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N-Acetylcysteine slows down ageing and increases the life span of *Drosophila melanogaster*

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Abstract. Ageing can be defined as the time-dependent decline of physiological functions of an organism. The molecular causes for the ageing process are multiple, involving both genetic and environmental factors. It has been proposed that antioxidants may positively influence the ageing process, protecting the organism against free radical-induced damage. Here we show that the antioxidant *N*-acetylcysteine (NAC) has a life-extending effect on *Drosophila melanogaster*. Dietary uptake of NAC results in a dose-dependent increase in median and maximum life span. Flies fed on 1 mg/ml NAC food live 16.6% longer; at 10 mg/ml, life span increases by 26.6%. We have examined the effect of NAC treatment on protein and RNA levels: we observe an NAC-dependent increase in absolute amounts of total RNA and ribosomal RNA, but no differences in protein levels. The NAC effect on longevity may involve differential expression of specific mRNA genes, as suggested by RNA fingerprinting experiments.

Key words. *Drosophila melanogaster*; ageing; longevity; antioxidant; *N*-acetylcysteine; differential gene expression.

Ageing can be defined as the time-related decline of physiological functions of an organism. Homeostatic maintenance functions at all levels of organization decrease with age, resulting in the progressive accumulation of changes at the organ, tissue, cell and molecular level [1]. Homeostatic maintenance mechanisms at the molecular level include DNA synthesis and repair, protein synthesis and turnover, stress defence, and redox and ion homeostasis. Impairments of these functions are particularly relevant for postmitotic tissues that have no replicative capacity to replace damaged cells. As an example, the balance between pro-oxidant generation and antioxidant defence mechanisms changes with age, either as a result of a decrease in antioxidant defence molecules or an increased rate of pro-oxidant generation [2]. As a consequence, the cells in an ageing organism are exposed to an increasing load of oxidative stress that can influence the rate of ageing. Considerable variations in these parameters have been observed, depending on the enzymes, the tissue and the species examined [2, 3].

The fruit fly *Drosophila melanogaster* and other insects have been widely used as model organisms for ageing research (for recent reviews see [4, 5]). Because the fly is composed almost exclusively of postmitotic cells, it is well suited as a model to study ageing mechanisms in postmitotic tissues. Experiments carried out with house flies support the hypothesis that oxidative stress is involved in the ageing process [6]. Modulation of antioxidant enzyme expression levels in transgenic *Drosophila* was proposed to influence the life span [7]. Several insect studies have also presented evidence that ageing is

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accompanied by a gradual decrease in cysteine and glutathione (GSH) levels [6, 8–10]. GSH constitutes the most important nonenzymatic antioxidant molecule in the cell. At the same time, the glutathione system constitutes a powerful intracellular redox buffer. Changes in cysteine or GSH levels might, therefore, not only affect the antioxidant capacity of cells, but might also influence the activity of genes that are regulated by redox-sensitive transcription factors [6]. The concept that free radicals may play a role in the ageing process has been proposed and extensively discussed [2, 11].

The antioxidant *N*-acetylcysteine (NAC) is known as a radical scavenger and/or as a cysteine donor and GSH precursor [12, 13]. In the present work we show that NAC has an antiageing effect on *D. melanogaster*. Feeding fruit flies with NAC results in a dose-dependent increase in mean and maximum life span. This effect on longevity may be correlated with altered gene activity.

Materials and methods

Fly culture and life span experiments. *D. melanogaster* Oregon R stock cultures were maintained at 25 °C in 500-ml bottles on standard corn meal food as described [14]. For the life span studies, newly eclosed males were transferred to glass vials (25 flies per vial) containing the test food. Every 2–3 days, dead flies were counted and survivors were transferred to freshly made-up food. Most experiments were done with Ready Mix Drosophila Dry Food (Philip Harris, Lichfield, England). The instant powder was mixed with H_2O or NAC dilutions as indicated below.

