Chapter 5

Beating in the cold: autophagy in the heart during arousal in natural hibernators

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ABSTRACT

Hibernation consists of torpor periods with reduced metabolic rate, low body temperature and gross changes in physiology, which are interspersed by periods of arousal in which these physiological functions are restored to normal euthermic levels. While heart rate reduces dramatically during torpor from 400 to 5-10 bpm, the heart still functions properly. Cardiac function is highly dependent on avoiding cellular stress through maintenance of an accurate protein homeostasis by the protein quality control (PQC) system. The PQC system encompasses the endoplasmic reticulum (ER) unfolded protein response (UPR\textsubscript{ER}), heat shock response and autophagy. We hypothesized cardiac cellular stress to accumulate during early arousal, reflecting the rapid and dramatic physiological changes. Thus, we examined the various components of the cardiac PQC system in Syrian hamsters during early and late torpor and early and late arousal. Our results show that the PQC system is mainly activated around the transition from torpor to arousal. During late torpor the UPR\textsubscript{ER} shows signs of activation, while autophagy is inhibited and sumoylation of proteins is increased. While the UPR\textsubscript{ER} seems also to be activated during early arousal, this phase is particularly characterized by a strong activation of autophagy and an increase in protein ubiquitination. These results indicate that cellular stress builds up during torpor and is cleared by regulated activation of the PQC system — particularly autophagy — during arousal. This regulated activation likely serves to relieve the heart of aroused animals from aberrant proteins that have been formed during torpor, thus enabling the huge ramp-up of cardiac function during the transition from torpor to arousal.
INTRODUCTION
Hibernation allows animals to survive harsh environmental conditions, such as low temperatures and food shortage, by conserving energy during periods of torpor (lasting several days), which are alternated with arousal bouts (lasting up to one day). During torpor, metabolic rate reduces to <5% of euthermic rates, resulting in a reduction of body temperature from 37°C to a few degrees above ambient temperature (which often is as low as 0-5°C). In addition, key physiological functions undergo drastic changes during torpor, which are restored to normal euthermic levels during arousal.1,2 Changes in cardiac function constitute an important adaptation of physiology during hibernation, as exemplified by the reduction of heart rate from 400 to 5-10 beats per minute during torpor. Despite this dramatic reduction of heart rate during torpor, the heart still needs to function properly at very low body temperatures, which is accomplished by various adaptations in the Ca\(^{2+}\) homeostasis ensuring a low intracellular Ca\(^{2+}\) concentration. This renders hibernating animals resistant to developing arrhythmias,3-6 such as atrial fibrillation (AF), which are associated with an intracellular Ca\(^{2+}\) overload.

In AF, the most common clinical tachyarrhythmia, we recently identified the need for a proper maintenance of proteostasis, i.e. the synthesis, folding, trafficking and degradation of proteins, to prevent cardiac dysfunction and arrhythmogenicity.7-9 The condition of proteostasis is monitored by the protein quality control (PQC) system, which responds to cellular stress by posttranslational modifications, autophagic-lysosomal and ubiquitin-proteasomal degradation of proteins and by mounting the heat shock and unfolded protein responses.10,11 Maintaining proteostasis is challenged in (early) arousal especially, as the associated rapid and dramatic changes in physiological functions are likely to cause cellular stress, which may be due to an altered redox homeostasis and protein damage.1 The vast reduction in body temperature during torpor has shown to influence proteostasis, by inhibiting protein synthesis, changing protein binding properties, protein stability and enzyme function and modifying protein function by switching the phosphorylation status.12-14 Furthermore, proteomic studies in hibernators showed an induction of several stress-related proteins, including heat shock proteins, during late torpor or arousal in liver;15 brain16 and skeletal muscle.17 Also, ubiquitination was found increased during torpor in liver, however, proteasomal degradation was decreased.18 Moreover, HSPA5 expression, a protein involved in the endoplasmic reticulum (ER) unfolded protein response (UPR\(_{\text{ER}}\)), was found altered in several tissues between torpor and arousal.19
Thus, we hypothesized that the activity of the PQC system in heart tissue is reduced during torpor, but highly increased during arousal in the hibernating Syrian hamster. Therefore, we examined the cardiac UPR\textsubscript{ER} by measuring HSPA5 protein expression, the phosphorylation of eIF2α and HSPA5, ATF4, XBP1-s and ATG12 mRNA expression, and the heat shock response (HSR) by quantifying the expression of HSF1 and HSPB1. In addition, we examined cardiac macroautophagy (further referred to as ‘autophagy’), a lysosomal degradation pathway of damaged or aged proteins, macromolecules and organelles. Examination of autophagy was performed by measuring phosphorylation of mTOR (Ser2448), a major switch controlling the activation of autophagy\textsuperscript{20,21} and the expression levels of LC3B, representing the autophagic flux.\textsuperscript{22} Finally, we determined posttranslational protein modifications, including ubiquitination, acetylation and sumoylation, which represent key regulators of protein function.

