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Out Cold: Biochemical Regulation of Mammalian Hibernation – A Mini-Review

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Metabolic rate depression · Hypometabolism · Torpor · Protein phosphorylation, reversible · Unfolded protein response · Antioxidant defense

Abstract
Hibernating mammals offer an intriguing example of natural torpor and illustrate the regulatory mechanisms that control metabolic rate depression and the cell preservation strategies that support long-term viability in a hypometabolic state. These suggest applied strategies for improving the hypothermic preservation of human organs for transplant, and guidelines that could aid the development of torpor as an intervention strategy in human medicine. Recent advances in hibernation research have illustrated mechanisms that contribute to metabolic depression by orchestrating the global suppression of ATP-expensive transcription and translation including multiple forms of post-translational modification of proteins/enzymes (phosphorylation, acetylation, SUMOylation), mRNA storage mechanisms, and differential expression of microRNA species. DNA-screening technologies have also contributed new advances in understanding the range of cell functions that are impacted during torpor and point out some critical preservation strategies that aid long-term viability in a torpid state. These include antioxidant defenses, chaperones and the implementation of the unfolded protein response, and the enhancement of serpins (serine protease inhibitors) to control the actions of extracellular proteases in clotting and inflammation responses.

Introduction

Mankind has always been interested in life extension including not just living longer but ways to remain much healthier into late life and to lessen the impact of injury and disease. One way that could potentially extend lifespan is to enter a state of torpor, where physiological and biochemical activities are suppressed and energy expenditure is minimized, and then arouse back to normal life at a future date. Indeed, torpor is a critical natural strategy for animal survival under many environmental extremes. Existence in a virtual state of suspended animation is not really what most people have in mind when they think of life extension but the ability to induce torpor could have multiple applications that help to extend human life in other ways. For example, current practice in transplant medicine relies on either cold ischemia (packing in ice) or the recently developed normoxic perfusion methods, but both are time-limited and require rapid transfer of donor organs to recipients [1, 2]; methodology could be improved by using inducible torpor to

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lengthen the time that organ explants can remain viable. Inducible torpor could also be a valuable aid to various surgeries and is of interest to the military for use with seriously injured personnel who have to be transported over a long distance to medical care. The mechanisms used in natural torpor to prevent degeneration of vital functions during long periods of inactivity also have applications to other medical concerns such as how to minimize skeletal muscle atrophy during long-term bed rest. Ultimately, inducible torpor will likely also be necessary for human exploration of outer space. Hence, there is keen interest in understanding the biochemistry of natural states of torpor and dormancy.

The ability to enter a hypometabolic state actually occurs widely across the animal kingdom and allows many vertebrate and invertebrate species to put life on hold when environmental conditions are inhospitable for normal life [3, 4]. Entry into a hypometabolic state has a range of uses. Shallow nightly torpor is critical to overnight survival by many small mammals and birds that live on a very tight energy budget [5]. Much longer seasonal dormancies are used by other species to hibernate through the winter or estivate through the hot dry months of summer [5, 6]. Developmental arrest (e.g. diapause, dauer state) preserves viability in stressful environments and is also employed by some insects to synchronize populations so that adult forms all emerge at once to facilitate breeding [7, 8]. Animals that live in environments with variable oxygen supply use hypometabolism when oxygen is unavailable; by suppressing metabolic rate by >90% they compensate for the interruption of oxidative phosphorylation and reduce their energy needs to a level that can be sustained by the ATP produced from carbohydrate fermentation alone [4]. Indeed, given the widespread use of hypometabolism across phylogeny, humans might almost be viewed as being remarkable for their near lack of this capacity, although some evidence of what may be an ancestral capacity probably still exists in deep meditative states or in the reduction of thermogenesis during starvation, and may also tie into clinical depression and other mood disorders [9, 10]. Human newborns also exhibit a hypometabolic response to hypoxia, in common with other infant mammals [11].

Research in my laboratory has focused on the biochemistry of natural hypometabolism for many years and we have studied animal models of hibernation, estivation, anaerobiosis, and freeze tolerance to discover the common principles of metabolic arrest across phylogeny [3, 4]. Mammalian hibernation is perhaps the most complex form of natural hypometabolism because it involves not just a torpid state but also the inhibition of thermogenesis so that core body temperature (Tb) can fall to near 0°C. However, being mammals, the metabolic challenges encountered by hibernators and their solutions to them are probably the most relevant to a goal of developing inducible torpor as a treatment strategy for humans and their organs. This review discusses some recent advances in understanding the biochemistry of mammalian hibernation with a particular focus on regulatory and preservation strategies that contribute to long-term viability in the hypometabolic state.

