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Torpor-arousal cycles in Syrian hamster heart are associated with transient activation of the protein quality control system

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ABSTRACT

Hibernation consists of torpor, with marked suppression of metabolism and physiological functions, alternated with arousal periods featuring their full restoration. The heart is particularly challenged, exemplified by its rate reduction from 400 to 5–10 beats per minute during torpor in Syrian hamsters. In addition, during arousals, the heart needs to accommodate the very rapid return to normal function, which lead to our hypothesis that cardiac function during hibernation is supported by maintenance of protein homeostasis through adaptations in the protein quality control (PQC) system. Hereto, we examined autophagy, the endoplasmic reticulum (ER) unfolded protein (UPR_{ER}) response and the heat shock response (HSR) in Syrian hamster hearts during torpor and arousal. Transition from torpor to arousal (1.5 h) was associated with stimulation of the PQC system during early arousal, demonstrated by induction of autophagosomes, as shown by an increase in LC3B-II protein abundance, likely related to the activation of the UPR_{ER} during late torpor in response to proteotoxic stress. The HSR was not activated during torpor or arousal. Our results demonstrate activation of the cardiac PQC system – particularly autophagosomal degradation – in early arousal in response to cardiac stress, to clear excess aberrant or damaged proteins, being gradually formed during the torpor bout and/or the rapid increase in heart rate during the transition from torpor to arousal. This mechanism may enable the large gain in cardiac function during the transition from torpor to arousal, which may hold promise to further understand 'hibernation' of cardiomyocytes in human heart disease.

1. Introduction

Hibernation allows mammals to survive challenging environmental conditions, such as low temperatures and food shortage, by limiting energy expenditure through lowering of metabolism during periods of torpor. Torpor phases, lasting from several days to weeks, are alternated with much shorter arousal periods (interbout arousal), during which metabolism is restored. During torpor, metabolic rate is suppressed > 95% of euthermic (summer) rates, resulting in a reduction of body temperature (T_b) from 37 °C to a few degrees above ambient temperature (T_a), often as low as 0–5 °C. In addition, key physiological functions undergo drastic changes during torpor, which are restored to levels even exceeding those observed in summer animals during arousal (Carey et al., 2003; Geiser, 2004; McArthur and Milsom, 1991; Zatzman, 1984).

The mammalian heart is particularly challenged during hibernation. During torpor, the heart needs to balance its suppressed metabolism with providing sufficient cardiac output in the face of a largely increased peripheral vascular resistance (Heinis et al., 2015), whilst precluding arrhythmias and cardiac arrest due to low T_b (Johansson, 1996). In addition, during interbout arousals, the heart needs to accommodate the very rapid return to normal function, which markedly exceeds baseline summer function because of low T_a, thermogenesis and shivering. Adaptations of cardiac physiology and morphology during torpor include a reduction of heart rate from 400 to 5–10 beats per minute, an increase in contractility and cardiac hypertrophy (Nelson and Rourke, 2013). Various adaptations in Ca^{2+} homeostasis ensure a low intracellular Ca^{2+} concentration, which confers resistance of torpid hearts to arrhythmias provoked by an intracellular Ca^{2+} overload (Fedorov et al., 2008; Giroud et al., 2013; Johansson, 1996; Li et al., 2011; Nakipova et al., 2017; Yatani et al., 2004). Molecular studies have documented cardiac expression changes mainly during

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preparation for hibernation to adapt metabolism towards beta-oxidation, harness the cardiomyocytes by deploying a foetal expression program (gene expression and molecular changes resembling those during foetal cardiac development, including the preference of carbohydrates over fatty acids as substrates for ATP generation in a hypoxic environment) and increase oxidant defence (Grabek et al., 2011; Heinis et al., 2015; Nelson and Rourke, 2013; Vermillion et al., 2015).

