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Lifespan extension and elevated *hsp* gene expression in *Drosophila* caused by histone deacetylase inhibitors

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Summary

The heat shock proteins (Hsps) play a positive role in lifespan determination, and histone acetylation has been shown to be involved in transcription of *hsp* genes in *Drosophila*. To further determine if *hsp22* and *hsp70* expression is correlated with lifespan, and if histone acetylation participates in this process, RNA levels for *hsp22* and *hsp70* were analyzed throughout the lifespan in the long-lived and short-lived iso-female lines. The results showed that *hsp22* and *hsp70* RNA levels were higher in long-lived line than in short-lived line and that the long-lived flies responded more rapidly to heat but were more tolerant to high temperature. Moreover, we investigated the influences of histone acetylation modification on longevity and on *hsp* gene expression by using histone deacetylase (HDAC) inhibitors TSA and BuA. The results demonstrated that both inhibitors were able to extend the lifespan and promote *hsp22* and *hsp70* expression. However, the optimal concentrations of these inhibitors, and probably the mechanisms of their actions, vary with the genetic background. In addition, we showed that HDAC inhibitors caused the hyperacetylation of core histone H3, implicating the involvement of chromatin modulation in *hsp* gene transcription. These data suggested a close correlation among histone acetylation, *hsp* gene expression and longevity in *D. melanogaster*.

Key words: *hsp* gene, lifespan, histone acetylation, histone deacetylase inhibitor, *Drosophila melanogaster*.

Introduction

The lifespan of an organism is influenced by both genetic and environmental factors. To date, our understanding of the mechanisms of aging is limited because of the biological complexities of the process. There has been evidence to support the hypothesis that aging is associated with the accumulation of abnormal and/or malfolded proteins and oxidatively damaged proteins in many organisms, including nematodes, flies and mammals (Gershon and Gershon, 1970; Stadtman, 1992). The heat shock proteins (Hsps) are induced in response to protein damage caused by heat and other stresses (Parsell and Lindquist, 1993). Induction of *hsp* genes during *Drosophila* aging might have a beneficial effect on the lifespan of flies (Wheeler et al., 1995; King and Tower, 1999). Mild heat stress of *Drosophila* transgenic for extra copies of the *hsp70* gene produced increased expression of the gene and extended the lifespan (Tatar et al., 1997; Tatar, 1999). Heat-induced expression of *hsp70* may reduce age-related mortality rates (Minois et al., 2001) and *hsp22* and *hsp23* genes were upregulated in selected lines for increased longevity (Kurapati et al., 2000), implicating the role of Hsp proteins in longevity determination. Molecular chaperones must be playing important roles in maintaining cellular functions during aging, through promoting protein renaturation and preventing proteins from aggregating and denaturing (Hartl, 1996; Marin et al., 1993).

Expression of *hsp* genes represents a special model of gene regulation involving basal and inducible expression. The acetylation/deacetylation modifications of N-terminal tails of core histones play critical roles in activation/repression of many eukaryotic genes, including *hsp* genes. *In vitro*, histone acetylation has been shown to be involved in *hsp* gene activation (Nightingale et al., 1998; Reid et al., 2000). Li et al. (1998) demonstrated that in *Xenopus* oocytes, p300 participated in the inducible transcription of the *hsp70* gene both as a coactivator and an acetyltransferase. During *Xenopus* development, the presence of histone deacetylase (HDAC) inhibitors enhanced the heat shock-induced accumulation of both *hsp70* and *hsp30* mRNA in post-midblastula transition (MBT) staged embryos and resulted in the expression of *hsp30* immediately after MBT rather than at the late neurula/early tailbud stage under normal conditions (Ovakim and Heikkila, 2003). In a previous study, we showed that HDAC inhibitors trichostatin A (TSA) and sodium butyrate (BuA) were able to affect the chromatin structure at the site where the *hsp70* gene is located along the polytene chromosome and significantly enhanced both the basal and

the inducible expression of *hsp70* gene in *Drosophila* (Chen et al., 2002).

Histone acetylation has also been shown to be involved in lifespan determination. In *Saccharomyces cerevisiae*, *sir2* and *rpd3*, which encode two important histone deacetylases, were found to be involved in the regulation of lifespan, and deletion of *sir2* shortened the lifespan (Kaeberlein et al., 1999), while the *rpd3* knockout increased lifespan (Kim et al., 1999). Additional copies of *sir2* in *Caenorhabditis elegans* also increased the lifespan of the worm (Tissenbaum and Guarente, 2001). The lifespan extension was achieved by feeding the HDAC inhibitor phenylbutyrate (Kang et al., 2002), or by a hypomorphic mutation of *rpd3* (Rogina et al., 2002) in *Drosophila*. These results indicate that histone acetylation is associated with lifespan. But the molecular mechanisms of HDAC-dependent changes in aging and lifespan remain unclear.

The aim of this study was to investigate the roles of histone hyperacetylation in expression of *hsp* genes during aging and longevity determination in *Drosophila*. Our results show that the increase in acetylation level of histone H3 enhances the basal and inducible expression of *hsp22* and *hsp70* during aging, and extends both mean and maximum lifespan, to variable extents, in different lines of *Drosophila*. This strongly implicates a correlation among histone acetylation, *hsp* gene expression and longevity determination.

Materials and methods

Drosophila *stocks and culture*

Wild-type strain of *Drosophila melanogaster*, Canton-S, was used. Flies were raised on standard cornmeal/yeast/agar medium under a 12 h:12 h light:dark cycle.