The Phenomenon of Hibernation

Hibernation has been documented in eight different groups of mammals: monotremes, marsupials, rodents, bats, shrews, insectivores, primates (some lemurs), and carnivores (bears) [5]. Prevalence is greatest among rodent and bat species and these have received the greatest attention from researchers. Preparations for hibernation begin in late summer when animals go through a period of hyperphagia that greatly increases body fat (mainly stored as triglycerides in white adipose tissue) sometimes doubling body mass [12]. Many species use only body fuel reserves to survive the winter but others also have food caches in their burrows. Enzymatic adjustments enhance the potential for lipid catabolism by all organs, even the brain which switches to a strong reliance on ketone bodies (acetoacetate, β-hydroxybutyrate) as fuel during hibernation; these are produced from fatty acids by the liver [12]. The hibernating season consists of multiple bouts of deep torpor that can last for days or weeks interspersed with periods of arousal during which animals rewarm to the euthermic state for about a day (fig. 1). Small mammals in cold surroundings can reduce their metabolic rate by 95–99% when in full torpor compared with the basal rate in euthermia, and core Tb, normally 35–38°C, can fall to <5°C [5]. Vital functions are strongly suppressed; e.g., ground squirrels hibernating with a core Tb of 5°C have a heart rate of only 5–10 beats/min compared with 350–400 in euthermia, and the organ perfusion rate drops to <10% of normal. Breathing drops from >40 breaths/min to less than one. Overall, by hibernating, small mammals can save about 90% of the energy that would otherwise be needed to remain euthermic over the winter [13]. Indeed, energy savings would be much greater if animals did not undergo periodic arousals back to euthermia because this process requires massive thermogenesis from two sources: uncoupled respiration by the specialized mitochondria

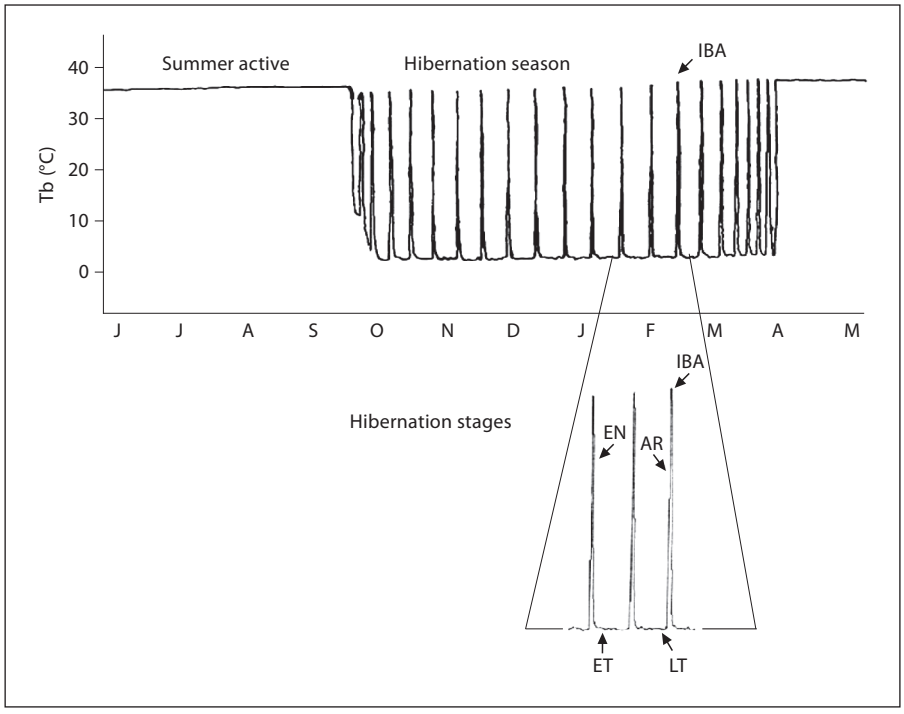

Fig. 1. Body temperature (Tb) of a golden-mantled ground squirrel *(Spermophilus lateralis)* monitored over a year from June to May. The inset shows an enlargement of three torpor-arousal cycles. Stages are: EN = entrance into torpor which lasts up to 12 h; ET = early torpor within 48 h of entering torpor; LT = late torpor; AR = arousal lasting about 2 h; IBA = interbout arousal lasting about 20 h. From Carey et al. [44] with permission.

found in brown adipose tissue and shivering by skeletal muscle. Brown adipose thermogenesis is achieved by the action of uncoupling protein-1 that short-circuits the proton motive force by allowing protons to cross the inner mitochondrial membrane without driving ATP synthesis by the F_1F_0-ATPase; hence, energy that should drive oxidative phosphorylation is dissipated as heat instead. Multiple theories about the purpose of periodic arousals are still being debated [14].