While pre-hibernation adaptations ensure proper heart function during torpor, the transitions between torpor and arousal may still require additional measures to cope with cardiac stress. 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 in models of acute cardiac stress induced by rapid pacing (Brundel et al., 2006; Meijering et al., 2012; Wiersma et al., 2017; Zhang et al., 2014). Patency of proteostasis is monitored by the protein quality control (PQC) system, which responds to cellular stress by mounting the heat shock and unfolded protein responses and by degrading aberrant proteins by the autophagic-lysosomal pathway (Meijering et al., 2015; Powers and Balch, 2013). Maintaining proteostasis is challenged in (early) arousal especially, as the associated rapid increase in cardiac activation rate and hemodynamic load likely induces cardiomyocyte stress, which may lead to imbalances in redox homeostasis and protein damage. In addition, the reduction in metabolism during torpor influences proteostasis, by inhibiting protein synthesis and changing protein stability, function and binding properties, partly conferred through modification of protein phosphorylation (Carey et al., 2003; Epperson et al., 2010a,b; Frerichs et al., 1998; Hindle et al., 2014; Storey, 1997; Whitten and Klain, 1968). Furthermore, proteomic studies in hibernating ground squirrels documented an induction of several stress-related proteins, including heat shock proteins, during late torpor or arousal in liver (Xu et al., 2013), brain (Epperson et al., 2010b) and skeletal muscle (Hindle et al., 2011), whereas heart has not been examined. Expression of GRP78/BiP, a protein involved in the endoplasmic reticulum (ER) unfolded protein response (UPR_{ER}), was found altered in several tissues, including heart, during torpor and arousal (Mamady and Storey, 2006).

Although changed activation of the PQC system has been found in hibernating mammals, these studies only document the differences of PQC activation between summer and torpid animals. Changes in the PQC system within the different hibernation phases (i.e. torpor and arousal) have never been examined. We hypothesized that the activity of the PQC system in heart tissue is reduced during torpor and increases during interbout arousal in the hibernating Syrian hamster. Hereto, we examined cardiac macroautophagy (further referred to as 'autophagy'), a lysosomal degradation pathway of damaged and/or aged proteins, macromolecules and organelles, by measuring the expression levels of LC3B-II, a protein correlating with the levels of autophagosomes (Kabeya et al., 2000) and phosphorylation of mTOR (Ser2448), a major switch controlling the activation of autophagy (Chou et al., 2012; Codogno and Meijer, 2005). Cardiac UPR_{ER} activation was measured by GRP78/BiP protein expression, phosphorylation of eIF2α and mRNA expression of ATF4 and ATG12. In addition, the heat shock response (HSR) was analysed by quantifying the expression of HSF1 and HSP25.

2. Material and methods

2.1. Ethical approval

Experiments were approved by the Institutional Animal Ethical Committee of the University Medical Center Groningen. Animal procedures were carried out in accordance with the European Directive on the Protection of Animals used for Scientific Purposes and Dutch legislation. Experiments were performed on Syrian hamsters (*Mesocricetus auratus*) from Envigo (Indianapolis, USA) with free access to food and water ad libitum. Euthanasia was performed by intraperitoneal injection of an overdose of pentobarbital (600 mg/kg), followed by exsanguination within 5 min.

Table 1
Body temperature and weight of Syrian hamsters at euthanization.

	n	T_b (°C)	T_m (°C)	Weight (g)
TE	10	5.8 ± 0.6	5.6 ± 0.6	109.2 ± 7.2
TL	16	6.5 ± 0.6	6.1 ± 0.4	102.8 ± 4.6
AE	15	$31.0 \pm 0.5^{TE,TL}$	$34.3 \pm 0.7^{TE,TL}$	90.9 ± 5.3
AL	14	$34.2 \pm 0.7^{TE,TL}$	$35.1 \pm 0.7^{TE,TL}$	101.9 ± 5.0

Values are presented as mean value ± SEM or number of animals (n). T_b: body temperature (core), T_m: temperature mouth. Significant differences ($P < .05$) between groups are indicated by the superscripts. (TE: early torpor, TL: late torpor, AE: early arousal, AL: late arousal.)

