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Effects of intrinsic aerobic capacity, aging and voluntary running on skeletal muscle sirtuins and heat shock proteins

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Abstract

AIM: Sirtuins are proteins that connect energy metabolism, oxidative stress and aging. Expression of heat shock proteins (Hsps) is regulated by heat shock factors (HSFs) in response to various environmental and physiological stresses, such as oxidative stress. Oxidative stress accumulates during aging which makes cells more prone to DNA damage. Although many experimental animal models have been designed to study the effects of knockdown or overexpression of sirtuins, HSFs and Hsps, little is known about how aging *per se* affects their expression. Here we study the impact of intrinsic aerobic capacity, aging and voluntary exercise on the levels of sirtuins, HSFs and Hsps in skeletal muscle.

METHODS: We studied the protein levels of sirtuins (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6 and SIRT7), HSF1, HSF2, Hsp10, Hsp27 and Hsp70 before and after one-year of voluntary running intervention of rat strains selectively bred for intrinsic aerobic exercise capacity; high capacity runners (HCR) and low capacity runners (LCR) differ by more than 30% for median lifespan. This setup enabled us to discern the effects of inborn aerobic capacity, aging and exercise activity on the protein levels of sirtuins, HSFs and Hsps in skeletal muscle.

RESULTS: Our results revealed that the longer lived HCR rats had higher SIRT3, HSF1 and HSF2 contents in skeletal muscle (*gastrocnemius*, $p < 0.05$) than LCRs. Neither aging nor voluntary running had a significant effect on the studied sirtuin proteins. Aging significantly increased the protein levels of HSF1, HSF2 and Hsp27 ($p < 0.05$).

CONCLUSION: Our finding of elevated SIRT3 levels in HCR rats is in line with previous studies; SIRT3 in general is linked to elevated fatty acid oxidation and oxidative phosphorylation, which previously have been associated with metabolic profile of HCRs. HSF1, HSF2 and Hsp27 levels increased with aging, showing that aged muscles responded to aging-related stress. Our study shows for the first time that SIRT3 protein level is linked to high inborn aerobic capacity, and may be directly interconnected to longevity.

Keywords: aging, oxidative stress, physical activity, sirtuin, skeletal muscle

1 Introduction

Aging is a physiological phenomenon during which a progressive decline of organ and tissue function is accompanied with the development of complex diseases. Since aging phenotypes at whole body level results from a complex set of changes at the cellular level, several theories have been formulated to address the underlying mechanisms behind this phenomenon. Telomere shortening and accumulation of oxidative stress are among the most prominent candidates of these theories (1-4). In yeast cells, the SIRT2 ortholog Sir2 was shown to promote longevity, which raised the interest towards a possible role of sirtuin proteins in mammalian aging (5).

Sirtuins are nicotinamide adenine dinucleotide (NAD)-dependent protein deacylases that link protein acylation, metabolism and aging (6). Mammals have seven sirtuins; SIRT3, SIRT4 and SIRT5 are mitochondrial, SIRT1, SIRT6 and SIRT7 are primarily nuclear, and SIRT2 is found both in the nucleus and the cytoplasm (7, 8). Generally, the sirtuins deacylate proteins that are involved either in i) response to oxidative stress or ii) control of metabolism (6). Sirtuins interact with telomeric chromatin and several components of the DNA repair machinery making them potential targets for studies on the mechanisms of oxidative stress in aging (9, 10). Sirtuins are also energy sensor proteins that respond strongly to caloric restriction, a well-recognized intervention to increase life span of lower model organisms and rodents as well as primates (11-13). Furthermore, sirtuins play a role in the selection of energy source by regulating fatty acid oxidation (14, 15) along with mitochondrial energy metabolism and ATP homeostasis (16, 17).

Downstream from sirtuins, heat shock factors (HSFs) are important transcription factors for protecting cells from protein-damaging stress associated with misfolded proteins, and therefore aging and aging related diseases (18, 19). HSFs activate the transcription of a large number of genes that regulate protein homeostasis, including genes encoding heat shock proteins Hsp27 and Hsp70 (19, 20). Hsp10 typically functions as chaperone in mitochondria together with Hsp60 (21). In general, Hsp proteins function as molecular chaperones by supporting the folding of newly synthesized polypeptides and the assembly of multi-protein complexes (22-24). SIRT1 has been shown to maintain the DNA-binding state of HSF1 for Hsp70 induction (25) whereas Hsp10 has proven to be a functional SIRT3 substrate (26).

Several experimental animal models have been genetically designed to study the effects of knockdown of sirtuin and HSF proteins. SIRT6-knockout mice have aging-like phenotype, including severe hypoglycemia and loss of

subcutaneous fat, leading to death at early age (27, 28). SIRT7-knockout mice display cardiac hypertrophy (29), whereas SIRT2-knockdown mice develop tumorigenesis, leading to the notion that SIRT2 acts as a tumor suppressor (30). Mice lacking both SIRT3 alleles have higher levels of fatty-acid oxidation intermediate products and triglycerides during fasting, associated with decreased levels of fatty-acid oxidation, compared to livers from wild-type mice (14). Interestingly, reduced SIRT4 levels significantly increase fatty acid oxidation and cellular respiration in myotubes, demonstrating that that SIRT4 inhibition increases fat oxidative capacity and mitochondrial function in muscle (15). SIRT5-knockout mice are hyperammonemic during fasting and SIRT5-overexpressing mice have conversely increased urea production (31). SIRT1-knockout mice are not viable in inbred strain backgrounds and show pleiotropic phenotypes in outcrossed lines, including small size, developmental defects and sterility (32). In mice overexpression of SIRT1 leads to similar phenotype as calorie-restricted diet: mice are leaner, more metabolically active and display reductions in blood cholesterol, insulin and fasting glucose compared to littermate controls (33). In mammals, HSFs are involved in several developmental pathways, and HSF1-knockout mice exhibit growth retardation, prenatal lethality and decreased lifespan (34). Taken together, the knockout animal models of sirtuins and HSFs show several divergent metabolic defects of which some lead to a shortened lifespan.

Previous studies have revealed that high aerobic capacity and/or high physical activity levels are strongly associated with long lifespan in humans (35, 36). Recently, it was also reported that high intrinsic aerobic capacity is strongly linked to longevity in rodents (37). Because of this strong statistical linkage between exercise capacity and survivability in both rats and humans, many mechanistic studies have concentrated on the effects of exercise on sirtuins and Hsps. It has been shown that prolonged exercise training increases SIRT1 activity in heart tissue in aged rats (38). SIRT3 has been reported to be upregulated by exercise and chronic muscle contractions (39, 40), and resistance training has been shown to increase Hsp levels in skeletal muscle of both young and old rats (41). To date, SIRT1 and SIRT6 have proven to be strong determinants of life span in mice (42, 43). However, only a few studies have addressed how healthy aging itself affects the expression of sirtuins, HSFs and Hsps. There are indications that the activity of sirtuins declines during aging (9), yet more animal and humans studies are needed to explore the effects of the whole sirtuin protein family on lifespan (44).