**MATERIALS AND METHODS**

**Animals**

Male and female Syrian hamsters (*Mesocricetus auratus*) were housed at an ambient temperature of 21°C and a light:dark-cycle (L:D-cycle) of 14:10 hours. Hibernation was induced by shortening the L:D-cycle to 8:16 hours for 10 weeks followed by housing at an ambient temperature of 5°C at continuous dim light (<5 Lux).\textsuperscript{23,24} The hibernation patterns of animals were determined by continuous assessment of general movement via infrared detectors connected to a computer. Syrian hamsters with >24h of inactivity were considered to be into torpor. Animals were euthanized during winter euthermia (animals that did not enter torpor), torpor or interbout arousal. The animal’s activity pattern accurately identified hibernating hamsters as being in torpor or arousal, as evidenced by mouth and body temperatures at sacrification (Table 1). Euthanasia was performed by intraperitoneal injection of an overdose of pentobarbital, followed by exsanguination. Hearts were removed, snap-frozen in liquid nitrogen and stored at -80°C. Experiments were approved by the Animal Care and Use Committee of the University Medical Center Groningen.

**Protein extraction, Western blot analysis and antibodies**

Syrian hamster heart tissue samples were lysed in radioimmunoprecipitation assay buffer, after which Western blot analysis was performed as described before.\textsuperscript{25,26} Briefly, equal amounts of proteins in SDS-PAGE sample buffer were homogenized, by use of a 26G needle and syringe, before separation on 4-20% Precise\textsuperscript{TM} Protein gels
(Thermo Scientific, USA). After transfer to nitrocellulose membranes (Stratagene, The Netherlands), membranes were incubated with primary antibodies and subsequently with horseradish-peroxidase-conjugated secondary antibodies. Signals were detected by the Western Lightning Ultra (PerkinElmer, USA) method and quantified by densitometry with the software GeneGnome and GeneTools (SynGene, UK). Primary antibodies used were: rabbit polyclonal anti-HSF1 (#4356), rabbit polyclonal anti-LC3B (#2775), rabbit polyclonal anti-phospho-mTOR (Ser2448, #2971), rabbit polyclonal anti-phospho-eIF2α (Ser51, #9721), rabbit polyclonal anti-acetylated-lysine (#9441) (Cell Signaling Technology, The Netherlands), rabbit polyclonal anti-HSP25 (HSPB1, ADI-SPA-801, Enzo Life Sciences, USA), rabbit polyclonal anti-HSPA5 (ab21685, Abcam, UK), mouse monoclonal anti-ubiquitin (#AM12039PU-N, Acris Antibodies, USA), goat polyclonal anti-SUMO1 (#AF3289, R&D systems, The Netherlands), rabbit polyclonal anti-SUMO2-3 (#07-2167, Millipore, The Netherlands) and mouse monoclonal anti-GAPDH (#10R-G109a, Fitzgerald, USA). Secondary antibodies used were horseradish-peroxidase-conjugated anti-mouse, anti-rabbit or anti-goat (Dako, Denmark), depending on the species origin of the primary antibody.