NAC food. Fresh stock solutions (20 mg/ml, or 50 mg/ml in H_2O) of *N*-acetyl-L-cysteine (Sigma) were prepared weekly, stored at 4 °C and diluted prior to use. The dry food powder was mixed at 5 g per 5 ml H_2O (control food) or 5-ml NAC dilutions (100 µg/ml, 1 mg/ml, 10 mg/ml, 20 mg/ml and 50 mg/ml), respectively.

RNA extractions. Flies were homogenized in guanidinium thiocyanate buffer at 10 flies/100 µl buffer as described previously [14]. Total RNA was purified over a CsTFA gradient [15, 16] and concentrations were determined by absorption measurements at A_{260}. RNAs were separated on 1.0% formaldehyde agarose gels containing ethidium bromide for visualization of rRNAs [14].

Differential RNA display. Total RNA (2–3 µg) was used for reverse transcription with Not dT(18) as primers and the Ready-To-Go You-Prime First-Strand Beads (both from Pharmacia) according to the instructions supplied. One-microlitre aliquots of the complementary DNA (cDNA) reactions were employed for differential polymerase chain reaction (PCR) amplifications with primers from the Delta RNA fingerprinting kit (Clontech) according to the modified protocol [17] supplied by Clontech. The Delta fingerprinting protocol uses a single cDNA reaction for the subsequent PCR amplifications. The primers are 25–30 nucleotides long, which allows PCR cycling under stringent conditions (annealing at 60 °C). The PCR products were separated on 15% polyacrylamide gels (GeneGel Clean, Pharmacia) with the GenePhor Electrophoresis Unit (Pharmacia). The gels were silver-stained using the PlusOne silver staining kit and the Hoefer Automated Gel Stainer (both from Pharmacia) and photographed with the EagleEye (Stratagene) still video system.

Results

NAC treatment increases the life span of *Drosophila*. First, pilot experiments were carried out with small fly numbers (50 flies per sample) and several NAC concentrations (from 10 µg/ml to 1 mg/ml) in order to determine whether the NAC treatment had any effect on life span (results not shown). After observing a positive effect, larger screens were performed with increasing NAC concentrations and larger fly numbers (100 and 200 flies, respectively). The survival curves presented in figure 1 show results from two independent experiments. Experiment A was carried out with 100 flies per NAC concentration (fig. 1A), experiment B with 200 flies per NAC concentration (fig. 1B). In both cases, we observed a dose-dependent effect on life span with increasing NAC concentrations. Both the median (LT_{50}) and the maximum (LT_{100}) life span are increased at NAC concentrations above 100 µg/ml. Higher NAC doses, however, are toxic: at 20 mg/ml NAC the flies live a much shorter time (fig. 1B), and at 50 mg/ml they die within 2 days (not shown). Using the same batch of food, experiment B was repeated twice and gave reproducible results. Flies from a parallel experiment were used for RNA extractions (see next section).

The median life span was calculated for each vial. Table 1 summarizes the mean values with standard deviations from the experiment presented in figure 1B. The differences in median life span between control flies and NAC-treated flies is 5.7 days, 4.8 days and 9 days for 100 µg/ml, 1 mg/ml and 10 mg/ml NAC and correspond to an increase in median life span of 16.6, 14.04, and 26.6%, respectively. The values for 100 µg/ml and 10 mg/ml are significantly different from the control at 95% confidence value (Fischer test).