Here we use rat strains that differ from their intrinsic aerobic capacity; high capacity runners (HCR) and low capacity runners (LCR) (45) before and after one-year voluntary running intervention to measure the proteins levels of sirtuins, HSFs and Hsps. This setup enabled us to investigate the effects of inborn aerobic capacity,

aging *per se* and exercise on skeletal muscle sirtuin, HSF, and Hsp protein levels. This animal model is well suited for aging studies, since in addition to differing from their aerobic capacity HCRs have been shown to have 28-48% longer lifespan than LCRs (37). We hypothesized that HCRs display higher expression of sirtuin, HSF and Hsp proteins compared to LCRs due to their verified longer median lifespan that is tightly coupled to maximal aerobic capacity and their presumed better capability to compensate aging-related oxidative stress. We also hypothesized that the studied protein levels are higher in HCRs in response to exercise as HCRs have been reported to engage in more voluntary activity on a running wheel and to be spontaneously more active in novel environments than LCRs (37, 46, 47).

2 Materials and methods

2.1 Rat strains

The HCR/LCR contrasting rat model system was produced via two-way artificial selection, starting from a large founder population of genetically heterogeneous rats (N:NIH stock), as described previously (45). The phenotype for endurance running capacity was assessed at the University of Michigan (Ann Arbor, Michigan, USA) with a speed-ramped treadmill running test (15° slope, initial velocity of 10 m min^{-1}, increased 1 m min every 2 min) when the rats were 11 weeks of age. For this study, 60 female rats (30 HCRs and 30 LCRs) produced at generations 23-27 of selection were used. All rats were housed in an environment controlled facility (12/12 h light-dark cycle, 22˚C) and received water and standard rodent feed (R36, Labfor, Stockholm, Sweden) *ad libitum*.

2.2 Protocol

We conducted similar speed-ramped maximal treadmill running tests to the rats as described above at the age of 9 and 21 months and collected skeletal muscle samples (gastrocnemius muscle) at the same time points (Fig. 1). Before the first measurements and sampling, the rats were housed in pairs in standard cages. After the first measurements rats were divided evenly into weight and maximal running capacity matched groups (n = 10) and randomly assigned to control (standard cage) vs voluntary running groups (cage with an access to a running wheel). We collected samples from the non-trained animals at time points that coincided with before (age 9 months) and after one-year voluntary running intervention (age 21 months). All together, we had six different subgroups of rats; HCR_before (no intervention), LCR_before (no intervention), HCR-C_after (control), HCR-R_after (runner), LCR-C_after (control) and LCR-R_after (runner) (Fig. 1). During this one-year intervention the rats were housed 1/cage. Rats in the control groups had a wooden tunnel and all rats had nesting material to enrich their environment.

2.3 Body weight and energy intake

Body weight and energy intake of the rats were followed throughout the one-year intervention by weighing the rats and consumed food every second week. The energy intake was calculated from the feed energy content information provided by the manufacturer (Labfor).

2.4 Voluntary running distance and spontaneous activity

Voluntary running distance from the running wheels was followed throughout the one-year intervention with a computerized recording system. Running wheel was mounted on a cage (Techniplast 2154F0105, Buguggiate, Italy), and the wheel was connected to a computerized recording system (Acer Verinton 6900Pro, 32 bit processor produced by Intel, Windows XP). Total wheel laps were recorded continuously, and the total running distance per day was determined by multiplying the number of wheel rotations by the circumference of the running wheel (Ø 34.5 cm). From daily running distances we calculated an average daily running distance for every two-week period. Spontaneous activity was measured for four days at two time points; 13 and 15 months of age. For this purpose, we used ground reaction force recordings, as described previously (48). The absolute values of the differences between consecutive force values were calculated as described by Silvennoinen *et al* (48). The mean of the absolute values were calculated from every second from total 20 values per second. To obtain a single value for total spontaneous activity, the 1-s means were summed for the total measurement time and the sum was divided by the body mass (kg) of the measured rat. From that data, we calculated the activity index as a sum on three day activity per each month (48, 49).

2.5 Tissue processing

Gastrocnemius muscle (n = 10/group) samples were collected from HCR/LCR rats before and after one-year voluntary running intervention. Distal part of gastrocnemius muscle (2/3 of total muscle volume) was used for the analyses. The snap frozen samples were homogenized in liquid nitrogen and processed with either of the following protocols:

2.5.1 Sirtuins, Hsp10, PGC1a, cyt C and citrate synthase:

The homogenized muscle sample was dissolved in ice-cold buffer (20 mM HEPES [pH 7.4], 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 100 mM, b-glycerophosphate, 1 mM Na_3VO_4, 2 mM DTT, 1% NP-40 [nonyl phenoxypolyethoxylethanol], 0.2% sodium deoxycholate, and 3% protease and phosphatase inhibitor cocktail [P 78443; Pierce, Rockford, IL]). The muscle homogenate was then centrifuged at 10.000 x *g* for 10 min at 4°C. Total protein content was determined using the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL) with an automated KoneLab instrument (Thermo Scientific, Vantaa, Finland).

2.5.2 HSFs, Hsp27and Hsp70:

The muscle homogenates were lysed in lysis buffer (24 mM HEPES [pH 7.4], 100 mM NaCl, 5 mM EDTA, 0.5% Triton-X-100, 200 mM β-glycerolphosphate, 20 mM PNPP, 100 μM ortovanadate), and protein concentration was measured with the Bradford method.

2.6 Citrate synthase activity

Citrate synthase activity ($U \bullet \mu g^{-1} \bullet min^{-1}$) in the gastrocnemius muscle (n = 10/group) was studied as it is used as a marker of aerobic capacity and mitochondrial density in skeletal muscle (50). Citrate synthase activity was measured from the same muscle homogenates that were used to determine the total protein content (Citrate Synthase Assay Kit Sigma-Aldrich) with an automated KoneLab instrument (Thermo Scientific).