Readers of this journal may be interested in the question of whether hibernation impacts the longevity of a species. Hibernators may spend about half of their lives in torpor, so do these long periods in dormancy allow the periods of active life to be spread out over more years? For most hibernating species, this to difficult and/or impossible to test for a number of reasons including circannual rhythms of hibernation that cannot be broken, difficulty in maintaining some species in captivity, or extremely long lifespans that make data gathering impractical. However, interspecific comparisons generally conclude that hibernation has little effect on lifespan. Thus, within the rodent family Sciuridae, the longest-lived species are nonhibernating squirrels and not the many kinds of hibernators in this group (e.g. ground squirrels, marmots, chipmunks) [15]. As a group, bats live about 3.5 times longer than nonflying mammals of the same size and a small effect of hibernation seems to occur with hibernating species living about 5 years more than nonhibernating bats [15, 16]. However, hibernation cannot account for the full extent of bat longevity and it is proposed that the influence of hibernation has more to do with concealing bats from predators for many months of the year and/or retarding physiological deterioration while in cold torpor [16]. However, one study of Turkish hamsters showed a good correlation between the percentage of lifespan spent in torpor and longevity; this species is a short-lived facultative hibernator that enters torpor in the cold. Lyman et al. [17] grouped animals as poor, moderate, or good hibernators based on the amount of time spent in torpor (0–11, 12–18, or 19–33% of their lives) and these designations correlated well with mean lifespan which was 727, 916, or 1,093 days, respectively. Furthermore, room-temperature controls that never hibernated had a mean lifespan of 812 days compared with 914 days for all hibernating groups combined.

Regulation of Hibernation

Hibernation has a number of facets. Most prominent is the strong global suppression of metabolic rate that defines torpor and requires a regulated and coordinated re-

duction in the rates of all metabolic processes. Layered over this, differential controls are needed to allow varying degrees of suppression of different metabolic functions as well as meet organ-specific needs. Selective gene expression is called into play to enhance or induce specific proteins, particularly those that contribute to cell preservation during torpor, and adjustments are also needed to switch fuel metabolism to a lipid-based economy. Differential effects of low Tb on metabolic reactions may also be exploited to regulate some enzymes or compensatory adjustments may rebalance temperature-sensitive functions [18]. Indeed, a main underlying cause of the catastrophic injuries to human organs caused by hypothermia or by hypoxia/ischemia is the energy crisis that develops when stress-induced ATP limitation disrupts normally balanced cell functions; for example, a rapid consequence of reduced ATP availability is membrane depolarization when ATP-dependent ion pumps can no longer keep pace with oppositely directed ion movements through ion channels [19]. Mild hypothermia can have some benefits for human survival in selected situations. For example, rapid body cooling in some accident situations causing mild to moderate hypothermia (e.g. falling into cold water, avalanche burial) can have a neuroprotective benefit (although in avalanche burial, hypocapnia from rebreathing air accelerates cooling [20] so that lethal limits might occur sooner). Controlled hypothermia is also becoming widely used for medical treatment of traumatic brain injury or perinatal hypoxia-ischemia [21, 22] although the useful, noninjurious window for cooling of core Tb is only 2–4°C. Interestingly, summer-active hibernating species are as susceptible to metabolic damage from hypoxia or hypothermia insults as are nonhibernating mammals, but during the winter they can transition into cold torpor with ease, letting Tb fall to near 0°C. This is achieved by controlled metabolic rate depression and a regulated reduction of the hypothalamic Tb set point. What are some of the mechanisms involved?

Global Metabolic Arrest

Studies with a range of animal models and multiple forms of hypometabolism have shown that a critical universal mechanism of global metabolic suppression is reversible protein phosphorylation (RPP) [3, 4]. The addition or removal of covalently bound phosphate groups on proteins by the actions of protein kinases or protein phosphatases can produce major changes in the activity states of many enzymes and functional proteins, often providing virtually on/off control. RPP provides a fast, coordinated (i.e. one protein kinase can regulate multiple target proteins) and readily reversible mechanism for suppressing metabolic functions. Particularly important targets for global controls are the catabolic pathways that regulate ATP supply and the major ATP-consuming functions in cells including transmembrane ion transport, gene transcription and protein translation. There is now extensive evidence that RPP is the central mechanism that regulates these and other metabolic activities over cycles of hibernation/arousal, just as it does in many other systems of natural hypometabolism. Thus, hibernation-responsive RPP inhibits enzymes of carbohydrate catabolism (e.g. glycogen phosphorylase, hexokinase, pyruvate dehydrogenase) [3, 18] to spare carbohydrates. Phosphorylation of both Na^+/K^+-ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase reduces the activities of these membrane ion pumps by ~50% in tissues from hibernating animals as compared with euthermic controls (both measured at 25°C) [3]. Regulation of these pumps is also important to hibernation because elevated rates of transmembrane ion cycling, requiring high rates of ATP hydrolysis, is the main heat source that supports endothermy. Hence, inhibition of the pumps is integral to the fall in core Tb when animals enter torpor.