Quantitative Real-Time PCR analysis

Total RNA from Syrian hamster heart tissue samples was extracted using Trizol (Invitrogen, The Netherlands), according to manufacturer's instructions. First strand cDNA was generated by M-MLV reverse transcriptase (Promega, The Netherlands) and random hexamer primers (Promega, The Netherlands). Subsequently, the cDNA was used as a template for quantitative real-time PCR (qRT-PCR). Relative changes in transcription levels were determined using the CFX384 Real-time system C1000 Thermocycler (Bio-Rad, The Netherlands) in combination with SYBR green ROX-mix (Westburg, The Netherlands). mRNA levels were expressed in relative units on the basis of a standard curve (serial dilutions of a calibrator cDNA mixture). The mRNA expression level of XBP1-s (spliced) was calculated by subtraction of XBP1-u (unspliced) from XBP1-T (total) mRNA levels.27 Fold inductions were adjusted for GAPDH and β-actin levels and the PCR efficiencies for all primer pairs were between 90-110%.

Primer pairs used are: ATF4 fw: TCCTGAACAGCGAAGTGTTG and rv: GTGTCTGAGGCACTGACCAA, HSPA5 fw: TCGGTGGGTCTACTCGGATT and rv: GGTCATGACACCTCCCACTG, ATG12 fw: CGAACCATCCAAGGACTCAT and rv: TGTTCATCTGTGGCTCATCC, XBP1-T fw: CTCCAGAGACGGAGTCCAAG and rv: AAAGGGAGGCTGGTAAGGAA, XBP1-u fw: CTCCAGAGACGGAGTCCAAG and rv:
CAGAGGTGCACGTAGTCTGAGTGCTG, GAPDH fw: CATCAAGAAGGTGGTGAAGC and rv: ACCACCTGGTTGCTGTAG and β-actin fw: AGCTGAGAGGGAAATTGTGC and rv: GCAACGGAACCGCTCATT.

Table 1 Baseline characteristics of Syrian hamsters during hibernation

<table>
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<tr>
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<th>$T_b$ (°C)</th>
<th>$T_m$ (°C)</th>
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<tr>
<td>WE</td>
<td>7</td>
<td>$32.8 \pm 0.6_{TE,TL}$</td>
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<td>$104.5 \pm 5.6$</td>
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<td>TE</td>
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<td>TL</td>
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<td>$6.5 \pm 0.6$</td>
<td>$6.1 \pm 0.4$</td>
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<td>AE</td>
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<td>$31.0 \pm 0.5_{TE,TL}$</td>
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<td>$90.9 \pm 5.3$</td>
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<tr>
<td>AL</td>
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<td>$34.2 \pm 0.7_{TE,TL}$</td>
<td>$35.1 \pm 0.7_{TE,TL}$</td>
<td>$101.9 \pm 5.0$</td>
</tr>
</tbody>
</table>

Values are presented as mean value ± SEM or number of animals (N). $T_b$: body temperature (core), $T_m$: temperature mouth. Significant differences ($P<0.05$) between groups are indicated by the superscripts. (TE = torpor early, TL = torpor late).

**Statistical analysis**

Results are presented as mean ± standard error of the mean (SEM) for normally distributed data or as median and ranges for non-normally distributed data. For normally distributed data, a one-way ANOVA with a Bonferroni (when equal variances are assumed) or Games-Howell (when equal variances are not assumed) correction was performed. In the case of non-normally distributed data, a Kruskal-Wallis test followed by a Mann-Whitney U test was performed. All P values were two-sided and values of $P<0.05$ were considered statistically significant. SPSS version 22 was used for all statistical evaluations.