2.7 Western blot analyses

2.7.1 Sirtuins, Hsp10, PGC1a, cyt C and OXPHOS Cocktail:

Aliquots of muscle homogenate were solubilized in Laemmli sample buffer and heated at 95°C to denaturate proteins. Samples containing 30 μg of total protein were separated by SDS-PAGE for 60-90 min at 200 V using 4–20% gradient gels on Criterion electrophoresis cell (Bio-Rad Laboratories, Richmond, CA). Proteins were transferred to PVDF membranes at 300 mA constant current for 2 h on ice at 4°C. The homogeneity of protein loading was checked by staining the membrane with Ponceau S. Membranes were blocked in TBS with 0.1% Tween 20 (TBS-T) containing 5% nonfat dry milk for 2 h and then incubated overnight at 4°C with commercially available polyclonal primary antibodies to measure the following protein contents with stated dilutions: SIRT1 (1:2000; ab28170, Abcam), SIRT2 (1:500; ab75436, Abcam), SIRT3 (1:800; ab118334, Abcam), SIRT4 (1:1000; ab124521, Abcam), SIRT5 (1:1000; ab13697, Abcam) SIRT6 (1:500; ab62739, Abcam), SIRT7 (1:500; 13477, Cayman Chemical Company), Hsp10 (1:2000; SAB4501465, Sigma), PGC-1α (1:4000; 516557, Calbiochem), cytochrome C (cyt C) (1:500; sc-8385, Santa Cruz biotechnology, Inc.), Total OXPHOS Cocktail (1:1000; ab110413; Abcam) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:10000; ab9485, Abcam). After the primary antibody incubation membranes were washed in TBS-T, incubated with suitable secondary antibody diluted in TBS-T with 2.5% milk for 1 h followed by washing in TBS-T. Proteins were visualized by ECL according to the manufacturer's protocol (SuperSignal West femto maximum sensitivity substrate, Pierce Biotechnology) and quantified using ChemiDoc XRS in combination

with Quantity One software (version 4.6.3. Bio-Rad Laboratories). Sirtuin protein and OXPHOS Cocktail levels were normalized to corresponding Ponceau S stained actin band and after this the mitochondrial sirtuins (SIRT3, SIRT4 and SIRT5) were normalized to OXPHOS Cocktail level. Mitochondrial Hsp10 was first normalized to corresponding GAPDH band and thereafter to OXPHOS Cocktail level similarly as mitochondrial sirtuins. PGC-1α and cyt C were normalized to corresponding GAPDH band.

2.7.2 HSFs and Hsp27and Hsp70:

Samples containing 35 µg of total protein were separated by SDS-PAGE on 7.5 % gels (Bio-Rad Laboratories) and transferred to nitrocellulose membranes with the semi-dry method. Transfer efficiency was checked with Ponceau S staining, and the membranes were boiled for 15 min before being blocked with 5% milk in PBS-0.3%-Tween 20 and immunoblotted with the following primary antibodies: α-HSF1 (1:1000; SPA-901, Enzo Life Sciences), α-HSF2 (1:200; Clone 3E2, Cayman Chemicals), α-HSP70 (1:5000; SPA-810, Enzo Life Sciences), α-HSP27 (1:1000; SPA-800, Enzo Life Sciences), α-GAPDH (1: 15000; ab9485, Abcam). After the primary antibody incubation membranes were washed in PBS-T and incubated with suitable secondary antibody. The signal was developed using Amersham ECL Plus substrate (GE Healthcare) and quantified using ChemiDoc XRS in combination with Quantity One software (version 4.6.3. Bio-Rad Laboratories). HSF and Hsp protein levels were normalized to corresponding GAPDH band.

2.8 Ethics statement

This study was approved by the National Animal Experiment Board, Finland (Permit number ESAVI-2010-07989/Ym-23).

2.9 Conflict of interest

The authors declare no conflict of interest.

2.10 Statistical analyses

Statistical analyses for all variables were carried out using SPSS for Windows 22 statistical software (SPSS Inc., Chicago, IL, USA). The Shapiro-Wilk test was used to investigate within group normality for a given parameter of interest. Levene's test was conducted to assess the homogeneity of the variance assumption. When the normality or equality of variance assumptions were not met, statistical comparisons of parameters between LCR

and HCR groups were made using Mann-Whitney test. Univariate analysis was done by combining the data from both studied rat strains to analyze the strain (HCR or LCR), age (before [age 9 months] or after [age 21 months] the intervention) and running (access to running wheel or a standard cage) effects to the measured parameters. P-values less than 0.05 were considered statistically significant.

3 Results

3.1 Body weight and energy intake

LCRs were heavier during the whole intervention compared to the corresponding HCRs in both studied groups (LCR-C vs. HCR-C and LCR-R vs. HCR-R, $p < 0.05$, Fig. 2A). Energy intake was greater in runner groups compared to the corresponding controls in both rat strains ($p < 0.05$, Fig. 2B). In HCRs, HCR-R consumed more feed compared to HCR-C during 11.5-20 months, whereas in LCRs, LCR-R had higher energy intake during 11.5-12 and 13.5-20 months ($p < 0.05$). The control groups of both rat strains had a similar energy intake.

3.2 Voluntary running and spontaneous activity

HCRs run more voluntarily compared to the LCR runners during the whole intervention ($p < 0.05$, Fig. 3A). HCRs also had higher spontaneous activity compared to LCRs during control and running intervention, showing a strain effect at time point 15 months ($p < 0.050$, Fig. 3B). Rats from both strains that had the opportunity to voluntary run had a clearly higher spontaneous activity at both studied time points than the corresponding controls (Running effect $p < 0.05$, Fig. 3B).

3.3 Western blot analyses

Univariate analysis showed a strain effect for SIRT3 on the gastrocnemius muscle with HCRs having higher protein level compared to LCRs ($p < 0.050$, Fig. 4A, Table 1). Also, Hsp10 normalized to GAPDH showed a strain effect with HCRs having higher protein level than LCRs ($p < 0.010$, *data not shown*). Strain, aging or voluntary running had no significant effect on other studied sirtuins or Hsp10 normalized to OXPHOS Cocktail level (Fig. 4 and 5, Table 1).

Hsp27 levels in the gastrocnemius muscle increased with aging ($p < 0.001$, Fig. 6A, Table 1). No changes were detected for Hsp70 levels (Fig. 6B, Table 1). Univariate analysis further showed that both strain and age had a marked impact on HSF1 and HSF2 levels ($p < 0.050$, Fig. 6C and 6D, Table 1), with HCRs expressing higher protein levels of these transcription factors than LCRs and an increase in both proteins with aging.

There was a clear strain effect on both cyt C and PGC-1α contents in the gastrocnemius muscle with HCRs higher protein levels compared to LCRs ($p < 0.010$, Fig 7A and 7B, Table 1). Aging significantly increased PGC-1α level ($p < 0.05$, Fig. 7B, Table 1).

3.4 Citrate synthase activity

HCRs had higher citrate synthase activity compared to LCRs (strain effect $p < 0.05$). Aging or voluntary running had no significant effect on citrate synthase activity (HCR_before 2939±679 vs. HCR_after 3267±1081 and HCR-R_after 3554±835; LCR_before 2767±866 vs. LCR_after 2863±917 and LCR-R_after 3545±1328 [U•μg^{-1}•min^{-1}, mean ± SD]).

4 Discussion

Our results revealed that HCR rats had higher SIRT3 protein content in skeletal muscle compared to LCR rats. Also, HSF1 and HSF2 proteins showed a similar strain effect with HCRs having higher levels compared to LCRs. Aging increased the protein levels of HSF1, HSF2 and Hsp27.