Strong suppression of transcription and translation is another feature of hibernation [23, 24] although the upregulation of selected individual genes occurs against this background. Multiple global controls on these energy-expensive cell functions have been identified in recent research. First of all, global inhibition of the action of transcription factors (Tfs) occurs by conjugation of Tfs with small ubiquitin-related modifier (SUMO) proteins. In ground squirrels, the amount of SUMO-conjugated protein rose dramatically in the brain, liver and kidney during torpor with an opposite reduction in free SUMO [25]; this modification was reversed when animals aroused. Furthermore, immunohistochemistry showed that SUMO-1 protein was distributed throughout neuronal cell bodies in euthermic squirrels but was highly concentrated in the nucleus of torpid animals, arguing for the importance of SUMOylation in inhibiting Tf action during torpor. SUMOylation has also been linked with cellular responses to other stresses including hypoxia/ischemia, oxidative, osmotic and genotoxic stress [26].

Chromatin level controls also provide global inhibition of transcription in hibernation. Histone proteins alter chromatin structure to gate access to DNA by the transcriptional machinery and multiple post-transla-

tional modifications of histones modulate that access. For example, methylation of lysine residues leads to a closed chromatin structure whereas both acetylation and phosphorylation open up the DNA-protein structure to allow binding by Tfs and the transcriptional machinery. Studies on ground squirrel muscle showed modifications during hibernation that would suppress transcription: a 25% decrease in the content of acetylated histone H3 (Lys 23) and a 40% reduction in phosphorylated histone H3 (Ser 10) content as compared with the euthermic state [27]. Reduced histone acetylation was also linked with greater amounts of histone deacetylase (HDAC) protein in hibernator muscle and a strong (82%) increase in HDAC activity. Direct control over RNA polymerase II (Pol II) activity is also a factor; measured enzyme activity was 42% lower in muscle extracts from hibernating versus euthermic animals [27]. Furthermore, Pol II suppression may be another example of enzyme control by RPP since an antibody that detects phospho-Ser 5 in the heptapeptide repeats (YSPTSPS) in the C-terminal domain showed a 79% higher phospho-Pol II content in muscle of hibernating versus euthermic animals. However, there is still debate about the functional consequences of phosphorylation at this site.

Once transcription is complete, gene transcripts are subject to various controls that determine when/if they are translated and several of these controls are proving relevant in hibernation. Transcript maturation within the nucleus includes splicing to remove introns, changes to the 3′ end by cleavage factors and polyadenylation factors, and binding into various protein complexes for storage or export. Studies with dormice documented the appearance of nuclear bodies containing transcription and splicing factors in association with pre-mRNAs during torpor; these storage bodies disappeared again upon arousal [28]. Immunocytochemistry also showed that transcriptional, splicing and cleavage factors were redistributed inside the nucleus during torpor, being stored in domains where they are not usually found in euthermia [28]. Hence, this is good evidence that transcripts are stabilized and stored during torpor, remaining available for a rapid resumption of protein synthesis when animals arouse back to euthermia.

Outside the nucleus mRNA transcripts are subject to even more controls and all evidence points to global suppression of translational activity and storage of mRNA transcripts during torpor. Total mRNA transcript levels do not change significantly over hibernation/arousal cycles [23, 29], but their translation status does and several mechanisms contribute to this. One of the hottest new topics in metabolic regulation is the control of mRNA expression by microRNA (miRNA). miRNAs are small non-coding transcripts (19–25 nucleotides long) that bind to target mRNAs; a perfect sequence match between a miRNA species and its target typically directs the mRNA into degradation pathways, whereas an imperfect match leads to translational inhibition via storage in cytoplasmic P-bodies [30]. Regulation by miRNA control over mRNA translation is now known to be particularly important in the regulation of development, and aberrant miRNA expression patterns have been linked with multiple diseases [31]. Given that the suppression of growth, development and proliferation responses must be integral parts of hypometabolism, it would be expected that differential regulation of miRNA action would be a part of this process. Indeed, the first example of this was the demonstration that developmental arrest in the L1 phase in *Caenorhabditis elegans* is linked with suppression of the expression of miRNA lin-4 which is under the control of the DAF16/FOXO Tf [32].