Isofemale lines screening and lifespan determination

Eggs from a single female fly were collected and used for the screening of iso-female lines, and ten iso-female lines were established and the lifespan was measured. The long-lived and short-lived lines were then screened from these lines. To obtain flies of defined age, newly eclosed flies were collected and maintained at 25°C at a density of 50 per vial and were transferred to fresh vials every 3 days to prevent growth of bacteria or mold, and 200 flies for each line were cultured. The number of dead flies was counted every day.

HDAC inhibitor treatment and heat shock induction

The HDAC inhibitors trichostain A (TSA; Sigma, MI, USA) and sodium butyrate (BuA; Sigma) were used at final concentrations of 10 μmol l^{-1} and 10 mmol l^{-1}, respectively. The third instars larvae were divided into equal aliquots for different treatments. Part of the larvae were cultured for 5 h in physiological brine (130 mmol l^{-1} NaCl, 4.7 mmol l^{-1} KCl and 1.9 mmol l^{-1} $CaCl_2$) and then allowed to develop directly into flies. For the repeated mild heat shock (RMHS), the flies were induced at 32°C for 1 h every 3 days. Some of the larvae were heat shocked (HS) at 37°C for 30 min before they were allowed to develop into flies. For HDAC inhibitor treatment, the larvae were cultured in physiological brine with TSA or BuA and then allowed to develop into flies either without additional inhibitor feeding (o.TSA and o.BuA) or were continuously cultured on medium containing inhibitors (c.TSA and c.BuA). The remaining flies were treated with both HDAC inhibitors and RMHS (TSA-RMHS and BuA-RMHS).

Real-time quantitative PCR analysis of hsp mRNAs

The flies were cultured and treated as described above until 6 and 30 days old, or induced at 37°C for 2, 5, 10, 20 and 40 min. Total RNA was extracted from flies using the RNA extraction kit supplied by Promega. The reverse transcription (RT) reaction was performed by using a RT system (Promega) following the manufacturer's protocol. Quantification of mRNA was performed using an ABI PRISM® 7700 sequence Detection System (PE Applied Biosystems, Weiterstadt, USA) and SYBR® Green (PE Applied Biosystems) as a double-stranded DNA-specific fluorescent dye. *Rp49* (ribosomal protein 49) was used as a housekeeping gene for standardizing *hsp* mRNA expression.

Amplification mix (50 μl) contained 4 μl cDNA solution, 10× SYBR® Green buffer, 0.24 mmol l^{-1} $MgCl_2$, dNTP (0.2 mmol l^{-1} dATP, dCTP, dGTP, 0.4 mmol l^{-1} dUTP), 1.25 U AmpliTaq Gold DNA polymerase, 0.5 U Amp Erase UNG and 200 nmol l^{-1} DNA primers. Amplification primers for *hsp22* were 5′-ctttcacgccttcttcc-3′ and 5′-gcggttttgtcttttgg-3′; for *hsp70* were 5′-agccgtgccaggtttg-3′ and 5′-cgtt-cgccctcataca-3′; and for *rp49* were 5′-agcacttcatccgccacc-3′ and 5′-atctcgccgcagtaaacg-3′. Samples were incubated for 2 min at 50°C to inhibit carryover of contamination. Thermal cycling conditions consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. All PCR reactions were run in duplicate.

The Ct (threshold cycle) was defined as the number of cycles required for the fluorescence signal to exceed the detection threshold. Data were analyzed by using the $2^{-\Delta\Delta Ct}$ method, which is a convenient way to analyze the relative changes in gene expression (Livak and Schmittgen, 2001). Quantitative results are given as means ± S.E.M.

Western blot analysis

Flies were cultured and treated as described above. Nuclear protein was extracted as described by Giancotti et al. (1984), or whole flies were homogenized directly into lysis buffer [50 mmol l^{-1} Tris-HCl pH 8.8, 300 mmol l^{-1} NaCl, 1% NP-40, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} dithiothreitol (DTT), 1 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF)] to obtain total proteins. Following 15% SDS-polyacrylamide gel electrophoresis (PAGE), protein was transferred to nitrocellulose membranes, then the membranes were incubated with antibodies to acetylated histones H3 and H4 (Upstate Biotechnology, Lake Placid, NY, USA), to Hsp22 (produced in rabbit), and to actin (Santa Cruz, CA, USA), respectively.

Actin was used as a housekeeping gene product for normalizing the loading materials.

Results

Screening of the long- and short-lived lines

Longevity is dependent on both genetic and environmental factors. Under the same environmental conditions, diversity of longevity occurs among the different individuals. We established 10 iso-female lines of *Drosophila melanogaster*, and the number of dead flies was counted every day. From these lines, we selected the long-lived line iso2 and the short-lived line iso4. The maximum and mean lifespan of the long-lived iso2 line were 73 days and 45.53 days, respectively (Fig. 1). In comparison, the maximum and mean lifespan of the short-lived iso4 line were 62 days and 38.47 days, respectively (Fig. 1).

Basal expression of hsp *genes during aging in long- and short-lived lines*

A real-time quantitative PCR procedure was used to analyze the RNA expression of *hsp* genes in long- and short-lived lines during aging. It is clear from Fig. 2 that the *hsp22* (Fig. 2A) and *hsp70* (Fig. 2B) basal expression declined with aging and this decline was more distinct in the long-lived line than in the short-lived line (Fig. 2). On the other hand, *hsp22* and *hsp70* mRNA levels were higher in the long-lived line relative to the short-lived line in young flies (Fig. 2), but became less distinct in old flies (Fig. 2). No prominent differences were seen in the expression of other *hsp* genes, such as *hsp26*, during aging and between long- and short-lived lines (data not shown).