The main finding from this study is that HCRs have higher SIRT3 protein content in skeletal muscle compared to LCRs (Fig. 4A, Table 1). SIRT3 in general is linked to elevated metabolism, fatty-acid oxidation and oxidative phosphorylation (14, 51), which have been established in HCRs (52-54). A recent study of Overmyer *et al* showed that HCRs have lower mitochondrial protein acetylation in gastrocnemius muscle during rest and exercise than LCRs; however, they did not find a difference in the SIRT3 level (54). Since the rats utilized in Overmyer's study were three month old male rats, whereas our rats were nine month old females, it is possible that the age and gender difference may contribute to the different results. Nonetheless, SIRT3 has been proposed to be the most prominent deacetylase in the mitochondria (55), and Overmyer *et al* suggested that the clear difference seen in the acetylation level might be due to SIRT3 activation, but not protein level *per se*. The higher SIRT3 level and/or activation of HCRs may promote increased deacetylation in mitochondria, thereby increasing the activity of enzymes of oxidative pathways. Thus, compared to LCRs, HCRs are able to more efficiently continue ATP production through oxidative phosphorylation especially during exercise (54).

Furthermore, in a previous study with humans, the strongest association between sirtuins and aging has been found in the SIRT3 gene polymorphism in mitochondria (56). Rose *et al* searched for variability in the evolutionary conserved domain of the SIRT3 gene and identified a silent G/T transversion at the position 477 of the coding region (G477T polymorphism). They found that the TT genotype increased survival in the elderly even though this variant does not alter the amino acid sequence (56). In our study, we found that HCRs, that are shown to have longer lifespan than LCRs, expressed higher levels of SIRT3 protein, indicating that not only the SIRT3 variant but also the protein level may be linked to a longer lifespan.

In addition to SIRT3, the muscle tissue level of its target protein Hsp10 (after normalization to GAPDH) was higher in HCRs than in LCRs (strain effect, $p < 0.010$, *data not shown*). Though originally identified as a mitochondrial chaperone, Hsp10 is also known to be present in cytosol, cell surface, extracellular space and peripheral blood and to have various other cellular functions (21, 57). The ability of HSP10 to change its location is believed to be related to the protection of cells from various kinds of stressors such as infections and

inflammation that are potential threats for healthy aging (21). It may be speculated, that cellular Hsp10 shares a role in healthy aging beside SIRT3.

Unlike what we hypothesized, there were no differences in the protein levels of other studied sirtuins than SIRT3 between the rat strains. In previous studies SIRT1 deacetylase activity has been shown to be similar in non-trained and trained HCR and LCR rats when the training consisted of 12 week treadmill running, whereas SIRT4 level was decreased with exercise in gastrocnemius muscle (58, 59). It was speculated, that decreased SIRT4 level resulted in increased free fatty acid utilization. However, in these studies the results of HCR and LCR rats were reported separately, so there was no comparison between the rat strains on the studied sirtuins. SIRT1 is known to deacetylate PGC-1α, which links SIRT1 to oxidative metabolism (6). Indeed, the activity of SIRT1, but not its expression, has been observed to regulate mitochondrial biogenesis both in human and rat skeletal muscle (60, 61). Another study found that acute endurance exercise increases the levels of SIRT1 and PGC-1α in rat skeletal muscle (62). In our study, we did not detect an increase in SIRT1, either caused by aging or long-term voluntary running, but HCRs displayed higher PGC-1α and cyt C levels than LCRs (strain effect, $p < 0.010$, Fig. 7). Our results from elevated levels of PGC-1α, cyt C and citrate synthase activity in HCRs skeletal muscle are consistent with previous findings of increased mitochondrial copy number, protein expression of mitochondrial respiratory complexes and citrate synthase activity of HCRs in soleus and EDL muscles compared to LCRs (63). Since PGC-1α plays a key role in mitochondrial biogenesis and cyt C is an indicator of mitochondrial density, the elevated PGC-1α and cyt C levels may aid HCRs to maintain their high muscle oxidative capacity during aging (64, 65).

Although we did not detect an aging-related effect on sirtuins, aging significantly increased Hsp27, HSF1 and HSF2 levels (Table 1, Fig. 6). In non-stressed cells, the proteins of many Hsps are hardly detectable and they increase dramatically in response to acute stress. Once induced, Hsps directly modulate the execution of the apoptotic signaling pathway (24, 66). Thus, Hsps have a cytoprotective function: they allow the cells to adapt to gradual changes in their environment and to survive under otherwise lethal conditions (66). Aging in general is linked to accumulation of oxidative stress (1), and it appears also to attenuate the heat shock response in the myocardium of old animals (67, 68). However, in skeletal muscle, old animals seem to retain their capability to accumulate Hsps when subjected to heat shock (69). HSF1 and HSF2 are important for protecting cells from protein-damaging stress associated with misfolded proteins and aging as well as aging-related diseases (18, 19). Interestingly, Hsp27 has been shown to increase the cellular anti-oxidant defense (70). Elevated HSF1, HSF2

and Hsp27 levels reported here may indicate that muscle cells of aged animals respond to aging-related accumulation of oxidative stress and attempt to compensate the stress by increasing the amount of components required for the protective molecular machinery (41). Moreover, aging increased the level of PGC-1α and had a tendency to increase cyt C level (Fig. 7, Table 1), not showing the expected aging-related decrease of mitochondrial function and content that has been established previously (71, 72). One reason for these unexpected results may be the fact that aging-related loss of muscle fibers is associated with a selective atrophy of type 2 (mainly glycolytic) muscle fibers, resulting in higher relative amount of type 1 (oxidative) fibers in aged muscle (73, 74). Hence aging-related changes in skeletal muscle, especially ones in relation to oxidative capacity, are controversially reported in the literature (75). It may be speculated that the shift in fiber-type ratio is also affecting our results. Furthermore, it is possible, that there would have been an aging effect in mitochondrial markers if we had chosen a later time point.

Our second hypothesis was that the exercise response to the studied protein levels would be more profound in HCRs than in LCRs, due to their higher running activity. As expected, HCRs did run significantly more than LCRs (Fig. 3A). Surprisingly, voluntary running had no significant effect on the studied proteins. It has been established in previous studies, that endurance training increases PGC-1α and cyt C levels as well as mitochondrial content and respiratory capacity in skeletal muscle (76). It seems that in gastrocnemius muscle, there was no pressure to increase the oxidative capacity to significant extent, at least not at the very late time point chosen in our study. PGC-1α is known to respond to exercise acutely (77), so as in our study the running distance decreased gradually over time during the one year intervention (Fig. 3A), also the pressure for change decreased. It is possible that we would have seen an adaptive increase in the studied mitochondrial markers if we had chosen an earlier time point or used other muscle instead of gastrocnemius to measure these parameters.