New work by my laboratory has shown that changes in the levels of miRNA species also occur during ground squirrel hibernation [33]. For example, mir-24 levels were reduced by 30–50% in heart and skeletal muscle, whereas levels of mir-1 and mir-21 increased 2.0- and 1.3-fold in kidney during torpor. In other systems, mir-1 has been linked with the control of gene transcripts involved in cell proliferation and expression of HDAC-4 that is involved in gene repression whereas mir-21 is linked to anti-apoptotic properties [34, 35]. Although there is much more work to be done to determine the actual roles of these miRNA species in hibernation, there is clearly potential for miRNA to be a significant regulatory mechanism in both global and selective gene expression during hibernation.

Other controls are directed at the ribosomal level. Multiple studies have documented polysome dissociation during torpor with a corresponding increase in mRNA transcripts stored in monosome and ribonuclear protein fractions [3]. Protein synthesis is also directly inhibited by RPP action affecting at least three key sites: (a) the ribosomal initiation factor eIF2α that controls entry of the initiating methionine residue; (b) the inhibitory binding protein 4E-BP1 that controls eIF4E to regulate mRNA entry to the assembling ribosome, and (c) the elongation factor eEF2 that is the focus of controls affecting the elongation phase of protein synthesis [3, 24]. Exceptions to these controls are transcripts that are actively translated during torpor and remain in polysome fractions; transcripts of fatty acid-binding proteins are one example [3].

Interestingly, the formation of stress granules – transient cytoplasmic foci that contain HSPs, translationally stalled mRNA and mRNA-binding proteins – is a known response to various cellular stresses and phosphorylated eIF2α is a key stimulator of stress granule formation [36]. Thus, it would appear that this reversible mechanism is put to use in natural torpor to preserve valuable mRNA transcripts throughout the hypometabolic episode until they are needed again when animals arouse.

Hibernation Responsive Gene Expression

Despite the global suppression of transcription and translation, a small percentage of genes are actually upregulated when animals enter a torpor bout and these genes and their protein products are particularly important because they give vital clues to metabolic functions that are important for long-term survival in cold torpor. Methods of gene discovery that have been used include selective studies on individual genes/proteins, cDNA library construction and screening, DNA array screening (both homologous and heterologous), and proteomic approaches (e.g. 2D gel electrophoresis, peptide identification via mass spectrometry) [3, 29, 37–39]. In particular, the recent use of DNA array screening has made huge advances in our understanding of the gene expression responses that support hibernation, identifying not just individual genes or gene families but also related functional groups of genes that are coordinately controlled. From this work a number of classes of cellular processes have emerged as particularly critical to hibernation. Themes include elevation of expression of genes/proteins involved in reorganization of fuel metabolism (fatty acid transport and catabolism; inhibition of carbohydrate catabolism), antioxidant defense, protein chaperones, clotting inhibition, muscle restructuring, transmembrane transporters, system regulators (growth, cell cycle, apoptosis, atrophy) and support for nonshivering thermogenesis. Several of these themes have to do with the protection and preservation of macromolecules during long-term torpor and the remainder of this article will explore some of these.

Preservation Mechanisms

Entry into a hypometabolic state is an excellent way for organisms to maximize the length of time that they can survive under unfavorable environmental conditions. However, in a torpid state, long-term viability could be challenged if cells fall into disrepair due to energy restrictions on synthesis, repair, and degradative processes. Hence, organisms need to improve their cell preservation strategies so that macromolecules remain functional despite their much lower rate of turnover. Furthermore, mechanisms need to be in place that deal with or anticipate the stresses that are encountered during the hypometabolic period. For example, for hibernators, this can include the reality that metabolic homeostasis needs to be maintained at a very low Tb for a mammal and that in order to arouse back to the euthermic state a tremendous increase in oxygen consumption must occur, a situation that is tied to a comparable rise in the generation of reactive oxygen species (ROS). So, every form of hypometabolism must also have contingency plans that deal with the existing or anticipated cellular conditions that occur both during hypometabolism and when animals break torpor. For hibernating mammals, these contingency plans seem to be put in place in two ways – pre-hibernation adjustments versus strategies that are implemented during the torpor bout. With respect to human applications, these natural contingency plans also illustrate principles of life extension that need to be addressed.