Differences in response to heat induction and in the level of expression of hsp between long- and short-lived lines

The *hsp* RNA expression after 37°C heat induction for different periods of time was also examined by real-time quantitative PCR. As shown in Fig. 3, differences in the response to heat induction and in the level of expression of

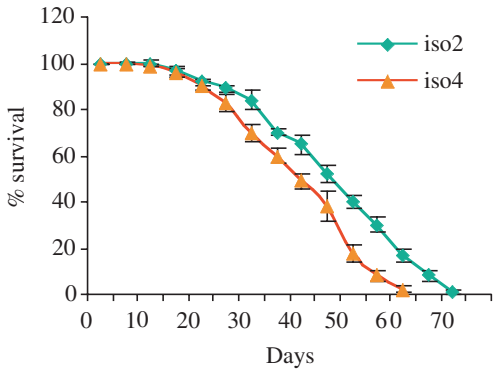

Fig. 1. Survival curves of adult *Drosophila*. iso2: the long-lived iso-female line, maximum lifespan 73 days, mean lifespan 45.53 days; iso4: the short-lived iso-female line, maximum lifespan 62 days, mean lifespan 38.47 days. The differences were significant (P<0.01). Values shown are the means ± S.E.M. of four parallel experiments.

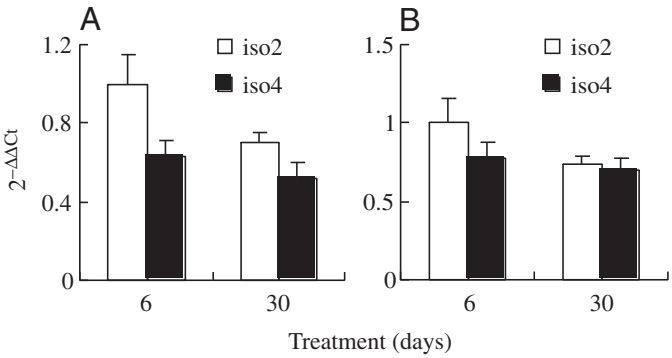

Fig. 2. Difference in basal expression of *hsp22* (A) and *hsp70* (B) genes during aging between long- and short-lived lines. Total RNA was isolated from flies, which were cultured and treated as described in the Materials and methods until 6 days and 30 days. Quantitative real-time PCR was performed to determine the mRNA expression levels of *hsp* genes (N=3). Values are means ± S.E.M.

hsp22 (Fig. 3A) and *hsp70* (Fig. 3B) gene products in fly strains with different lifespan were observed. The long-lived line was more sensitive to heat shock than the short-lived line. Heat shock for 2 min caused rapid increase in the expression of *hsp22* and *hsp70* in long-lived line (Fig. 3, iso2), but in the short-lived line, a detectable change in *hsp22* and *hsp70* expression did not occur until 5 min of heat shock (Fig. 3, iso4). Also, *hsp22* expression increased by approximate 130-fold in short-lived flies at 40 min of induction, while only an approximate 80-fold increase was detected in long-lived flies at the same time (Fig. 3A). It can also be seen from Fig. 3B that the intensity of *hsp70* gene did not change significantly upon heat treatment.

Difference in stress resistance between long- and short-lived lines

The experimental results described above revealed a correlation between *hsp* gene expression and longevity in flies. We then investigated the tolerance of flies to persistent high temperature, and we found that the long-lived flies had a greater resistance to high temperature stress. Within 30 min of the treatment at 37°C rapid death occurred in the short-lived line, whereas in long-lived flies, the rapid death occurred after 60 min of the heat treatment (Fig. 4).

Influence of heat shock and HDAC inhibitors on the lifespan of flies

To test the action of HDAC inhibitors (TSA and BuA) and heat shock in flies with different longevity, we cultured and treated flies as described in Materials and methods, and the lifespan was measured. The repeated mild heat shock (RMHS) resulted in an extension of both the mean and maximum lifespan of the flies (Fig. 5A,E), but the degrees of extension were different between the long- and short-lived lines. The maximum lifespan increased only slightly, by 5.6%, in long-lived flies, and moderately, by 11.5%, in short-lived flies; the mean lifespan increased moderately, by 10.3%, in long-lived

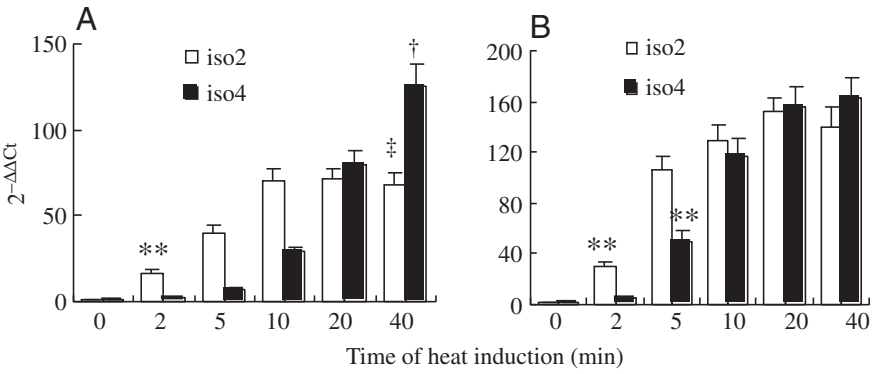

Fig. 3. Difference in response to heat induction and in the level of expression of *hsp22* (A) and *hsp70* (B) genes between long- and short-lived lines. The flies were heat induced at 37°C for 2, 5, 10, 20 and 40 min before RNA was extracted for reverse transcription. Quantitative PCR was performed to determine the inducible mRNA expression levels of *hsp* genes (N=3). Values are means ± S.E.M. **P<0.01 *versus* control; †130-fold increase compared to the control; ‡80-fold increase compared to the control.