Effect of NAC treatment on RNA levels. In our previous work [4, 14, 16] we have shown that ageing in *Drosophila* is correlated with a decrease in the steady-state levels of total RNA, rRNA, tRNA, as well as many specific mRNAs. The total soluble protein levels

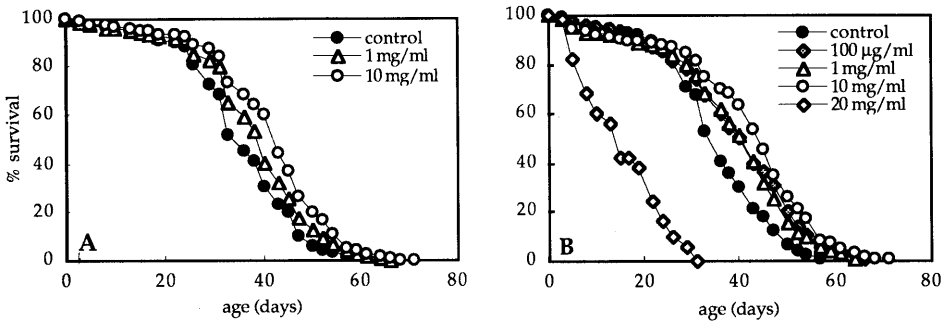

Figure 1. Survival curves for male *D. melanogaster* raised on control food and NAC-containing food. (*A*) Curves calculated from 100 flies (four vials with 25 flies). (*B*) 200 flies (eight vials with 25 flies) were used for each NAC concentration; the curves represent average values from eight individual vials with 25 flies.

do not change with age. Here we have investigated whether NAC treatment has an effect on age-related RNA changes and on the differential expression of specific genes.

In a first experiment, total RNA was extracted from flies that had grown for various time periods on control food or NAC food, and the amounts of total RNA and protein were determined. The long-term NAC treatment resulted in a net increase in the absolute amounts of RNA (fig. 2A). There was no significant change in total soluble protein per fly after the NAC treatment (not shown).

In addition to the increase in RNA per fly, we also observed an effect on the age-related decline in total RNA. When the data are expressed in relative values, it is obvious that the decrease in RNA levels occurring in the first 10 days of adult flies is less pronounced after NAC treatment (fig. 2B).

The quantitative differences in total RNA levels are mainly due to an increase in steady-state levels of ribosomal RNAs. When RNA extracted from NAC-treated flies is separated on agarose gels and stained with ethidium bromide, the band corresponding to rRNAs is stronger in samples from NAC-treated flies (fig. 3).

We next tested the effect of short-term NAC diet. Flies that had grown on control food for 5 days, 15 days and 30 days were transferred to NAC food (1 mg/ml), and total RNA was extracted after 48 h. Short-term NAC treatment resulted in a considerable increase in total RNA in 5-day-old and 15-day-old flies, while the effect on 30-day-old flies was less pronounced (fig. 4).

Short-term NAC diet results in differential gene expression. To determine whether short-term NAC treatment influences specific gene expression, we carried out RNA fingerprinting experiments on these RNA samples (aliquots from the RNA samples used in fig. 4). Total RNAs extracted from 5-day-old flies that had been treated for 48 h with 1 mg/ml NAC and from control flies were reverse-transcribed and submitted to a differential RNA fingerprinting or DDRT-PCR protocol (differential display reverse transcription PCR). We have used several different primer pairs under stringent PCR amplification conditions [17]. In almost all cases we observe both qualitative as well as quantitative differences between the PCR products obtained from control and NAC cDNA samples. Representative results are given in figure 5. They show that each primer pair results in a distinct pattern of PCR amplicons.

Fingerprinting experiments were then carried out also with the RNAs extracted from flies of different ages submitted to a short-term NAC diet. The example presented in figure 6 shows that NAC induces differential gene expression in flies of all ages tested. When the patterns from 5-, 15- and 30-day-old flies are compared, we can detect – in addition to the NAC-induced differences – age-related differences in PCR products (fig. 6).

Taken together, the present results show that the life-extending functions of NAC include alterations in specific gene expression. Flies of all ages tested are subject to NAC-induced changes in mRNA expression. This is consistent with the observations made in other experimental systems, which suggested that NAC may positively or negatively influence the expression of genes

Table 1. Effect of NAC on median (LT_{50}) and maximum (LT_{100}) life spans of male *Drosophila melanogaster*.