Plasma Protease Inhibitors

Conditions of low blood flow (due to slow heart beat) and elevated blood viscosity (due to low Tb) should increase the risk of thrombosis in the microvasculature of hibernating animals. However, this does not happen and an understanding of how hibernators deal with this issue has application to organ transplant technology and hypothermic medicine. The answer is that hibernators implement multiple strategies that reduce the clotting response. Indeed, clotting time was ~50% longer in hibernating versus active ground squirrels, and Srere et al. [40] showed that this was linked to upregulation of α_2-macroglobulin production by liver; this secreted plasma protein inhibits the Xa clotting factor. Circulating levels of platelets are also reduced (and sequestered into spleen) and other clotting factors are suppressed [41]. DNA array screening also revealed the upregulation several types of serine protease inhibitors (serpins) during hibernation [42]. Serpins are a superfamily of proteins with 16 clades; most are plasma proteins that are irreversible covalent inhibitors of proteases that cleave specific proteins [43]. Hibernation-responsive serpins include Serpin C1 that inhibits thrombin and Serpins E2 and F2 that participate in the inhibition of fibrin (clot) breakdown. Serpins A1 (α_1-an-

titrypsin) and A3 (α_1-antichymotrypsin), the two most abundant plasma serpins, are also upregulated. These act to suppress the action of circulating proteases that are normally involved in inflammation responses. Immune and inflammation responses are also suppressed during hibernation and serpin action may be part of the mechanism. Indeed, it has been suggested that one reason why animals arouse periodically from torpor is to reactivate a dormant immune system to combat any pathogens that entered the body during the torpor bout [14].

Chaperones and the Unfolded Protein Response

Chaperones are proteins that assist other proteins to fold into their correct conformation. They participate in the folding of nascent proteins and in the refolding of improperly folded proteins that accumulate under stress. Several conditions could constitute stress during hibernation including low Tb, ischemia in some organs, and oxidative stress during arousal. During hypometabolism, the emphasis should be on preservation/repair of existing macromolecules to avoid the need for replacement of these cellular components via ATP-expensive synthesis/degradation. Recent evidence shows that some kinds of chaperones are involved. Heat shock proteins (HSPs) are the best-known chaperones but a comprehensive evaluation of HSP responses during hibernation has yet to be published. For example, Carey et al. [44] reported that HSP70 protein levels in ground squirrel intestine varied over the torpor bout, being highest during entrance and early hibernation and up to 50% lower during arousal and inter-bout periods. It was suggested that cellular conditions during entrance into torpor may compromise proper protein folding leading to HSP70 induction or that HSP70 induction during entrance may increase general stress tolerance with benefits for the later stages of torpor or arousal. Organ-specific responses by HSPs also appear to occur; e.g., a new study of hibernating ground squirrels by my laboratory found a 2-fold increase in HSP90 in kidney, a 50% increase in HSP70 in skeletal muscle, and 2- to 3-fold increases in HSP40 in brown adipose and heart [Yan K and Storey KB, unpublished].

Another class of chaperones is also important to hibernation success, the glucose-regulated proteins (GRPs). These residents of the endoplasmic reticulum (ER) work to fold proteins that are destined for secretion or for insertion into membranes. Stresses that overwhelm ER-folding capacity trigger a phylogenetically conserved set of actions, known as the unfolded protein response (UPR). In nonhibernating mammals, such stresses include hypothermia, oxidative stress and ischemia so it is predictable that hibernators would include the UPR as a cytoprotective mechanism during torpor. The UPR has three main parts: (1) increase protein folding capacity by synthesis of more ER-resident chaperones; (2) reduce folding demand by lowering the input of nascent proteins via translational suppression, and (3) increase clearance of slowly folding or misfolded proteins by enhancing the ER-associated protein degradation pathway [45]. Sensing of ER stress and stimulation of the UPR is accomplished by proteins that span the ER membrane including the protein kinase PERK, the protein kinase endoribonuclease ERN/IRE and several bZIP Tfs such as activating Tf 6 (ATF6). One model for how ER stress is sensed involves the main ER-resident chaperone, GRP78. In this model, GRP78 in unstressed cells binds to the domains on sensor proteins that project into the ER lumen but when folding demand is high, GRP78 is called into action and dissociates, leading to activation of the sensors in the cytoplasm. One main action of ATF6 is the upregulation of genes coding for ER chaperones, enhancing production of GRP78 and GRP94. Among other actions, PERK is critical to suppressing protein synthesis by phosphorylating eIF2α, and also phosphorylates the Nrf2 Tf which in turn upregulates various antioxidant proteins (discussed later).

