the mitochondrial protein carbonyl content (23, 24). Enhanced physical activity by flies has been previously shown by us to cause an elevation in the rate of mitochondrial $O_2^{\cdot-}$ and H_2O_2 generation as well as in the level of protein oxidative damage, as measured by protein carbonyl content (11, 23). Thus, the present finding that experimental regimes—namely, hyperoxia and high level of physical activity—that enhance the *in vivo* rate of mitochondrial reactive oxygen species generation also induce an increase in 8OHdG concentration to a greater extent in mitochondria than in the nucleus supports the view that the mitochondrial

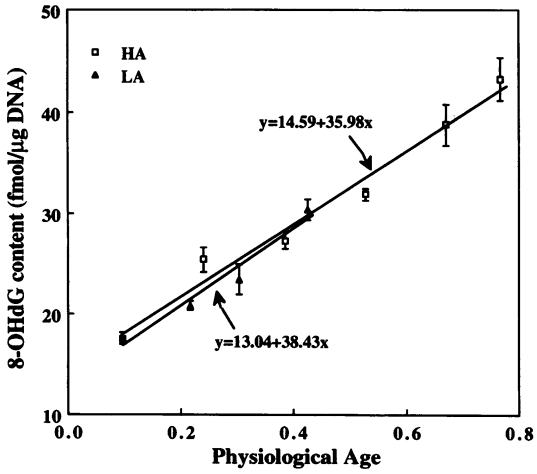

FIG. 4. Relationship between physiological age and concentration of 8OHdG in total DNA of flies. Physiological age of HA and LA flies was calculated by dividing chronological age by their respective average life-span.

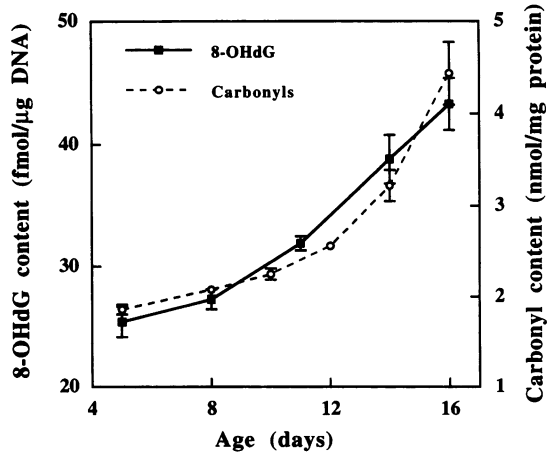

FIG. 5. Comparison of age-related accumulation of 8OHdG in total DNA and protein carbonyl content in whole body homogenates of houseflies. Carbonyl content was determined in a previous study (11).

genome is more vulnerable to *in vivo* oxidative damage than the nuclear genome, as first suggested by Richter *et al.* (14).

The observed age-related increase in 8OHdG content in total DNA is in agreement with previous studies in mammals (15, 24, 25). The present study extends this concept by showing that the 8OHdG accumulation in mitochondrial DNA also increases with age at a rate that is 3-fold greater than in total DNA. Thus, mitochondrial DNA not only displays oxidative damage that is higher than in the total DNA, but this damage increases with age more steeply in the former than in the latter. The underlying reason for the age-related increase in 8OHdG content can be hypothesized to be the corresponding increase in the *in vivo* level of oxidative stress. Our previous studies have shown that the rates of mitochondrial $O_2^{\cdot-}$ and H_2O_2 generation increase with the age of the houseflies (13, 26). Other indications of age-related increases in oxidative stress in the housefly include (*i*) a prooxidizing trend in the redox couples such as glutathione/glutathione disulfide, NADH/NAD^+, and NADPH/$NADP^+$ with age (27); (*ii*) an increase in the protein carbonyl content in mitochondria as well as homogenates of the flies with age (11, 23); and (*iii*) an age-related increase in the *in vivo* exhalation of *n*-pentane (28), a known product of lipid peroxidation. The age-related increase in susceptibility of flies to experimentally induced (x-rays) oxidative damage to DNA, as shown here (Fig. 2*A*), and to proteins, as reported earlier (29), suggests a corresponding decline in antioxidative or repair potential.

An interesting finding of the present study is that the rate of 8OHdG accumulation is related to the rate of metabolism and physiological rate of aging of the flies. Previous studies in this laboratory have demonstrated that metabolic rate is a key factor in the determination of life-span of poikilotherms (10, 30). Flies kept under conditions of relatively low levels of physical activity (i.e., those prevented from flying) undergo a slower rate of aging than those kept under conditions of relatively high levels of physical activity. This is indicated by the longer mortality rate doubling times (calculated from the slopes of the Gompertz plots) and extension of maximum life-spans in the LA groups as compared to the HA groups (11). These two parameters are presently believed to be the most reliable indicators of the rates of aging in populations (31).

Currently, a fundamental question in gerontology is whether oxidative damage is indeed a causal factor in the aging process or a secondary coincidental phenomenon. As pointed out by Stadtman (7) and Ames (2), it would be difficult to envision that the extensive damage to proteins and DNA observed during aging would be functionally inconsequential. In the housefly model system, both the DNA and the protein oxidative damage are correlated not only with the life expectancy of flies but also with each other. Such a correlation reinforces the relationship between oxidative molecular damage and the aging process. This concept is further supported by our recent finding that the extension of life-span in *D. melanogaster* by the overexpression of superoxide dismutase and catalase correlates with a decreased level of protein oxidative damage (1). Altogether, it would seem that there is sufficient evidence to support the hypothesis that oxidative damage is a contributory factor in the aging process.

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