2.2. Animals

Male and female Syrian hamsters were housed at an ambient temperature of 21 °C and a light:dark-cycle (L:D-cycle) of 14:10 h. Hibernation was induced by shortening the L:D-cycle to 8:16 h for 10 weeks followed by housing at an ambient temperature of 5 °C at continuous dim light (< 5 Lux) (Bouma et al., 2011, 2013). The hibernation patterns of animals were determined by continuous assessment of movement via infrared detectors connected to a computer. Syrian hamsters with > 24 h of inactivity were considered torpid (Oklejewicz et al., 2001). Animals were euthanized during torpor or interbout arousal, the final duration of uninterrupted torpor bout lasted > 80 h for all animals. The animal's activity pattern accurately identified hibernating hamsters as being in torpor or arousal, as evidenced by mouth and core T_b at euthanization (Table 1). Early arousal was induced by handling of the animals, with animals being euthanized 90 min later. Movement was not used to asses arousal, but T_b measurement was. Hearts were removed; the lower 1/3 of the ventricles (mainly representing left ventricle free wall and septum) was snap-frozen in liquid nitrogen and stored at −80 °C.

2.3. Protein-extraction, Western blot analysis and antibodies

Heart tissue samples were lysed in radioimmunoprecipitation assay buffer, after which Western blot analysis was performed as described before (Brundel et al., 1999, 2001; Wiersma et al., 2017). 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™ 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, all Cell Signaling Technology, The Netherlands), rabbit polyclonal anti-HSP25 (ADI-SPA-801, Enzo Life Sciences, USA), rabbit polyclonal anti-GRP78/BiP (ab21685, Abcam, UK) and mouse monoclonal anti-β-actin (#sc47778, Santa Cruz Biotechnology, USA). Secondary antibodies used were horseradish-peroxidase-conjugated anti-mouse or anti-rabbit (Dako, Denmark).

2.4. Quantitative real time-PCR analysis

Total RNA from 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 and random hexamer primers (Promega, The Netherlands). Subsequently, 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 using a standard curve (serial dilutions of a calibrator cDNA mixture). Fold inductions were adjusted for GAPDH and β-actin levels and the PCR efficiencies for all primer pairs were between 90 and 110%.

Primer pairs used were: ATF4 fw: TCCTGAACAGCGAAGTGTTG and rv: GTGTCTGAGGCACTGACCAA, GRP78/BiP fw: TCGGTGGGTCTACT CGGATT and rv: GGTCATGACACCTCCCACTG, ATG12 fw: CGAACCA TCCAAGGACTCAT and rv: TGTTCATCTGTGGCTCATCC, GAPDH fw: CATCAAGAAGGTGGTGAAGC and rv: ACCACCCTGTTGCTGTAG and β-actin fw: AGCTGAGAGGGAAATTGTGCG and rv: GCAACGGAACCGCT CATT.

2.5. Statistical analysis

Results are presented as mean ± standard error of the mean (SEM), with dots in figures showing the values of individual animals. For normally distributed data, a one-way ANOVA with a Bonferroni correction for multiple group comparisons was performed. Multiple group comparisons for non-normally distributed data were performed with a Kruskal-Wallis test followed by a Mann-Whitney *U* test. All *P*-values were two-sided and values of $P < .05$ were considered statistically significant. SPSS version 22 was used for all statistical evaluations.

3. Results

3.1. Autophagy is induced during arousal

To characterize the activation of the cardiac PQC system during hibernation, cardiac samples of Syrian hamsters obtained from early torpor (TE, 20–24 h after entrance into torpor), late torpor (TL, > 72 h after entrance into torpor), early arousal (AE, 1.5 h after cessation of torpor) and late arousal (AL, 6–8 h after cessation of torpor) were used. As we hypothesized that hibernation leads to stimulation of the PQC system, we interrogated activation of autophagy by determining protein levels of LC3B-II, a protein incorporated in and necessary for the formation of autophagosomes (Kundu and Thompson, 2008; Rashid et al., 2015). Relative protein abundance of LC3B-II increased (non-significantly) from early to late torpor, peaked significantly in early arousal and returned to levels not different from early torpor in late arousal (Fig. 1A, Fig. S1), suggesting that torpor-arousal cycles are associated with a transient induction of autophagosome formation during early arousal. Although proteolysis can be suppressed in the cold, we did not see any difference in ubiquitin levels between aroused and torpid animals (Fig. S2), suggesting that autophagic activity is not suppressed in the cold. Next, we examined mTOR, a protein suppressing autophagic activation when phosphorylated at Ser2448. In keeping with our observation that arousal is associated with autophagosome formation, mTOR Ser2448 phosphorylation was significantly lowered in late torpor and early arousal (Fig. 1B). These observations suggest that the rapid transformation from torpor to arousal (1.5 h) is associated with autophagosome formation and a transient activation of autophagy.