flies and considerably, by 25.8%, in short-lived flies compared with the control. It can be seen from Fig. 5B,F that the HDAC inhibitor TSA strikingly influenced the lifespan in both long- and short-lived lines, but with variable degrees. For the long-lived flies, one-off TSA treatment (o.TSA) had no obvious effect on both mean and maximum lifespan, while continuous TSA treatment (c.TSA) only increased mean lifespan by 15.6% (Fig. 5B). For short-lived flies, o.TSA resulted in an extension of mean lifespan by 5%, and c.TSA significantly increased mean lifespan by 24.4% and maximum lifespan by 16.4% (Fig. 5F). Data in Fig. 5C,G demonstrated that BuA treatment only affected the lifespan of the short-lived line, as one-off BuA treatment (o.BuA) increased the mean lifespan by 25.8% and the maximum lifespan by 11.5%. When BuA was added continuously throughout their lifetime (c.BuA), the life extension effect became less obvious (Fig. 5G). When the flies were fed with HDAC inhibitor and RMHS was applied every 3 days, we found no significant differences among RMHS, TSA-RMHS and BuA-RMHS treatments, and a similar extension to that of the controls was seen (Fig. 5D,H).

Hyperacetylation of histone H3 following HDAC inhibitor treatment and heat shock induction

In order to test if changes in histone acetylation are involved in HDAC inhibitor-mediated lifespan extension and *hsp* gene induction, we examined the acetylation levels of core histones (H3 and H4) after HDAC inhibitor treatment and heat shock using antibodies against acetylated H3 and H4. As shown in Fig. 6, acetylated lysine of histone H3 were detected. The photodensitometric analysis of the bands revealed that both TSA and BuA caused an increase in the acetylation level of H3 in long-lived (Fig. 6A) and short-lived (Fig. 6B) lines. More specifically, o.TSA and o.BuA treatments moderately increased the acetyl-H3 level (P<0.05) (Fig. 6C, o.BuA and o.TSA). c.TSA treatments brought about a significant increase (P<0.01) (Fig. 6C, c.TSA), whereas c.BuA treatment moderately increased the acetyl-H3 level in long-lived flies (P<0.05), and significantly increased it in the short-lived line

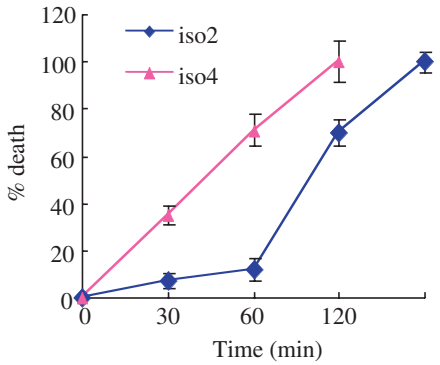

Fig. 4. The death curves of adult flies maintained under high temperature. One hundred flies 6 days after eclosion were kept at 37°C and the number of dead flies was counted at each of the indicated time points for long-lived (iso2) and short-lived (iso4) lines. Values shown are the means ± S.E.M. of four parallel experiments.

Fig. 5. Survival curves of adult *Drosophila* maintained at 25°C. (A) Influence of heat shock on lifespan in the long-lived line. RMHS extended mean lifespan by 11.5% and maximum lifespan by 5.6%. (B) Influence of the HDAC inhibitor TSA on lifespan in the long-lived line. Only c.TSA extended mean lifespan by 15.6%. (C) Influence of HDAC inhibitor BuA on lifespan in long-lived line. BuA treatment had no obvious effect on both mean and maximum lifespan. (D) Influence of heat shock and the HDAC inhibitors TSA or BuA on lifespan in the long-lived line. There was no significant differences among RMHS, TSA-RMHS and BuA-RMHS treatments. (E) Influence of heat shock on lifespan in short-lived line. RMHS extended mean lifespan by 25.8% and maximum lifespan by 11.5%, respectively. (F) Influence of the HDAC inhibitor TSA on lifespan in the short-lived line. o.TSA slightly increased mean lifespan by 5%, and c.TSA increased mean lifespan by 24.4% and maximum lifespan by 16.4%. (G) Influence of the HDAC inhibitor BuA on lifespan in the short-lived line. Only o.BuA increased mean lifespan by 25.8% and maximum lifespan by 11.5%, respectively. (H) Influence of heat shock and HDAC inhibitor TSA or BuA on lifespan in short-lived line. No significant differences existed among RMHS, TSA-RMHS and BuA-RMHS treatments. Values shown are the means ± S.E.M. of four parallel experiments. con, control without any treatment; HS, 37°C one-off heat shock induced for 30 minutes only in larvae; RMHS, 32°C repeated mild heat shock for 1 h every 3 days until death; o.TSA, 10 μmol l^{-1} TSA one-off treatment for 5 h only in larvae; c.TSA, continuous 10 μmol l^{-1} TSA treatment until death; o.BuA, 10 mmol l^{-1} BuA one-off treatment for 5 h only in larvae; c.BuA, continuous 10 mmol l^{-1} BuA treatment until death.

(P<0.01) (Fig. 6C, c.BuA). Moreover, RMHS also increased acetyl-H3 (P<0.01) (Fig. 6C, RMHS). When a combined treatment of both HDAC inhibitors and heat shock was applied, there was an additive effect (P<0.01) (Fig. 6C, TSARMHS and BuARMHS). However, no changes were detected in the acetylated lysine of histone H4 under the same treatments (data not shown).