Our data showed that high intrinsic aerobic capacity is linked to high SIRT3 protein levels in skeletal muscle. According to our former study at the age of 21 months all the HCRs in the control group (HCR-C) were surviving, whereas the survivability percent for the other studied groups were the following: 95% for HCR-R, 90% for LCR-C and 65% for LCR-R group (78). In our cross-sectional study we had same number of samples from both rat strains at time point 21 months randomly selected from a larger group size to compare the differences at muscular level. However, we did not detect any differences in the protein levels of the other studied sirtuins between HCR and LCR rats with aging or voluntary exercise. We were not able to measure the activities of sirtuins, and thus, it is possible that there are age-related or exercise-induced differences in the

activities of the proteins studied as well as other proteins that we were not able to detect with the methods utilized in this study. Since HSF1, HSF2 and Hsp27 levels increased with aging in both rat strains, our study suggests that aged muscles remain responsive to stress and can compensate for aging-related accumulative oxidative stress by increasing the amount of protective molecular machinery. To the best of our knowledge, this study provides the first evidence for a direct link between elevated SIRT3 protein levels and intrinsic aerobic capacity, which may be associated with an extended lifespan in mammals.

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Figure legends

Figure 1

Schematic representation of the study protocol. Before the voluntary running intervention we collected samples from HCR/LCR rats from untrained state (HCR_before and LCR_before, n=10/group). We had one-year voluntary running intervention with 4 sub-groups: HCR-C (control), HCR-R (runner), LCR-C (control) and LCR-R (runner) from which we collected samples after the intervention (n=10/group).

Figure 2

Body weight and energy intake during one-year intervention. n = 10/group. $^{*,\#}p < 0.05$, Values are expressed as mean ±SEM.

Figure 3

Voluntary running and spontaneous activity during one-year intervention. n = 7-10/group. Values are expressed as mean ± SEM.

Figure 4

Protein levels of mitochondrial sirtuins SIRT3, SIRT4 and SIRT5 and Hsp10 in gastrocnemius muscle. n = 9-10/group. Sirtuin protein and OXPHOS Cocktail levels were first normalized to corresponding Ponceau S stained actin band after which SIRT3, SIRT4 and SIRT5 were normalized to corresponding OXPHOS Cocktail level. Hsp10 was first normalized to GAPDH level and thereafter to corresponding PonceauS normalized OXPHOS Cocktail level. Values are expressed as arbitrary units (AU) mean ± SD.

Figure 5

Protein levels of nuclear (SIRT1, SIRT6 and SIRT7) and both nuclear and cytoplasmic (SIRT2) sirtuins in gastrocnemius muscle. n = 9-10/group. Values are expressed as arbitrary units (AU) mean ± SD.

Figure 6

Hsp and HSF protein levels in gastrocnemius muscle. n = 4-5/group. Values are expressed as arbitrary units (AU) mean ± SD.

Figure 7

Cyt C and PGC-1α protein levels in gastrocnemius muscle. n = 9-10/group. Values are expressed as arbitrary units (AU) mean ± SD.

Table

Table 1 Univariate analysis of strain, age and running effects of the studied protein levels.

	Variance analysis p		
Protein	Strain	Age	Running
SIRT1	0.412	0.326	0.727
SIRT2	0.232	0.825	0.677
SIRT3	0.020*	0.712	0.906
SIRT4	0.766	0.591	0.952
SIRT5	0.191	0.573	0.385
SIRT6	0.691	0.739	0.969
SIRT7	0.328	0.494	0.582
Hsp10	0.169	0.288	0.933
Hsp27	0.141	<0.001***	0.381
Hsp70	0.454	0.316	0.091
HSF1	<0.001***	0.003**	0.274
HSF2	0.005**	0.020*	0.114
PGC-1α	0.003**	0.049*	0.108
Cyt C	<0.001***	0.087	0.187