NAC concentration	Median life span (days ± SD)	Maximum life span (days ± SD)
0 (control)	34.0 3.7	53 6
100 µg/ml	39.7 5.9	61 6
1 mg/ml	38.8 3.3	61 5
10 mg/ml	43.1 2.4	66 7
20 mg/ml	17.2 2.3	31 3

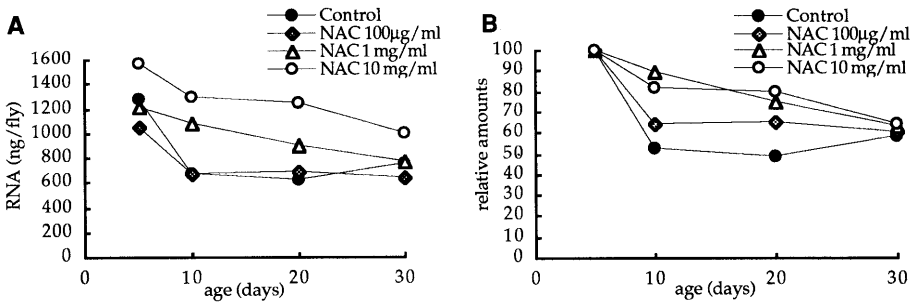

Figure 2. (*A*) Absolute amounts of total RNA (ng per fly) extracted from control and NAC-treated flies. The curves represent averaged values from three independent extractions. (*B*) The age-related decrease in total RNA is slowed down in NAC-treated flies. The values of 5-day-old flies are set at 100%.

that are regulated by redox-dependent transcription factors [18–20].

Discussion

In the present experiments we show that feeding fruit flies with NAC results in a dose-dependent extension of the median and maximum life span of *D. melanogaster*. The largest effect corresponding to ~30% life extension is obtained at NAC concentrations of 10 mg/ml food, which corresponds to ~60 mM. It is difficult to determine how much of the food is taken up by the flies and what intracellular NAC concentrations this amounts to. The fact that the antiageing effect is dosage-dependent and that higher NAC concentrations are toxic suggests that we are dealing with a true pharmacological effect and not a result of dietary restriction. It seems that NAC treatment does not influence the feeding behaviour nor the physical activity of flies. There is no change in the body weight nor in the amount of total protein per fly after NAC diet. As our preliminary data also indicate, NAC has no effect on the walking activity of flies (data not shown).

An additional observation deserves mention. In order to obtain reproducible results in life span studies, it was important to use the same batch of food prepared under identical conditions. Because the production of the powdered instant food from Harris was discontinued in

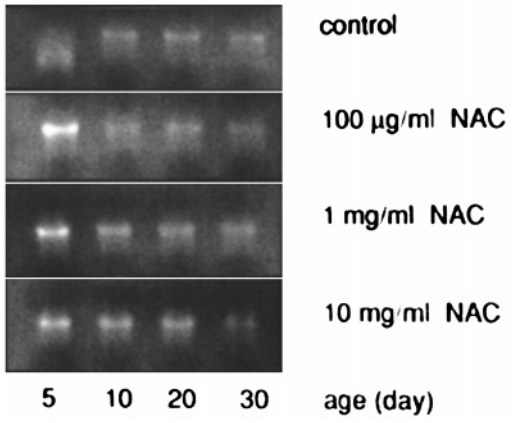

Figure 3. Ribosomal RNA levels are elevated in NAC-treated flies. Total RNA equivalent to two flies was separated on 1% agarose-formaldehyde gels and stained with ethidium bromide. The main band corresponds to rRNAs [14].