Influence of histone H3 hyperacetylation on hsp gene expression during aging

Next, we wanted to know whether the hyperacetylation of histone H3 influences *hsp* expression during aging. The *hsp* mRNA levels were assayed by quantitative real-time PCR, and it was found that after c.TSA and o.BuA treatments, *hsp22* and *hsp70* basal expression increased moderately in young flies (6-

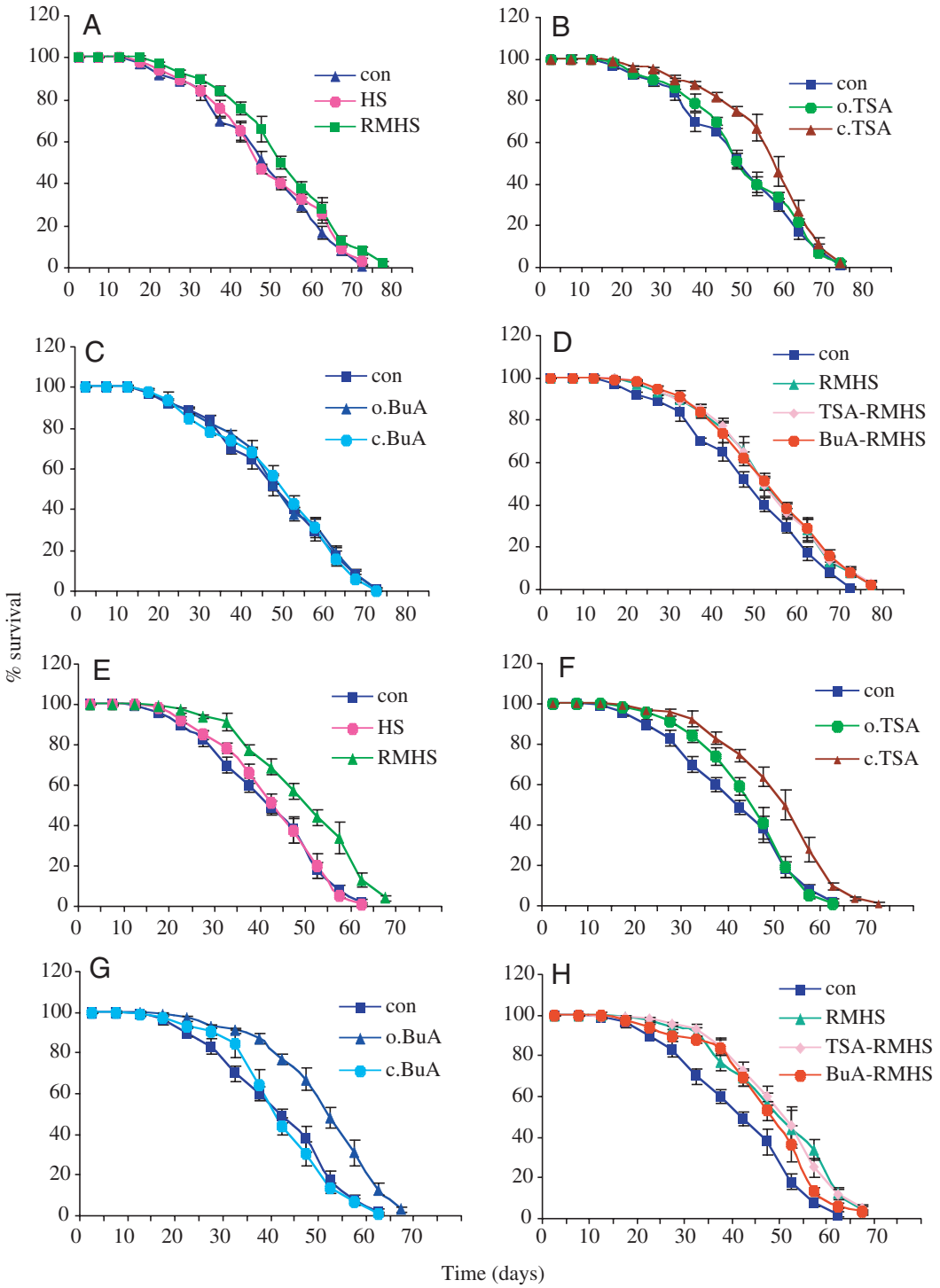

Fig. 5.

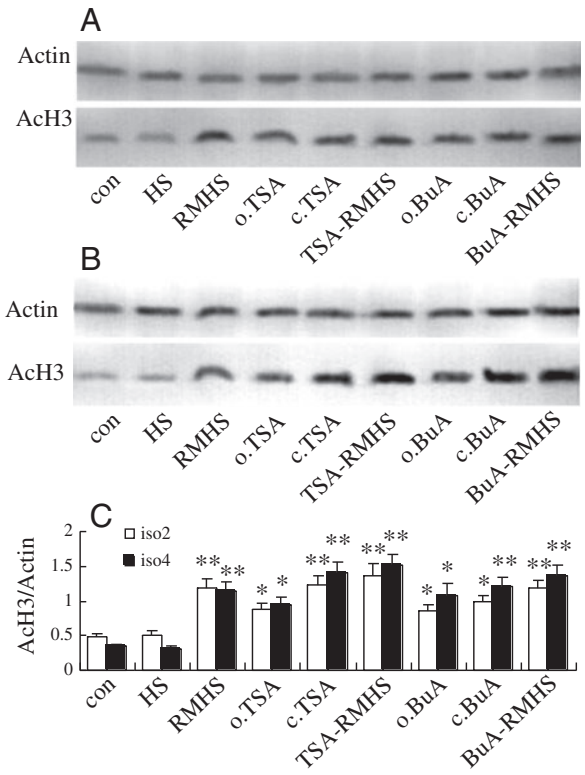

Fig. 6. Hyperacetylation of histone H3 following HDAC inhibitor treatment and heat shock induction. (A,B) Western blots of the acetylated histone H3 (AcH3) in long-lived and in short-lived flies, respectively. The results of photodensitometric analysis of A and B are shown in C. The upper bands are the internal reference actin, and the lower bands are acetylated histone H3. Values shown are the mean ± S.D. of three independent experiments. *Significant (P<0.05); **highly significant (P<0.01). Abbreviations as in Fig. 5.