3.2. The UPR_{ER} is activated during hibernation and is associated with autophagy activation

Next, we explored whether transient induction of autophagosomes is related to increase in the UPR_{ER}, which is activated by ER stress upon proteotoxic stress. Activation of the UPR_{ER} was determined by measuring protein expression of the ER-resident molecular chaperone GRP78/BiP and of phosphorylated eIF2α, an important ER stress regulator. The relative protein abundance of the ER-resident molecular chaperone GRP78/BiP remained unchanged throughout torpor and arousal (Fig. 2A). While mRNA levels of GRP78/BiP increased (Fig. 2B), differences did not reach statistical significance. The relative protein abundance of phosphorylated eIF2α increased from early torpor to late torpor and returned to the level of early torpor during arousal (Fig. 2C), suggesting presence of ER stress in late torpor. Phosphorylation of eIF2α induces transcription of the transcription factor ATF4, involved in the UPR_{ER}, and ATG12, a protein crucial in autophagosome formation (Hetz et al., 2015; Rashid et al., 2015; Verfaillie et al., 2010). Consequently, our data indicates that mRNA levels of both ATF4 and ATG12 were significantly increased during early arousal (Fig. 2D and E), matching the observed higher expression of phosphorylated eIF2α in late torpor. The increased expression of both phosphorylated eIF2α and ATF4 mRNA suggests UPR_{ER} activation during the transition from torpor to arousal, while the increased expression of ATG12 mRNA suggests a transient activation of autophagosome formation. Collectively, these results indicate that the rapid transition from torpor to arousal (1.5 h) is associated with induction of autophagosome formation and possibly the activation of autophagy, likely related to activation of the UPR_{ER} due to cellular stress.

3.3. Hibernation does not activate the HSR

To characterize activation of the HSR, we determined expression of HSF1 and HSP25, both key proteins involved in the HSR in heart. The relative protein abundance of HSF1 and HSP25 did not change throughout torpor and arousal (Fig. 3), suggesting that the HSR is not activated during hibernation.

4. Discussion

In the present study, we show for the first time that torpor-arousal cycles in the hibernating Syrian hamster are associated with a transient activation of the PQC system. In contrast, previous studies only

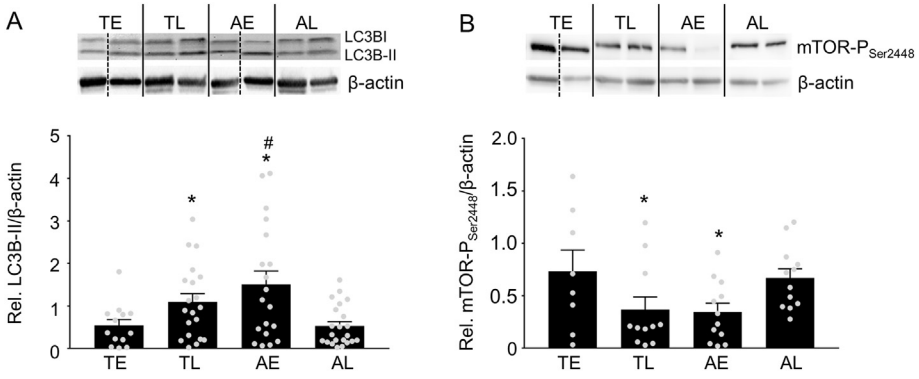

Fig. 1. Autophagy is inhibited during torpor, but activated during early arousal. (A) LC3B-II expression, indicating activation of autophagy, is increased during early arousal. (B) Phosphorylation of mTOR (Ser2448), which negatively regulates autophagy, is elevated in late arousal. Top panels represent Western blots of proteins and lower panels reveal quantified data normalized for basal protein levels (β-actin). $^{*}P < .05$ vs AL, $^{\#}P < .05$ vs TE.