**Figure 1** The HSR is not activated during torpor or arousal. Top panels represent Western blots of proteins involved in the HSR and lower panels reveal quantified data normalized for basal protein levels (GAPDH). **A)** HSF1 and **B)** HSBP1 for the indicated hibernation phases.
Figure 2 The UPR_{ER} is presumably activated during late torpor and early arousal. A) Representative Western blots of HSPA5 and phosphorylated eIF2α protein expression normalized for basal protein levels (GAPDH) for the hibernation phases as indicated. B) Quantified HSPA5 protein expression levels, which show a nonsignificant increased expression during late torpor. C) Quantified phosphorylated eIF2α protein levels, which show elevated, although not significant, expression in early arousal. D) mRNA expression of HSPA5 is significantly upregulated during late torpor. mRNA expression of E) ATF4, F) spliced XBP1 and G) ATG12 show higher, but not significant, expression during early arousal. *P<0.05, **P<0.01.
RESULTS
Unchanged HSR and potential activation of UPR_{ER} during torpor and arousal
To characterize cellular stress, first the activation of the HSR and the UPR_{ER} was measured in cardiac samples of Syrian hamsters obtained from early torpor (TE, 24 hours), late torpor (TL, 72 hours), early arousal (AE, 1.5 hours after cessation of torpor) and late arousal (AL, 8 hours after cessation of torpor). The expression of HSF1 and HSPB1, which are involved in the HSR as mediator and target, respectively, did not change throughout torpor and arousal (Figure 1). Next, to measure activation of the UPR_{ER}, HSPA5 and phosphorylated eIF2α protein expression and HSPA5, ATF4, an transcription factor transcribed during eIF2α phosphorylation,\textsuperscript{28} and XBP1-s, a transcription factor which is spliced and, thereby activated upon UPR_{ER} activation,\textsuperscript{28} mRNA expression were determined. Despite observed changes in mean values between groups for some of the expression levels (Figure 2), the large variance precluded any of these factors to reach statistical significance, except in the case of HSPA5 mRNA which was increased in TL (Figure 2D). Lastly, the mRNA expression of ATG12 was determined. ATG12, a crucial protein for the formation of the autophagosomes, is induced by ER stress through phosphorylation of eIF2α and ATF4 transcription.\textsuperscript{29,30} ATG12 mRNA levels showed a higher expression, although not significant, at AE (Figure 2G), which is concomitant to the observed higher expression of eIF2α phosphorylation and ATF4 transcription during AE. Thus, the timing and direction of changes in UPR_{ER} associated proteins and mRNA levels suggests an accumulation of cellular stress in the cardiac ER during torpor, which, however, does not lead to activation of the HSR.

Figure 3 Autophagy is inhibited during late torpor, but activated during early arousal. Top panels represent Western blots of proteins and lower panels reveal quantified data normalized for basal protein levels (GAPDH). A) Phosphorylation of mTOR (Ser2448), which negatively regulates autophagy, is elevated in late torpor. B) The LC3B-II/LC3B-I ratio, indicating activation of autophagy, is increased during early arousal. *P<0.05, **P<0.01, ***P<0.001.
Figure 4 Posttranslational modifications contribute to reducing cellular stress. Top panels represent Western blots of total proteins gels and lower panels reveal quantified data normalized for basal protein levels (GAPDH). A) Total ubiquitination is increased from early torpor to early arousal and decreased during late arousal (P=0.07). B) Total lysine-acetylation is significantly reduced during late torpor. C) SUMO1 is significantly increased during early and late torpor and D) SUMO2-3 is significantly increased during early and late torpor and early arousal compared to late arousal. *P<0.05, **P<0.01 and ***P<0.001.

Activation of autophagy during arousal
Our data suggest that late torpor presumably results in ER stress, which may be cleared via autophagy in the arousal phase. Therefore, the activation of autophagy was examined, by measuring the levels of mTOR phosphorylation and LC3B-II/LC3B-I ratio. We found a significant increase in mTOR phosphorylation at Ser2448 in TL (Figure
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3A), indicating that autophagy is inhibited in this phase. The phosphorylation of mTOR is reduced to basal level in AE, suggesting activation of the autophagic pathway. In accord, there was a significant increase in the LC3B-II/LC3B-I ratio in AE, returning to normal levels in AL (Figure 3B). These results suggest that the rapid transition from torpor to arousal (1.5 h) is associated with activation of autophagy, possibly by the activation of the UPR_{ER}.