day old) of the long-lived line (P<0.05) (Fig. 7A,C, iso2), while it increased considerably in young flies (6-day old) of the short-lived line (P<0.01) (Fig. 7A,C, iso4). With the aging of the flies, the degree of increase in these gene products declined gradually. In 30-day old short-lived flies, only the basal transcription of *hsp22* increased moderately compared with control (P<0.05) (Fig. 7B, iso4). For the inducible expression of *hsp* genes, only TSA treatment gave rise to the noticeable effect (Fig. 7E-H). In both long- and short-lived young flies, TSA-RMHS treatment caused a higher expression of *hsp22* and *hsp70* than RMHS (Fig. 7E,G). Similarly, the increase in inducible *hsp* expression declined gradually during aging (Fig. 7E–H). Until 30 days after eclosion, TSA-RMHS treatment only increased *hsp22* expression in the short-lived line (Fig. 7F, iso4), while no increase in *hsp70* expression was detected (Fig. 7H). In addition, to determine whether changes in mRNA levels actually reflect changes in the relative levels of proteins, we assayed Hsp22 protein content in young flies after HDAC inhibitor treatment and heat shock. Total protein was isolated from 6-day-old flies and Hsp22 protein was detected by western blot (Fig. 8). Consistent with changes in the *hsp22* mRNA level, Hsp22 protein expression was up-regulated by the HDAC inhibitors (Fig. 8, c.TSA and o.BuA). Similarly, continuous TSA treatment (c.TSA) and one-off BuA treatment (o.BuA) strikingly increased Hsp22 protein basal expression and only TSA treatment produced a rise in inducible expression of Hsp22 (Fig. 8, TSA-RMHS). Additionally, it is noticeable that the HDAC inhibitor-induced up-regulation of Hsp expression was more prominent in the short-lived line than in the long-lived line (Fig. 8, iso2 and iso4).

In conclusion, this study clarifies the correlation between the elevated expression of *hsp* genes and the longevity in *Drosophila melanogaster*. The results revealed the higher *hsp* basal expression level, higher thermotolerance and higher response to heat shock, but lower *hsp22* induction under continuous high temperature in long-lived than in short-lived flies. *Drosophila* lifespan could be extended by feeding the flies with HDAC inhibitors TSA and BuA, and/or repeated mild heat shock. The range of lifespan extension differed between long- and short-lived lines. HDAC inhibitor treatment promoted *hsp* basal expression, but with the time of aging, the extent of this increase declined gradually. Chromatin modulation may be involved in HDAC inhibitor-mediated *hsp* gene activation, since hyperacetylation was detected in core histone H3 upon TSA and BuA treatments. Thus, a close correlation among histone acetylation, *hsp* gene regulation and aging in *D. melanogaster* can be established.

Discussion

Correlation between hsp *gene expression and longevity*

It has been shown that aging is associated with the accumulation of inactive enzymes, partially denatured and/or damaged proteins (Gershon and Gershon, 1970; Stadtman, 1992). Heat shock proteins are thought to reduce protein denaturation and aggregation, facilitate re-folding of partly denatured proteins, direct the entry of damaged proteins into proteolytic pathways and protect the organisms from additional stress (Wheeler et al., 1995; Parsell and Lindquist, 1993). Results presented in this report indicated that *hsp22* and *hsp70* genes were upregulated and more promptly induced in the long-lived iso-female line (Figs 2 and 3). Also, the thermotolerance was higher in the long-lived line (Fig. 4). Lin et al. (1998) demonstrated that the long-lived *D. melanogaster* mutant displayed resistance to starvation, high temperature and oxidative stress. A similar increased resistance to heat was observed in long-lived *C. elegans* lines (Lithgow et al., 1995). It is possible that higher expression of *hsp* genes may help maintaining cellular functions during aging and prolong the lifespan of the fly. Meanwhile, in long-lived flies, the higher response to heat shock perhaps results in the higher thermotolerance. On the other hand, both the basal expression and induction speed of *hsp* genes in short-lived flies were low. When the flies were kept in harsh conditions, such as high temperature, more Hsp22 was induced to repair the damage (Fig. 3A). This process presumably consumes more energy and therefore results in quicker death.

Previously, two patterns of *hsp* expression during aging were reported. With Oregon-R wild-type and W^{118} flies cultured at low density in culture bottles, *hsp22* RNA levels were low or undetectable in young flies, but *hsp22, hsp23, hsp70* were upregulated with age (King and Tower, 1999). In contrast, when the same Oregon-R flies were cultured at high density using population cage protocol, the starting level of *hsp22* RNA in young flies was high, and the increase in *hsp22* RNA during aging was minor or undetectable (Kurapati et al., 2000). In the experiments of this study, Canton-S wild-type flies were cultured using the high-density population cage protocol. In contrast to the previous observations, we found that *hsp22* and *hsp70* mRNA levels were relatively higher in young flies, and declined with age (Fig. 2). We presume that this may be partially due to the different strain used. Also, it is important to note that this difference was observed under conditions that were more stressful than the optimum.

Moreover, data in this study revealed that the response of *hsp* genes to heat induction varied in flies with different longevity. In the long-lived flies, *hsp* genes were more sensitive to heat than in the short-lived line. In addition, different *hsp* genes exhibited a variable response to heat, i.e. *hsp22* was more responsive to heat than *hsp70*. This suggests that a more positive correlation may exit between *hsp22* and longevity, while *hsp70* may affect longevity to a lesser extent. In contrast, the expression of *hsp26* did not differ significantly in expression between long- and short-lived flies (data not shown), implying that it may not be closely related to longevity. These results implicate the existence of a mechanism for preferential induction of *hsp* genes during aging.