In studies with ground squirrels we found that the UPR is activated in some organs when animals hibernate. A strong 3- to 4-fold increase in *grp78* message levels occurred in brain and brown adipose during hibernation and, as a consequence, GRP78 protein increased 2-fold [46]. In bats, brain GRP78 protein also rose 1.7-fold within the first 30 min of arousal, compared with the torpid state [47]. Put together, these two results suggest that a focus for the strong increase in *grp78* mRNA in torpor may be to support GRP78 accumulation not just during torpor but also a rapid synthesis during arousal. The mitochondrial GRP75, which responds to energy or oxidative stress, also increased in selected tissues of torpid ground squirrels [44]. We analyzed the control of hibernation-responsive GRP78 upregulation in ground squirrel tissues [48], the data suggesting that control may reside with the PERK cascade. PERK phosphorylates eIF2α which in turn stimulates the ATF4 Tf that, in combination with its cofactor CREB, upregulates genes including *grp78*. ATF4 levels increased by 2- to 2.5-fold in hibernator organs and the amount of active phosphorylated CREB1 (Ser133) rose by 2.6- to 7.4-fold [48]. Furthermore, both ATF4 and pCREB1 moved into the nucleus; indeed,

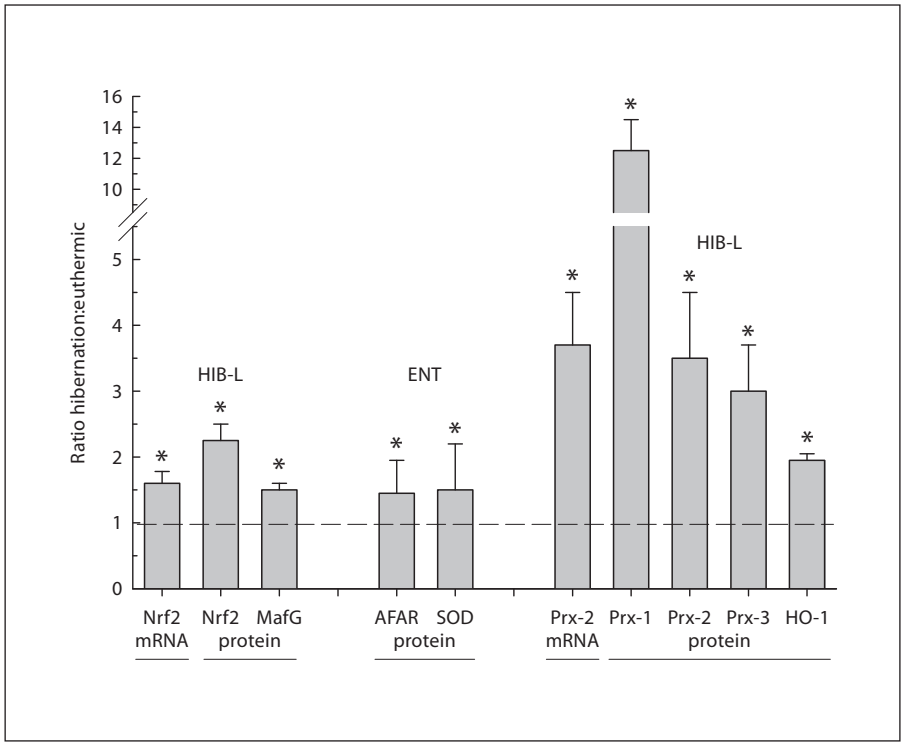

Fig. 2. Nrf2 transcription factor and the responses of downstream antioxidant genes during hibernation in hearts of hibernating ground squirrels *(Spermophilus tridecemlineatus)*. Both *nrf2* mRNA and Nrf2 protein levels increased along with the Nrf2-binding partner, MafG. Protein levels of genes under Nrf2 control were elevated during entrance into torpor (ENT) or in long-term torpor (HIB-L; 3–5 days a stable body temperature of ~5–7°C) including aflatoxin aldehyde reductase (AFAR), Cu/Zn superoxide dismutase (SOD), peroxiredoxin (Prx) isoforms 1–3, and heme oxygenase 1 (HO-1). Prx2 mRNA levels were also upregulated. Data are means ± SEM (n = 3–7). * Hibernator values are significantly higher than euthermic: $p < 0.05$. Compiled from Morin et al. [56] and Morin and Storey [57].

nuclear pCREB1 content skyrocketed by 38-fold in the brain and 25-fold in the muscle of torpid animals, providing clear evidence that the ATF4/CREB1 pathway mediates GRP expression in hibernation. Overall, then, the data indicate that not only is the UPR employed as part of the mechanism of metabolic suppression when animals enter torpor, but the enhancement of ER chaperones appears to be a necessary aid to maintaining the long-term conformation stability of cellular proteins during hypometabolism.