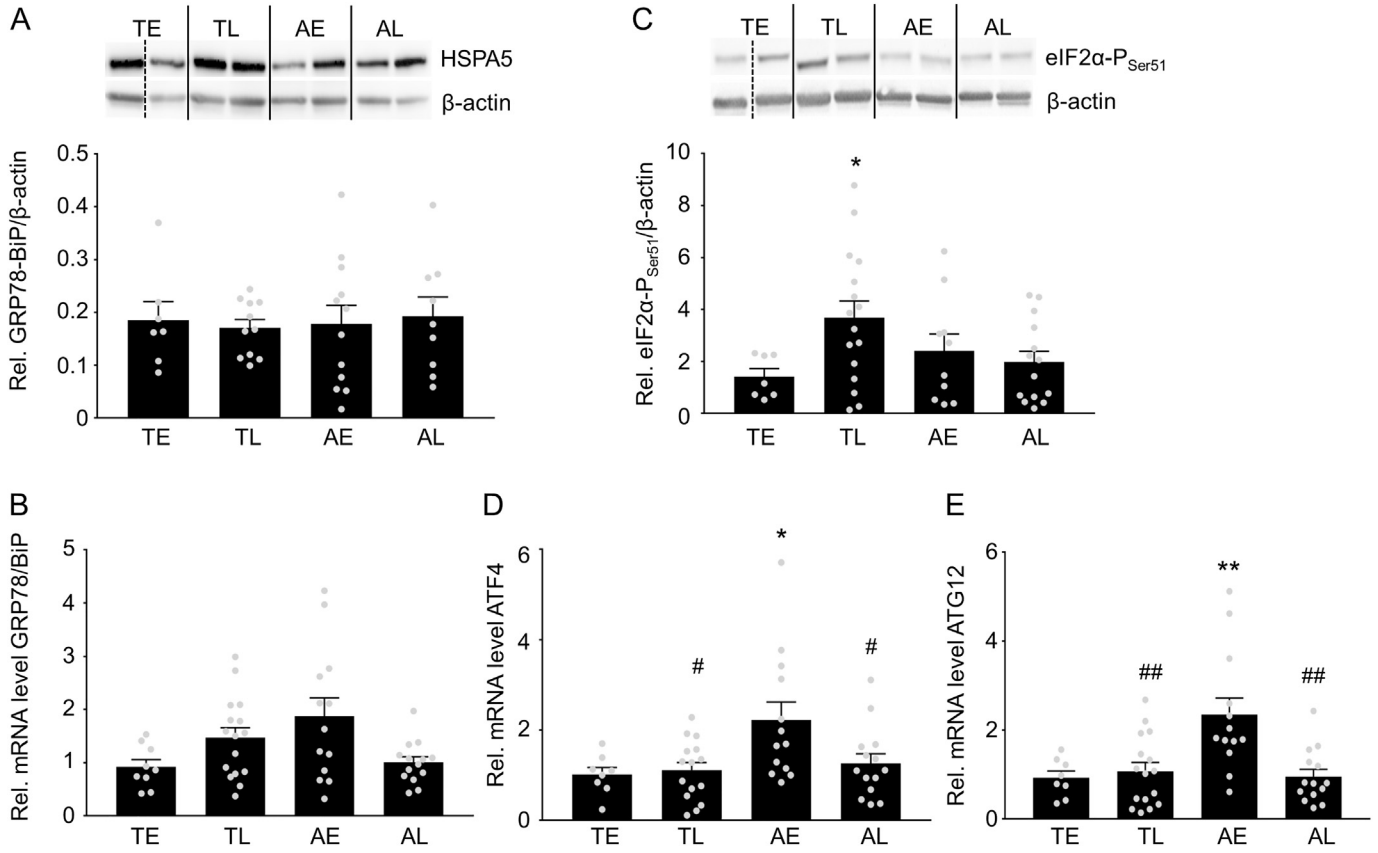

Fig. 2. The UPR$_{ER}$ is activated during late torpor. (A) Top panel represents Western blot of GRP78/BiP and lower panel reveals quantified data normalized for basal protein levels (β-actin) for the indicated hibernation phases. (B) mRNA expression of GRP78/BiP for the indicated hibernation phases. (C) Top panel represents Western blot of phosphorylated eIF2α and lower panel reveals quantified data normalized for basal protein levels (β-actin) for the indicated hibernation phases. (D) mRNA expression of ATF4 is significantly upregulated during early arousal. (E) mRNA expression of ATG12 is significantly upregulated during early arousal. $^{*}P < .05$, $^{**}P < .01$ vs TE, $^{\#}P < .05$, $^{\#\#}P < .01$ vs AE.