Histone acetylation/deacetylation modifications and expression of hsp *genes*

The mechanisms of *hsp* gene induction by heat stress in *Drosophila* have been studied in some detail (Lis and Wu, 1993). Binding sites for the GAGA transcription factor and other *cis*-acting sequences at *hsp* promoters are required for generation of an accessible promoter structure in unstressed cells, which involves a transcriptionally engaged RNA polymerase paused ~25 bp downstream of the start site for transcription (Gilmour and Lis, 1986; Lee et al., 1992; Rougvie and Lis, 1988). Upon heat shock, the heat shock factor (HSF) is converted into an active trimer from inactive monomers, and

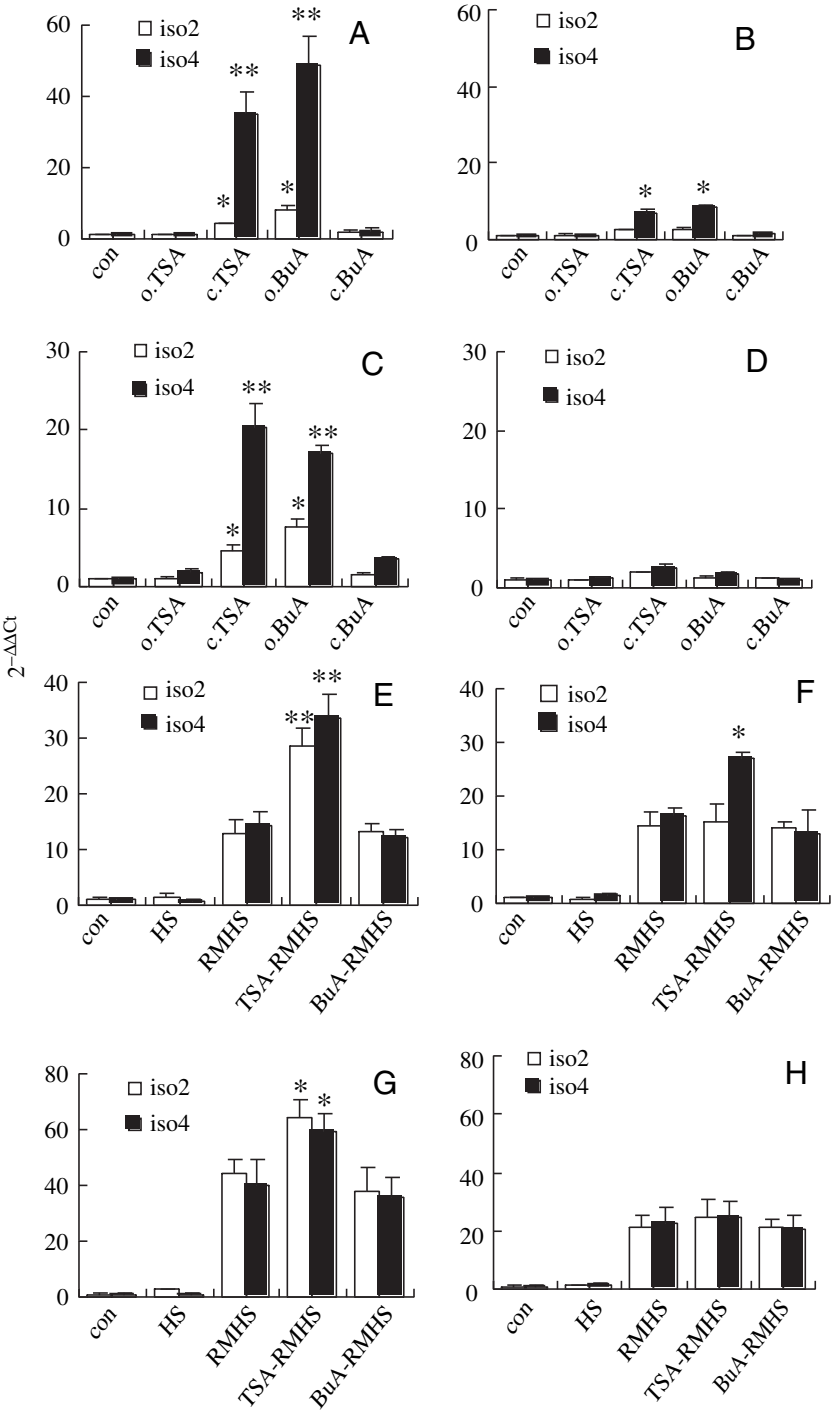

Fig. 7. Influence of HDAC inhibitor-induced histone H3 hyperacetylation on *hsp* mRNA expression during aging. (A,B) *hsp22* mRNA basal expression in young flies (6-day old; A) and old flies (30-day old; B) after HDAC inhibitor treatment. (C,D) *hsp70* mRNA basal expression in young flies (6-day old; C) and old flies (30-day old; D) after HDAC inhibitor treatment. (E,F) *hsp22* mRNA inducible expression in young flies (6-day old; E) and old flies (30-day old; F) after HDAC inhibitor treatment. (G,H) *hsp70* mRNA inducible expression in young flies (6-day old; G) and old flies (30-day old; H) after HDAC inhibitor treatment. Experiments were performed in triplicate. Bars represent means ± S.E.M. For basal expression (A–D), **P*<0.05 and ***P*<0.01, *versus* untreated control. For inducible expression (E–H), **P*<0.05 and ***P*<0.01, *versus* RMHS. Abbreviations as in Fig. 5.