Posttranslational modifications during torpor and early arousal

Furthermore, we determined the role of posttranslational modifications, such as ubiquitination, acetylation and sumoylation, in cellular stress. No significant changes were found in expression of total ubiquitinated proteins during torpor and arousal (Figure 4A). However, total ubiquitination is lowest at AL and there is a trend towards a subsequent increase in total ubiquitination from TE to AE (P=0.07 vs AL). Also, there is a trend towards increased ubiquitination during TL and AE of specific protein sizes (Figure S1). Increased ubiquitination in TL and AE suggests that the ubiquitin-proteasome system is activated to clear cellular stress developed during these phases. The expression of total lysine-acetylation shows a significant decrease in TL (Figure 4B). This seems to reflect an overall change in acetylation levels, as lysine-acetylation of specific protein sizes did not show significant changes (Figure S2). Lastly, the expression of SUMO1 and SUMO2-3 was measured. Expression of both SUMO1 and SUMO2-3 is upregulated during torpor and reduced in arousal (Figure 4C and D). Also, sumoylation of specific protein sizes shows upregulation during torpor, especially in proteins with sizes higher than 50kDa (Figure S3 and S4). These results indicate that different posttranslational modifications are induced during torpor and may help to reduce cellular stress.

DISCUSSION

In the present study, we show substantial changes in various elements of the cardiac PQC system during torpor-arousal cycles in the hibernating Syrian hamster. Our data demonstrate that the PQC system is activated mainly around the transition of torpor to arousal. Late torpor displays signs of cellular stress, i.e. activation of the UPR_{ER} and increased sumoylation of proteins. Moreover, animals in late torpor show a substantial increase in phosphorylation of mTOR Ser2448, leading to inhibition of autophagy. Subsequently, during early arousal at 1.5 h following torpor, activation of the UPR_{ER} remains, but is accompanied by increased protein ubiquitination and the
During the transition from late torpor to early arousal, which takes only 1.5 h, the heart encounters cellular stress, resulting from the vast physiological and metabolic changes. This cellular stress leads to the activation of the protein quality control (PQC) system, which is characterized by activation of the UPR$_{ER}$ and autophagy, increased protein ubiquitination and decreased protein sumoylation. The activation of the PQC system may remove aberrant proteins formed during torpor, which enables the heart to safely increase its cardiac function during arousal.

Together, these results indicate that cellular stress is build up in the heart during torpor, but successively cleared during arousal by the regulated activation of the PQC system. This regulated activation of the PQC system likely serves to relief the heart of aroused animals from aberrant proteins that have been formed during torpor, thus enabling the huge ramp-up of cardiac function during the transition from torpor to arousal (Figure 5).
Activation of the PQC system during the transition of torpor to arousal