documented differences in the PQC system between summer and hibernating (torpor-arousal) animals (Epperson et al., 2010a, b; Frerichs et al., 1998; Grabek et al., 2011; Hindle et al., 2011; Mamady and Storey, 2006; van der Harg et al., 2014). Particularly, early arousal is associated with autophagosome formation, as shown by increases in ATG12 mRNA and LC3B-II protein abundance and a decrease in mTOR phosphorylation. This may be due to activation of the UPR$_{ER}$ in late torpor and early arousal. Our data suggests that hibernation is associated with the accumulation of cellular stress in specific cardiac cellular compartments, as the UPR$_{ER}$ but not the HSR is activated. Furthermore, our data suggests that cellular stress in the heart accumulates mainly around the transition (1.5 h) from torpor to arousal, but is cleared very early during arousal by the regulated activation of the PQC system. This mechanism may serve to relieve the heart of aroused animals from aberrant proteins that have been formed during torpor, thus enabling the large gain in cardiac function during the transition from torpor to arousal.

4.1. Activation of the PQC system during the transition of torpor to arousal

Our data suggests that cellular stress accumulates in the heart of hibernating hamsters during the torpor phase, which might subsequently be reduced by activation of the PQC system during early arousal. Our data principally demonstrates induction of autophagosome

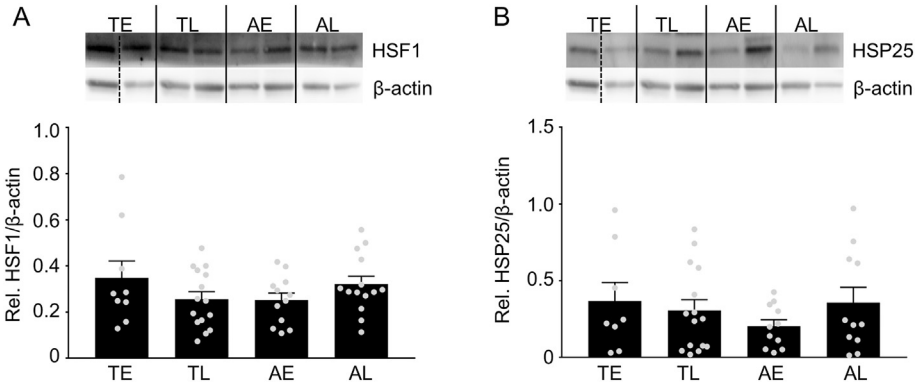

Fig. 3. The HSR is not activated during torpor or arousal. Top panels represent Western blots of proteins involved in the HSR and lower panels reveal quantified data normalized for basal protein levels (β-actin). (A) HSF1 and (B) HSP25 for the indicated hibernation phases.

formation in the hibernator's heart during early arousal, as evidenced by an increased LC3B-II protein abundance (Kabeya et al., 2000). Autophagy is a lysosomal degradation pathway of dysfunctional cytosolic components, serving to be re-used by the cell. Autophagy is activated during cellular stress, such as starvation and proteotoxic stress (Kundu and Thompson, 2008), and phosphorylated eIF2α can activate the autophagic pathway whereas the presence of GRP78/BiP is necessary for autophagosome formation (B'Chir et al., 2013; Li et al., 2008). In addition, phosphorylation and desphosphorylation of mTOR at Ser2448 occurs during a large number of cellular processes, including autophagy. Dephosphorylation of mTOR at Ser2448 activates autophagy, as observed during starvation (Datan et al., 2014). Decreased mTOR phosphorylation found in heart during late torpor and early arousal may thus be permissive in the stimulation of autophagosome formation. Successively, mTOR phosphorylation increased during late arousal, consistent with the inactivation of autophagosome formation during this phase. Autophagosome formation during early arousal may serve to clear damaged proteins with the additional advantage of generation of energy and recyclable amino acids and fatty acids. Interestingly, autophagosome formation is absent by late arousal, suggesting that the arousal duration suffices to fully clear cellular stress and restores a healthy cardiac function. However, decreased mTOR phosphorylation may suggest a role for starvation in the hibernating Syrian hamster heart (Datan et al., 2014).