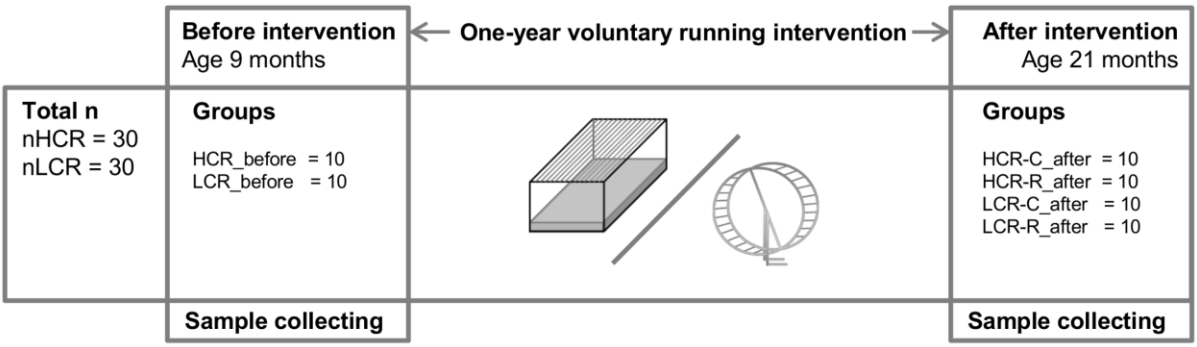


Fig. 1

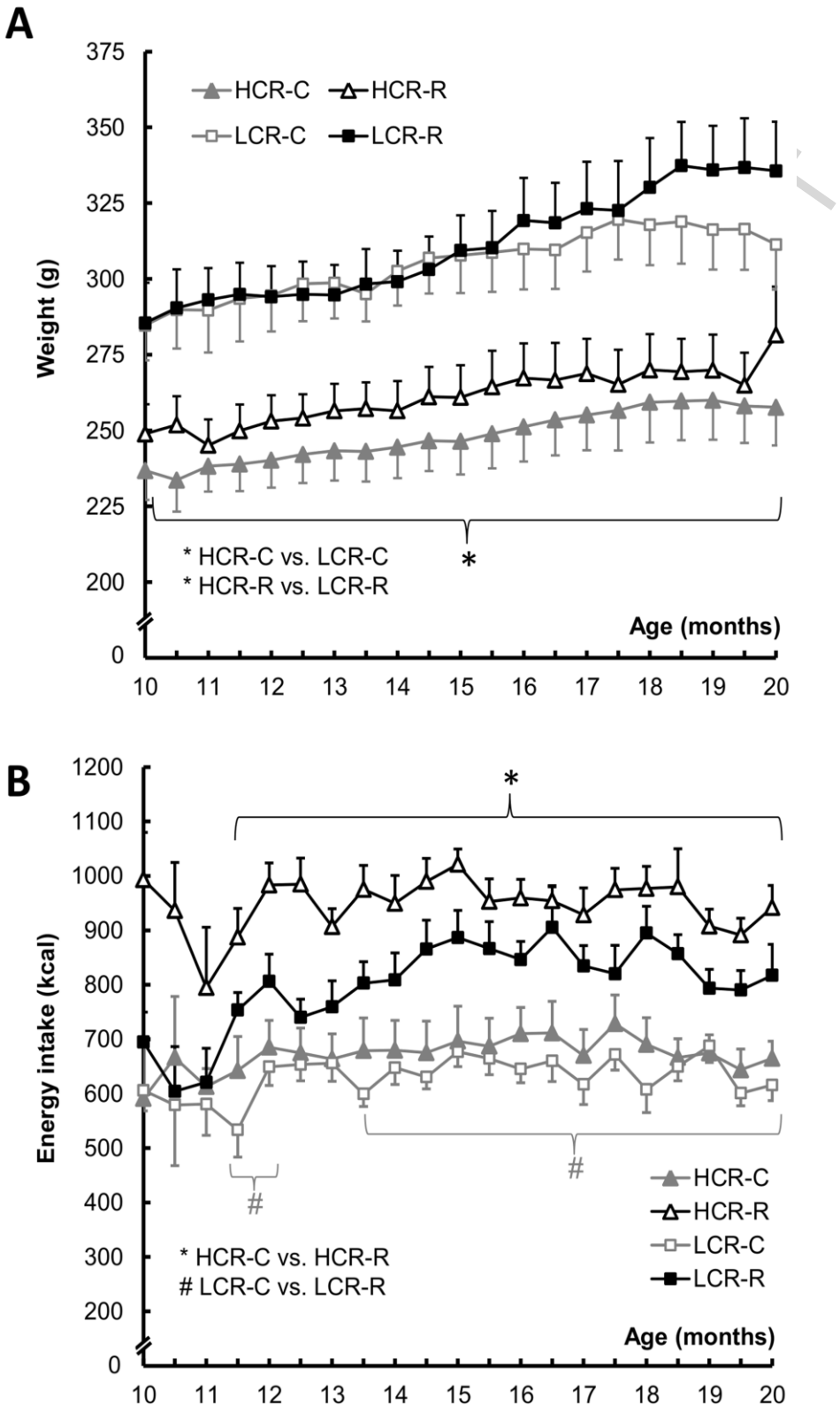


Fig. 2

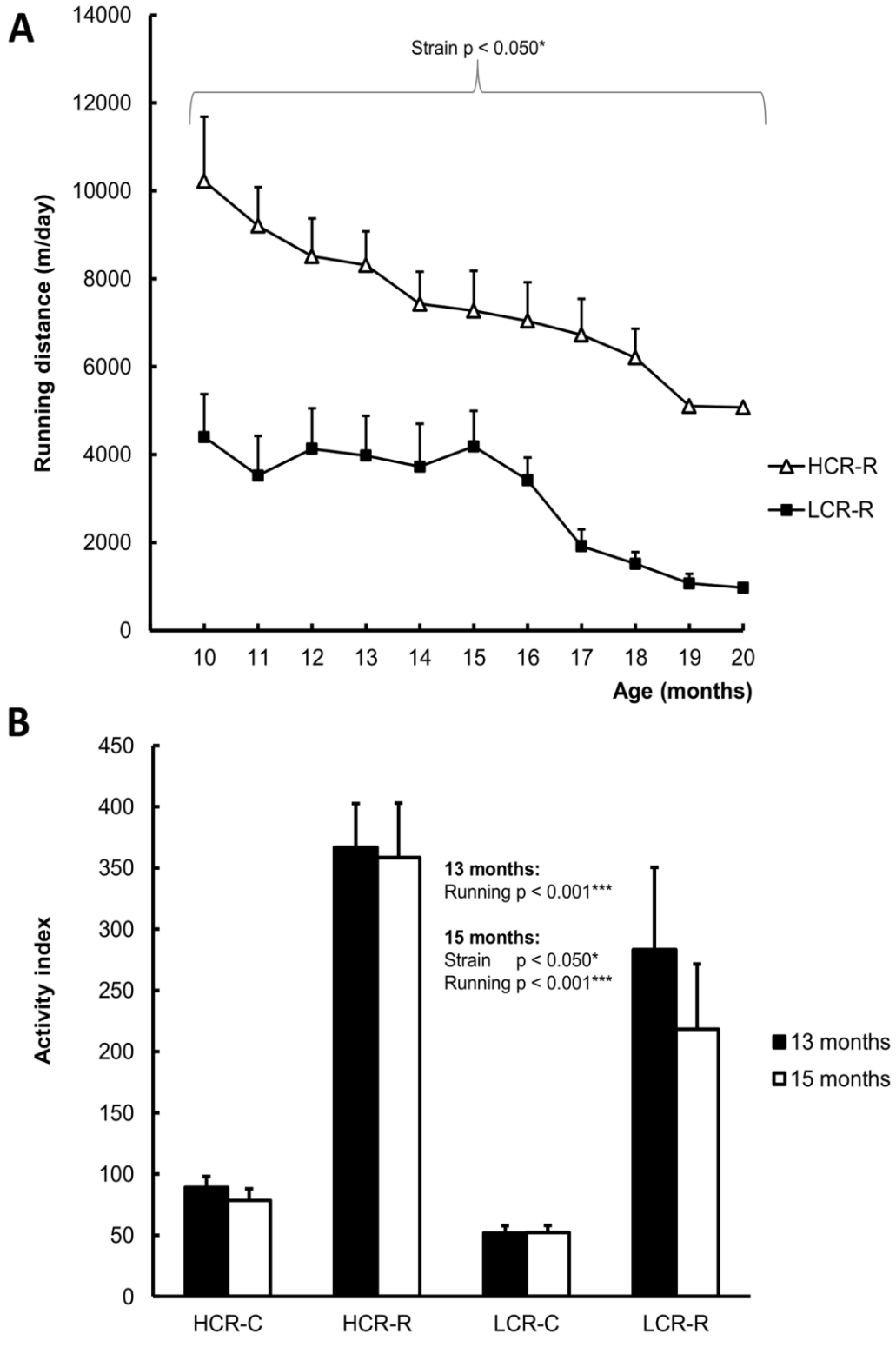


Fig. 3

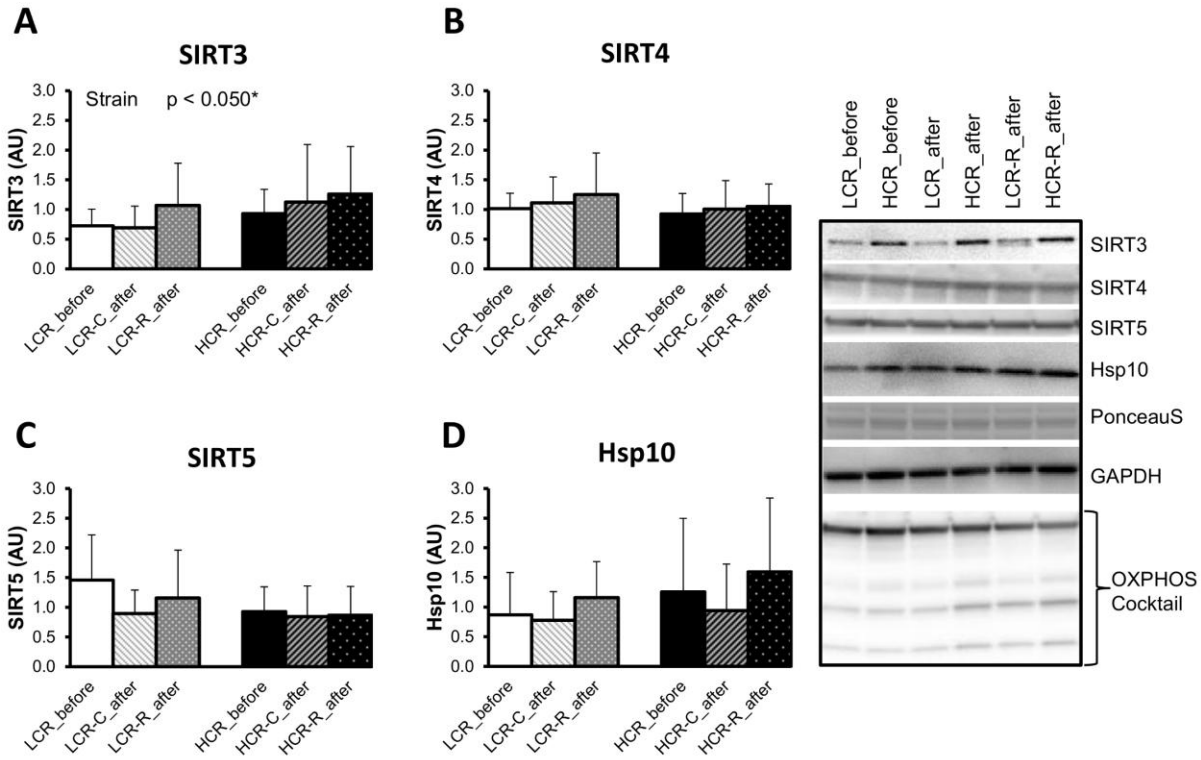


Fig. 4

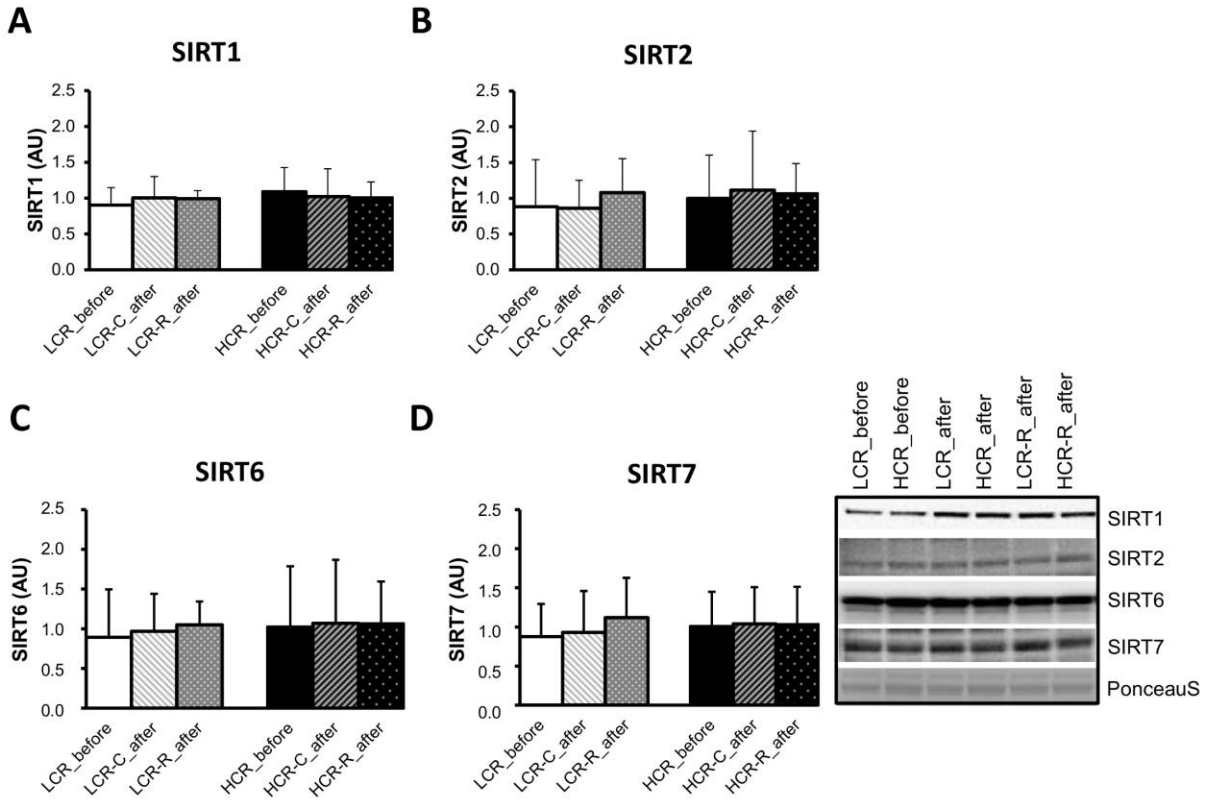


Fig. 5