Antioxidant Defense

Enhancement of antioxidant defenses is proving to be a universal feature of hypometabolism across phylogeny [49] and serves two purposes: (a) protection of macromolecules from oxidative damage during the hypometabolic excursion, a time when organisms cannot afford high ATP expenditures associated with repairing, degrading or resynthesizing macromolecules that are damaged by ROS, and (b) providing defense against the rapid rise in ROS generation that occurs when oxygen-based metabolic rate increases rapidly during arousal. The increase in oxygen consumption during arousal can be dramatic, for ground squirrels as much as 36-fold higher than the previous rate in torpor and 3-fold higher than in summer-active animals [50]. Much of the total increase in oxygen consumption is in fact localized to the massive increase in oxygen consumption by triglyceride-fueled, uncoupled respiration in brown fat that drives thermogenesis. Indeed, Buzadzic et al. [51] first reported elevated antioxidant defenses in brown adipose tissue of hibernators and made the link between high rates of oxygen consumption during arousal and high rates of ROS generation. However, there is now good evidence that oxidative stress during arousal is a whole body phenomenon requiring enhancement of antioxidant defenses in both extra- and intracellular compartments. In extracellular compartments, ascorbate is particularly important; both ground squirrels and hamsters elevate plasma ascorbate while in torpor and then levels plummet during arousal [52]. Another metabolite with antioxidant properties in plasma is melatonin which appears to play an important role in natural systems of ischemia/recovery including diving, hibernation and birth [53]. Transient high levels of melatonin occur during arousal from hibernation and, in this situation, melatonin does not appear to be controlled by light and seems to be produced by multiple tissues, not just the pineal gland. Plasma defenses also in-

clude superoxide dismutase and catalase activities that rise by 3- to 4-fold during arousal to enhance ROS scavenging [54, 55].

Intracellular antioxidant defenses of hibernators also include both metabolites, such as glutathione [44], and enzymes. Indeed, DNA array screening has implicated selective enhancement of multiple antioxidant enzymes; e.g., screening of ground squirrel and bat tissues using nylon macroarrays showed >2-fold upregulation of superoxide dismutase, glutathione peroxidase, and glutathione-*S*-transferase in the kidney whereas these plus peroxiredoxin and metallothionein were upregulated in the liver [44]. Expression of these genes is under the control of redox-sensitive Tfs such as NFκB [44] and Nrf2 [56]. For example, figure 2 shows data for Nrf2 signaling in the ground squirrel heart. During hibernation, *nrf2* mRNA transcript levels increased by 1.5-fold in the heart and Nrf2 protein rose 2.3-fold; levels of the MafG-binding partner of Nrf2 also increased 1.5-fold which would allow the pair to enhance gene transcription. As a result several genes under Nrf2 control were upregulated during torpor resulting in enhanced protein levels of Cu/Zn superoxide dismutase, heme oxygenase, aflatoxin aldehyde reductase, and peroxiredoxins [56, 57]. In particular, peroxiredoxins were strongly upregulated suggesting that they may be primary targets for Nrf2 action in hibernation. Peroxiredoxins are well known to be stress-responsive in other systems and reduce and detoxify a range of hydroperoxides using thioredoxin as the electron donor [58]. The data suggest that they are also key players in hibernation providing highly inducible intracellular antioxidant defense.

Concluding Remarks

With all of the regulatory adjustments and cell preservation events that are part of natural torpor, it can seem like a daunting task to develop methodologies to orchestrate inducible cold torpor for use with human organ explants or to extend human survival time under difficult circumstances. However, the more that we learn about the biochemistry that goes on inside hibernator cells, the more it becomes obvious that the molecular mechanisms that coordinate torpor and enhance cytoprotection during torpor represent common principles and mechanisms that occur across phylogeny [1, 3]. Indeed, studies of the dauer diapause state of *C. elegans* larvae provide some of the most novel advances in our understanding of hypometabolism and these mechanisms are also turning up in other systems [59]. For example, a central role for FOXO Tfs in regulating gene expression events that support dauer formation and enhance longevity is emerging and ongoing studies in my laboratory on several animal models, including mammalian hibernation, are showing that FOXO-mediated events are also integral to hypometabolism across phylogeny. Furthermore, the mechanisms discussed in this article are all actually known as responses to various stresses (e.g. ischemia, nutrient limitation, etc.) in humans but are not sufficiently intense to achieve the extreme degree of metabolic suppression needed for torpor and are not implemented fast enough to prevent metabolic damage during a stress-induced energy crisis. So, the potential to improve the longevity of human organ explants by mimicking the mechanisms used by hibernators appears to exist but we need to find ways to better induce cytoprotective actions (e.g. upregulate antioxidant defenses and/or chaperone levels) before organ retrieval, and/or trigger organ-wide post-translational events that strongly suppress ATP turnover. Ischemic preconditioning strategies have already been tested quite extensively with some transplantable organs (notably the liver) with variable effects on post-transplantation organ function [60], and both heat and cold preconditioning may also be effective with some organs [61]. Metabolic inhibitors and hormone mimics that send starvation, growth suppression or cell cycle inhibition signals to cells could prove important. Continuing studies on hibernators will have much to teach us.

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