In spite of autophagy in early arousal, not all elements of the PQC system, however, seem activated in the heart during torpor-arousal cycles. Notably, we did not find activation of the cardiac HSR. Possibly, absent or limited HSR during the hibernation cycle may result from earlier mounting of the HSR during preparation for the hibernating season, as suggested by studies in thirteen-lined ground squirrels (Epperson et al., 2010b; Grabek et al., 2011; Hindle et al., 2011). In contrast to the HSR, our data substantiate activation of the PQC system in a particular cellular compartment, i.e. the endoplasmic reticulum. Activation of the UPR_{ER} during the hibernation cycle was evidenced by the increase of phosphorylated eIF2α abundance, a protein that blocks protein translation and thereby reduces ER stress (Groenendyk et al., 2013), and increased ATF4 mRNA levels. It is important to note that spurious protein phosphorylation has been observed in the context of hibernation, as shown by Hindle et al. (2014) and van Breukelen et al. (2004). Nevertheless, a previous report comparing summer and torpid animals documented increased phosphorylation of eIF2α during torpor in brain of thirteen-lined squirrels (Frerichs et al., 1998). Moreover, eIF2α phosphorylation during torpor is indirectly evidenced by increased phosphorylation of its upstream kinase PERK in brain of torpid Syrian hamsters (van der Harg et al., 2014). However, Protein and mRNA expression of GRP78/BiP were unaltered. GRP78/BiP is an ER-resident chaperone protein involved in protein folding processes (Groenendyk et al., 2013), whose upregulation was shown in brown adipose tissue (Mamady and Storey, 2006), brain (Epperson et al., 2010b; Mamady and Storey, 2006) and liver (Epperson et al., 2010a) in thirteen-lined ground squirrels during late torpor compared to summer levels. Thus, our current study indicates that ER stress may occur during torpor in Syrian hamster heart, without stimulating the HSR. Collectively, our data suggest a gradual increase in autophagosome formation and possible initiation of autophagy during torpor, which is only fully deployed in early arousal via ER stress and dephosphorylation of mTOR.

4.2. Heart function in natural hibernators during stress and in human heart disease

Because cardiomyocytes are terminally differentiated cells, their PQC system is vital for normal cardiac function (Henning and Brundel, 2017). Various cardiac diseases feature a phenotypic change of cardiomyocytes described by clinicians as ‘cardiac hibernation’, as it encompasses key features of the heart of torpid hibernators, such as re-expression of the foetal gene program. However, human ‘cardiac hibernation’ is characterized by substantial degradation of myofilaments (myolysis) (Ausma et al., 1997; Elsasser et al., 2004; Maes et al., 1994), in contrast to its absence in natural hibernating animals (Skepper and Navaratnam, 1995). Absence of structural cardiac damage in hibernators, such as myolysis, may be rooted in their ability to regulate the PQC system to specifically clear aberrant proteins. Several cardiac diseases, such as atrial fibrillation (Wiersma et al., 2017), hypertensive heart disease (Wang et al., 2010), heart failure (Takemura et al., 2006; Zhu et al., 2007) and myocardial ischemia/reperfusion (Matsui et al., 2007), 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.

4.3. Current limitations

A limitation of this study is the unavailability of suitable antibodies with reactivity in hamster, including mTOR, eIF2α and p62, which is a limiting factor in interrogating the complete UPR_{ER} and autophagy pathways. However, we corrected the phosphorylated protein levels of mTOR and eIF2α for β-actin levels, which was stably expressed.

Competing interests

The authors have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpb.2018.06.001.

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