binds to the heat shock element (HSE), facilitating the transcription of *hsp* genes. However, the role of histone acetylation in transcription regulation of *hsp* genes is still obscure. In this study, we showed that the acetylation level of H3 was increased upon treatment with the HDAC inhibitors, TSA and BuA (Fig. 6). We also demonstrated that *hsp* genes were upregulated after HDAC inhibitor treatment (Fig. 7). Interestingly, we noticed that heat shock also increased the acetylation level of H3 (Fig. 6), further confirming the involvement of histone acetylation in *hsp* gene transcription in *D. melanogaster*. TSA and BuA are the two widely used HDAC inhibitors, but they may function through different mechanisms (Gibson, 2000; Finnin et al., 1999). TSA was originally identified as a fungal antibiotic with differentiation inducing properties, and it can strongly increase the acetylation level of histone H3 (Zhong et al., 2003). BuA is a simple chemical that can raise the acetylation level of H3 and H4 by suppressing HDAC activity via a noncompetitive mechanism (Gibson, 2000). TSA and BuA affected *hsp* gene expression differently in this study. TSA was able to induce the expression of *hsp22* and *hsp70* genes above their basal level, and it worked cooperatively with heat shock to raise the inducible transcription intensity of *hsp22* and *hsp70* genes (Fig. 7). BuA only promoted the basal expression of *hsp* genes (Fig. 7A,C). In addition, continuous TSA treatment (c.TSA) and one-off BuA treatment (o.BuA) increased *hsp22* and *hsp70* expression more in short-lived flies, and this result was consistent with the influences of these HDAC inhibitors on the longevity of the flies (Fig. 5F,G). Moreover, as aging proceeded, HDAC inhibitor treatments became less effective in promoting *hsp* gene expression. This may presumably be due to the increasingly inactive response and repair mechanisms in aged flies.

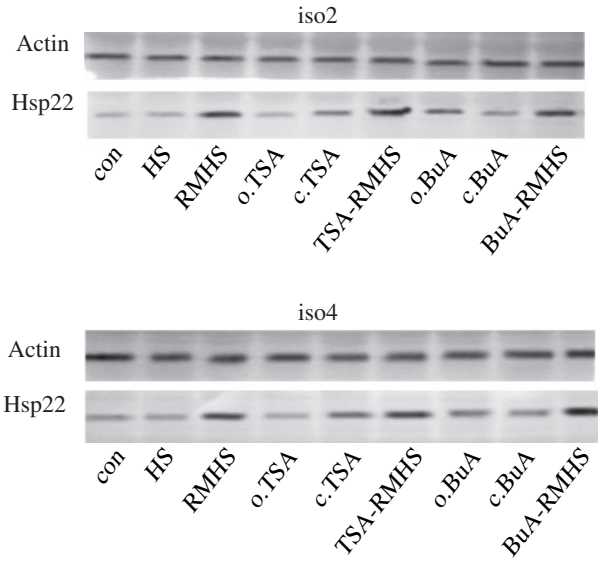

Fig. 8. Influence of HDAC inhibitor-induced histone H3 hyperacetylation on Hsp22 protein level. Western blot analysis of Hsp22 in long-lived (iso2) and short-lived flies (iso4). The upper bands are actin used as the internal reference and the lower bands are Hsp22 protein. Experiments were performed in triplicate. The abbreviations as in Fig. 5.

Histone acetylation/deacetylation modifications and the longevity

A balance of activation and repression of various genes regulates an optimal physiological and cellular environment for longevity. The acetylation/deacetylation modifications of histones play critical roles in activation/repression of many genes, and hence probably regulate the lifespan. In yeast and worms, the HDAC Sir2 and Rpd3 have been shown to be closely related to lifespan (Kaeberlein et al., 1999; Kim et al., 1999; Tissenbaum and Guarente, 2001), and feeding of the HDAC inhibitor phenylbutyrate (Kang et al., 2002) or a hypomorphic mutation of *rpd3* (Rogina et al., 2002) in the *Drosophila* extended the lifespan. However, mutation in *Drosophila sir2* (*dsir2*) gene did not shorten lifespan, as predicted from yeast and worms (Newman et al., 2002). It appears that the influence of histone acetylation on longevity is a complex process and may involve different pathways and mechanisms. We treated flies with a one-off and continuous feeding of HDAC inhibitors, heat shock, as well as combinations of both HDAC inhibitors and heat shock, in an attempt to elucidate the effects of HDAC inhibitor-mediated histone acetylation on longevity. The results showed that TSA strikingly extended the lifespan in different ways, as o.TSA treatment only increased the mean lifespan in the short-lived line, and c.TSA treatment extended the mean lifespan by 15.6% in the long-lived line; while in the short-lived line, it extended both the mean and maximum lifespan by 24.4% and 16.4%, respectively (Fig. 5B,F). Thus, a prolonged TSA action *in vivo* is probably necessary for longevity determination. However, BuA had less effect on lifespan. Only in the short-lived line did o.BuA treatment extend both the mean and maximum lifespan. When BuA was applied throughout the lifetime of the flies, its life-extension effect became insignificant (Fig. 5G). We presume that prolonged BuA feeding may be toxic, which reduced the survival rate. As a rule, both TSA and BuA were more effective in the short-lived line than in the long-lived line. It appears that different genetic background may determine the action as well as the type and optimal concentrations of the HDAC inhibitors. Furthermore, combined treatments of both inhibitors and heat shock (TSA-RMHS and BuA-RMHS) and heat shock alone (RMHS) had basically the same effect on life extension (Fig. 5D,H). We reason that the life extension by RMHS treatment may mask the influences of HDAC inhibitors. These results suggest that establishment of an altered cellular environment by HDAC inhibitors and heat shock changed the lifespan of flies, possibly through a pathway that involves the change in expression of certain genes resulting in inhibition of the accumulation of damaged proteins, and/or stimulation of repair mechanisms.

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