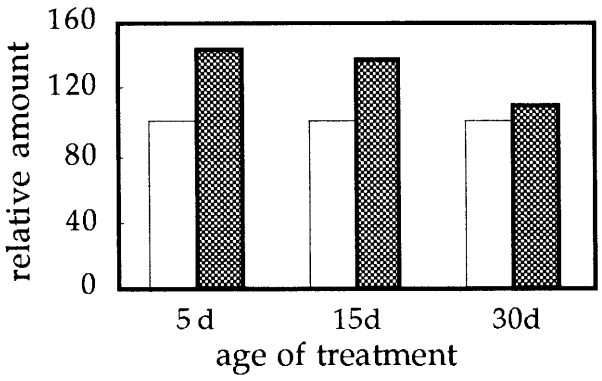

Figure 4. Total RNA increases after short-term (48 h) NAC treatment. Flies of different ages were transferred to control food or to food containing 1 mg/ml NAC. Values from NAC-treated flies (shaded columns) are compared with control values (white columns) set at 100%.

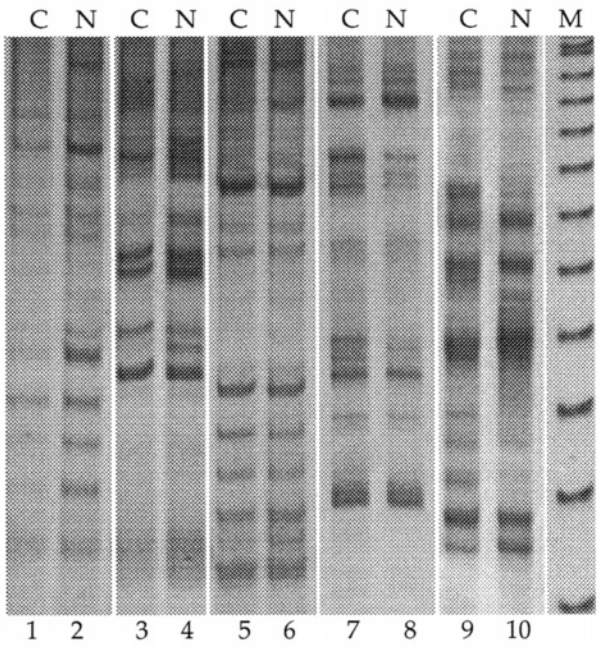

Figure 5. Differential RNA expression in NAC-treated flies. Template RNAs were from 5-day-old flies kept for 48 h on control food (C) or 1 mg/ml NAC (N). Lane M: DNA marker, 100-bp ladder. Lanes 1–10 show patterns obtained with five different primer pairs.

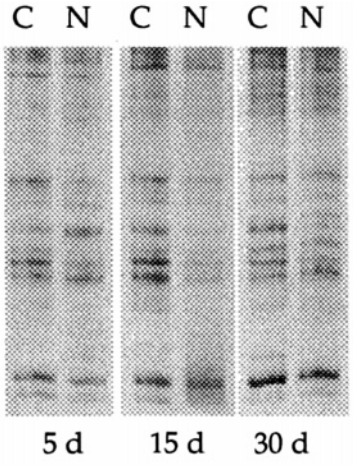

Figure 6. Differential mRNA expression in control and NAC-treated flies of increasing ages. Fingerprint experiments were carried out with the RNA samples shown in figure 4. Flies at the age of 5 days, 15 days and 30 days were transferred to 48 h to 1 mg/ml NAC (N) or control food (C).

the course of these investigations, we carried out one set of life span experiments with another brand of instant *Drosophila* food (Carolina Biological Supply). With this food, life extension was also dosage-dependent, but the NAC concentrations had to be titrated anew. Since the composition of the two brands of instant food is not defined, it is difficult to say whether and how NAC might interact with any of the components.