Our study indicates that in hibernating hamsters cellular stress accumulates during the torpor phase, which is subsequently reduced during the arousal phase, especially early arousal, by activation of the PQC system. However, not all elements of the PQC system seem activated in the heart. Notably, we did not find activation of the HSR during hibernation phases in Syrian hamsters, in contrast to observations in thirteen-lined and Arctic ground squirrels. Liver of Arctic ground squirrels showed an increased expression of HSPH1, HSPA8, HSP90AA1 and HSP90AB1 during torpor. Heart of thirteen-lined ground squirrels showed increased expression of HSP90AB1, HSPA4 and HSPB6 in torpor, while brain had increased HSPB1 and decreased HSPA4 and HSPA8 levels during early torpor. Skeletal muscle showed decreased HSPB1 and HSPB6 expression in late torpor and early arousal. As these studies show an opposite regulation of some HSPs, such as HSPA8 and HSPB1, in different organs of the same animal, activation of the HSR may well be dependent on the organ and/or animal species and may be directed by the severity of cellular stress encountered during hibernation. In addition to the lack of HSR, we found indications of the activation of proteins involved in the UPR. A clear trend towards an increase in HSPA5 expression and phosphorylated eIF2α was found in late torpor and early arousal, respectively. HSPA5 is an ER-resident chaperone protein that is involved in protein folding processes. HSPA5 upregulation during late torpor is in concordance with other studies that showed increased expression of HSPA5 during torpor in brown adipose tissue, brain and liver in thirteen-lined ground squirrels. Increased phosphorylation of eIF2α, a protein that blocks translation and thereby reduces ER stress, was also found during torpor in brain of thirteen-lined squirrels, and indirectly evidenced by increased phosphorylation of its upstream kinase PERK in brain of Syrian hamsters. Furthermore, mRNA levels of HSPA5, ATF4 and XBP1-s were determined. mRNA levels of HSPA5 are significantly increased at TL, which is simultaneously with the rise in HSPA5 protein levels. ATF4 mRNA levels show highest expression at AE, although not significant. Notably, ATF4 is a transcription factor which is transcribed during eIF2α phosphorylation and induced eIF2α phosphorylation was observed during AE. XBP1 is a transcription factor which is spliced and, thereby, activated upon UPR activation. Spliced XBP1 showed enhanced, however not significant, mRNA expression during AE. Although only HSPA5 mRNA levels show significant upregulation during TL, the notion that the other UPR markers, HSPA5 and phosphorylated eIF2α proteins, ATF4 and spliced XBP1 mRNA, also have an increased
expression during TL or AE may suggest that the UPR\textsubscript{ER} is activated. Thus, the current study shows increase in ER stress during torpor, but no activation of the HSR.

One particular interesting pathway, which is activated by ER stress, is autophagy, a lysosomal degradation pathway of cytosolic components and old or damaged organelles, which are degraded into ATP, amino acids and fatty acids to be re-used by the cell. Autophagy is activated during cellular stresses, such as starvation and proteotoxic stress,\textsuperscript{37} and both HSPA5 and phosphorylated eIF2α are involved in the activation of autophagy.\textsuperscript{38,39} In addition, dephosphorylation of mTOR at Ser2448, mainly due to starvation, activates autophagy.\textsuperscript{40} In our study, we found mTOR phosphorylation to be profoundly increased during late torpor, governing inhibition of autophagy during this phase. mTOR phosphorylation, however, returns to normal levels during early arousal, compatible with our finding of a significant activation of autophagy during early arousal, as evidenced by an increased LC3B-II/LC3B-I ratio, which represents activation of autophagy.\textsuperscript{22} Together, these data suggests a blockade of activation of autophagy during torpor, which is released during arousal via ER stress, possibly needed to clear damaged proteins with the advantage to generate additional energy and recyclable amino acids and fatty acids.

In addition, we found changes in posttranslational modifications of cardiac proteins during hibernation. First, total lysine-acetylation was significantly decreased during late torpor. As lysine-acetylation inhibits components of the autophagic pathway,\textsuperscript{41,42} decreased lysine-acetylation suggests a preparation for the activation of cardiac autophagy during early arousal. Second, total sumoylation was increased during torpor phases. Protein sumoylation serves various functions, of which major ones, including inhibition of transcription and promotion of protein stability,\textsuperscript{43,44} may possibly assist in maintaining cardiac function in torpor. Moreover, as sumoylation aids in the formation of autophagosomes,\textsuperscript{45} increased sumoylation during late torpor may prepare for autophagy which subsequently reduces cellular stress during early arousal. Finally, we found a trend towards an increase in total ubiquitination in arousal, indicating activation of the ubiquitin-proteasomal system. However, as the increase in total ubiquitination is at best quite modest, it seems that hibernating hamsters strongly favor autophagy over the proteasomal system to clear the cellular stress developed during torpor. Moreover, an increase of ubiquitin does not necessarily imply activation of the proteasome,\textsuperscript{18} as ubiquitinated proteins can also be cleared by autophagy.\textsuperscript{46,47}
Heart function in natural hibernators during stress and in human heart disease