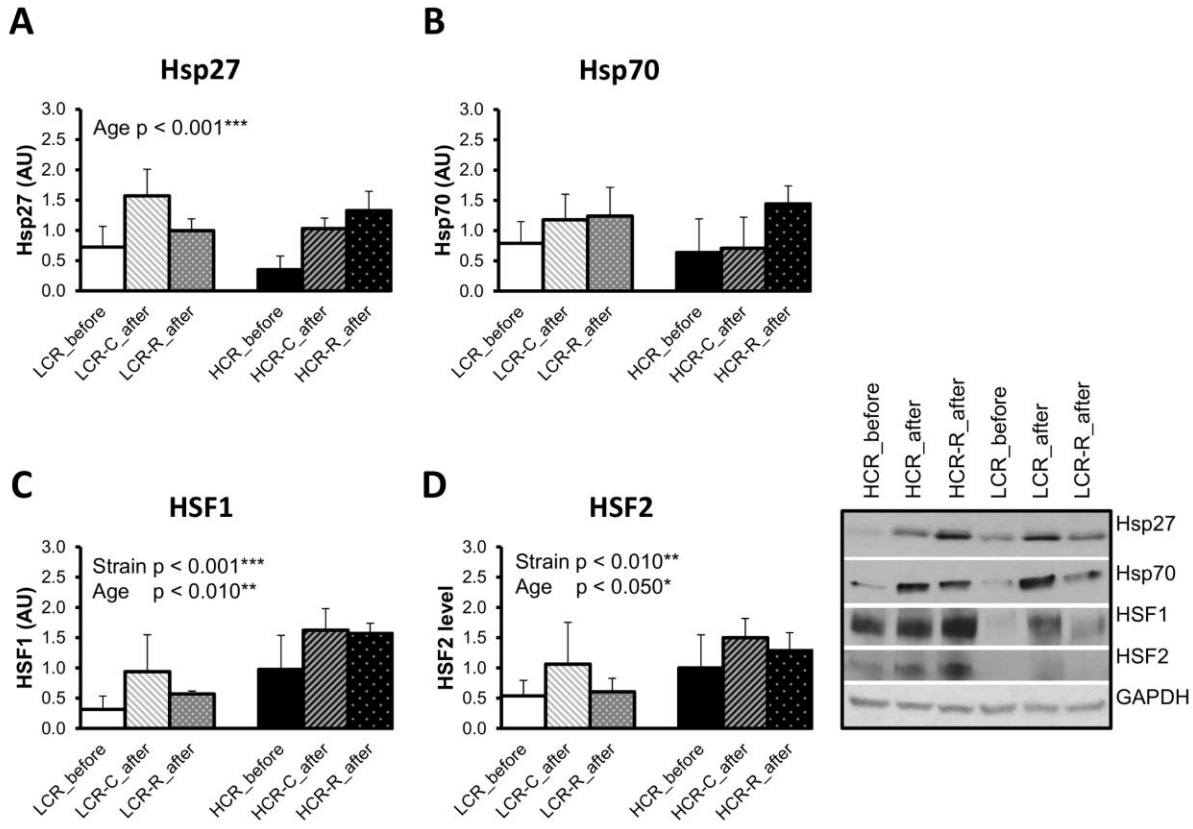


Fig. 6

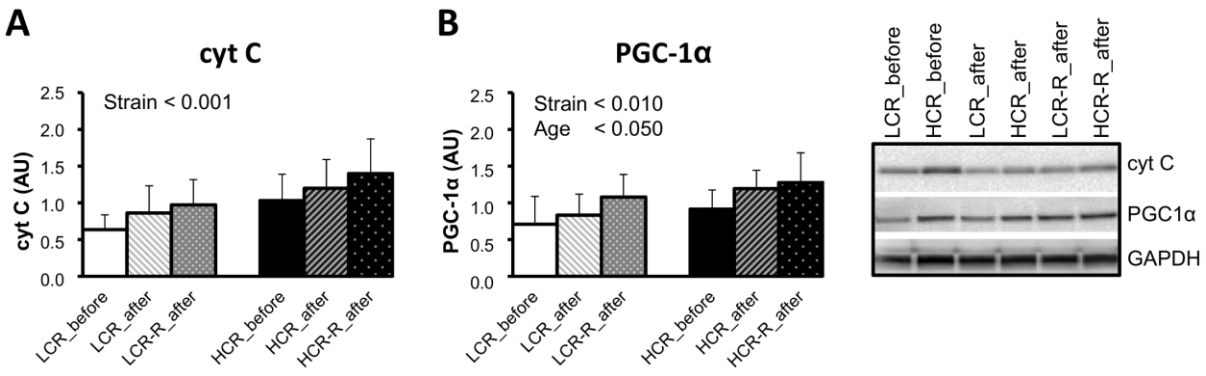
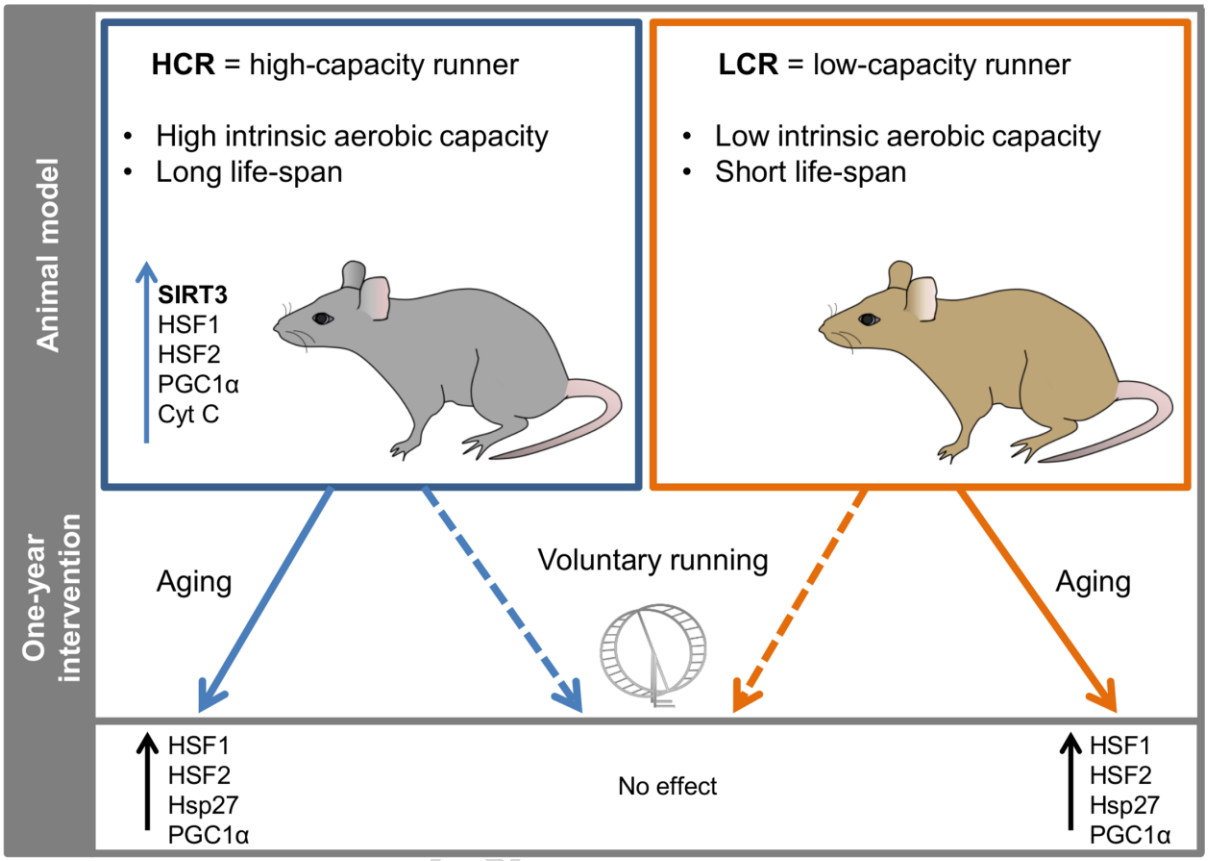


Fig. 7



Graphical abstract

Highlights

- We study the impact of intrinsic aerobic capacity and aging on sirtuins.
- Exploring the role of sirtuins in healthy aging is important for future studies.
- Our study shows that SIRT3 protein level is linked to high inborn aerobic capacity.
- Our study suggests that SIRT3 level possibly is interconnected to longevity.