The life-extending effect of NAC could be a result of its radical scavenger capacity, its reducing properties, or both. In any case, it also affects transcription activity. The results described above show that both the long-term diet as well as the 48-h NAC treatment results in changes in RNA levels. We have previously shown that ageing in *Drosophila* is accompanied by a gradual decline in steady-state RNA levels, including transcripts from RNA polymerase I, II and III genes [4, 14]. Here we present evidence that NAC treatment affects steady-state total RNA levels and results in a slowing down of the age-related decrease in rRNA. This could be either a result of higher RNA polymerase I transcription activity or of increased rRNA stability. The RNA fingerprinting experiments further reveal that the NAC diet results in distinct quantitative and qualitative changes in mRNA expression.

The present results give further support to the free radical theory of ageing [11]. They also agree with the unifying ageing theory proposed by Sohal and Allen [6]. According to these hypotheses, ageing results from the loss of the dynamic equilibrium between free radical generation, antioxidant defences and the expression of genes that are influenced by the redox state of the cell. They suggest that the main function of cellular antioxidants is the maintenance of the intracellular redox state. Since many housekeeping genes contain binding sites for redox-regulated transcription factors, it is possible that a molecule like NAC can have a profound effect on longevity by maintaining various homeostatic functions via redox homeostasis. Furthermore, multiple stress-induced genes under the control of redox-sensitive transcription factors could be influenced by the NAC diet. We have preliminary data suggesting that old NAC-treated flies are more resistant to paraquat.

In mammalian systems, NAC has been reported as having a wide variety of protective, antioxidative or antitoxic effects: protection of rat hepatocytes from bromobenzene toxicity [21], protection of mice against skin phototoxicity [22], prevention of apoptotic cell death after growth factor deprival [13] and inhibition of HIV (human immunodeficiency virus) replication [18, 23]. NAC has been shown to affect intracellular GSH levels [20] and to prevent apoptosis of neuronal cells by a mechanism that is transcription-dependent [13, 20]. The concentrations of NAC used in some of these experiments [13] are comparable to the ones used in the present study. We have not presented evidence for how much NAC is actually ingested by the flies; this would require the use of radioactive NAC. Therefore,

we can only extrapolate from the activities presented in other systems. Whether the same protective mechanisms also operate in flies remains to be shown.

Many of the protective activities of NAC have been attributed to the fact that it can serve as a cysteine donor and/or GSH precursor, thus affecting the intracellular thiol levels and the cellular redox state [18]. Redox changes can be induced by cysteine depletion, by many intracellular or extracellular stress factors, or by free radicals. They can trigger a signalling pathway that results in activation or repression of downstream genes dependent on redox-sensitive transcription factors. The best-known example is the transcription factor NF-κB: activation of NF-κB is required for HIV transcription, a process that is inhibited by NAC [18, 24, 25]. Several human lymphocyte functions are under control of NF-κB [24], and transcription of immune genes in insects is regulated by a homologue of NF-κB [26]. In both cases, these activities are influenced by NAC. A number of helix-loop-helix transcription factors (NF-κB, AP1 jun/fos) contain a cysteine residue in their DNA binding domain that is crucial for DNA binding activity [27].

There are, however, other insect experiments that have pointed to a correlation between intracellular thiol levels and longevity: Richie et al. have shown that in the mosquito ageing is correlated with a GSH loss that may be caused by a cysteine deficiency [9]. Feeding the mosquitoes with a cysteine precursor both restored GSH levels and extended longevity [8].

In conclusion, we have presented evidence that dietary uptake of the antioxidant *N*-acetylcysteine can influence the life span of *D. melanogaster*. Our results are consistent with the hypothesis that oxidative stress contributes to the ageing process and may be involved in longevity determination, at least in insects. The data further suggest that the effect of NAC on life span occurs via a mechanism that may depend on differential expression of specific genes, supporting the hypothesis proposed by Davies [2]. Numerous studies have presented evidence for the presence of an adaptive response to oxidative stress that involves alterations in gene expression (reviewed in [2, 11]). Future investigations will reveal whether the same genes that are induced by mild oxidative stress also respond to antioxidant (NAC) treatment and, most important, whether the same homeostatic antioxidant system is operating in all species.

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