In this study we characterized the PQC system in heart tissue during torpor and arousal in hibernating animals. We show that cellular stress accumulates during torpor and is completely cleared during the 1.5 h transition period from torpor to arousal, through increased activation of ER stress-induced autophagy. Moreover, in the course of a torpor bout, the PQC system is gradually mobilized and ready to be activated during early arousal. Because cardiomyocytes are terminally differentiated cells, the PQC system is vital for normal cardiac function. Dysfunction of the PQC system underlies accelerated aging of the heart and development of cardiac diseases, which are the most common cause of death worldwide.\textsuperscript{11,48,49} Interestingly, various cardiac diseases feature a phenotypic change of cardiomyocytes described as ‘cardiac hibernation’, as it resembles morphological key features of the heart of torpid hibernators. However, in contrast to natural hibernating animals, which do not show signs of degradation of myofilaments (myolysis),\textsuperscript{50} human cardiac hibernation is characterized by substantial myolysis.\textsuperscript{51-53} In fact, hibernating animals are resistant to developing arrhythmias and lethal cardiac arrest.\textsuperscript{3-6} So apparently, hibernating animals can regulate their PQC system in response to cellular stress to ensure timely clearance of aberrant proteins. This is in contrast to humans and animals with cardiac conditions, in which not only aberrant proteins but also healthy cardiac proteins are cleared, leading to myolysis. Particularly, in our study, we found that autophagy represents the major degradation pathway which becomes activated during AE. Several cardiac diseases, such as atrial fibrillation,\textsuperscript{54} hypertensive heart disease,\textsuperscript{55} heart failure\textsuperscript{56,57} and myocardial ischemia/reperfusion,\textsuperscript{58} show extensive activation of autophagy, which appears detrimental for cardiomyocytes in these conditions. Understanding how exactly hibernators regulate the activation of the PQC system in relation to cellular stress, especially regarding initiation and level of autophagy, may hold promise to further understand the ‘hibernation’ of cardiomyocytes in human heart diseases.

Current limitations

One major limitation of this study is the variability of the results, which presumably lies with the small number of animals per group included. Increasing the number of animals per group should minimize this variability. Another limitation is the inability of using the correct control for the phosphorylated mTOR and eIF2α proteins levels. In this study, the phosphorylated protein levels are corrected for GAPDH, instead of
unphosphorylated mTOR or eIF2α levels. This is due to the limited cross-reactivity of the antibodies with these hamster proteins. Finally, the autophagic flux could not be measured, as the animals were not treated with an autophagy inhibitor such as bafilomycin A1.

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References


22. Kabeya Y, Mizushima N, Ueno T, et al. LC3,


SUPPLEMENTAL FIGURES

Figure S1 Ubiquitination levels of specific proteins. Quantified data of ubiquitination levels of proteins with specific sizes normalized for basal protein levels (GAPDH). A) 70kDa, B) 50kDa, C) 45kDa and D) 24kDa. **P<0.01.

Figure S2 Lysine-acetylation levels of specific proteins. Quantified data of lysine-acetylation levels of proteins with specific sizes normalized for basal protein levels (GAPDH). A) 75kDa, B) 60kDa, C) 45kDa, D) 40kDa, E) 30kDa, F) 16kDa and G) 14kDa. *P<0.05.
Figure S3 Sumoylation (SUMO1) levels of specific proteins. Quantified data of SUMO1 sumoylation levels of proteins with specific sizes normalized for basal protein levels (GAPDH). A) 110kDa, B) 70kDa, C) 55kDa, D) 45kDa, E) 35kDa, F) 30kDa and G) 22kDa. *P<0.05, **P<0.01.

Figure S4 Sumoylation (SUMO2-3) levels of specific proteins. Quantified data of SUMO2-3 sumoylation levels of proteins with specific sizes normalized for basal protein levels (GAPDH). A) 200kDa, B) 100kDa, C) 75kDa, D) 70kDa, E) 55kDa, F) 45kDa, G) 40kDa, H) 37kDa, I) 35kDa and J) 24kDa. *P<0.05, **P<0.01.