Advances in molecular biology of hibernation in mammals

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Summary

Mammalian hibernation is characterized by profound reductions in metabolism, oxygen consumption and heart rate. As a result, the animal enters a state of suspended animation where core body temperatures can plummet as low as −2.9°C. Not only can hibernating mammals survive these physiological extremes, but they also return to a normothermic state of activity without reperfusion injury or other ill effects. This review examines recent findings on the genes, proteins and small molecules that control the induction and maintenance of hibernation in mammals. The molecular events involved with remodeling metabolism, inducing hypothermia and maintaining organ function are discussed and considered with respect to analogous processes in non-hibernating mammals such as mice and humans. The advent of sequenced genomes from three distantly related hibernators, a bat, hedgehog and ground squirrel, provides additional opportunities for molecular biologists to explore the mechanistic aspects of this biological adaptation in greater detail. ***BioEssays*** **29:431–440, 2007.**

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Funding agency: NIH grant HL-081100.
DOI 10.1002/bies.20560
Published online in Wiley InterScience (www.interscience.wiley.com).

Abbreviations: RQ, respiratory quotient; PDK4, pyruvate dehydrogenase kinase 4; ATP, adenosine triphosphate; IBA, interbout arousal; PPAR, peroxisome proliferator-activated receptor; PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator-1-alpha; Akt, protein kinase B; FOXO, forkhead box, sub-group O transcription factor; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; ERV, endogenous retrovirus; PTL, pancreatic triacylglycerol lipase; HSL, hormone sensitive lipase; GI, gastrointestinal; T_4, thyroxine; T_3, 3,5,3′-triiodothyronine; T_1AM, 3-iodothyronamine; TAAR, trace amine-associated receptor; TBG, thyroxine-binding globulin; HPc, hibernation-specific protein complex; CSF, cerebral spinal fluid; ARC, arcuate nucleus; *Npy*, neuropeptide Y gene; DD, constant darkness; LD, 12 h light:12 h dark; MAP2, microtubule-associated protein 2; PSD95, postsynaptic density protein 95; EST, expressed sequence tag; LTCC, l-type calcium channel; SR, sarcoplasmic reticulum; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; PLB, phospholamban; CaMKII, calcium-calmodulin protein kinase II; CaM, calmodulin; PKA, protein kinase A; WGS, whole genome shotgun sequence; ENCODE, ENCyclopedia of DNA elements; BAC, bacterial artificial chromosome; RyR, ryanodine receptor; NCX, Na^+/Ca^{2+} exchanger; NMR, nuclear magnetic resonance

Introduction

As with most biological processes that take place on our planet, the process of hibernation is directed by the expression of genes. Unlike embryonic development where genes direct the formation of a multicellular, multifunctional organism from a single fertilized egg, the genes controlling hibernation perform their duty within the existing framework of a fully differentiated animal. The hibernation phenotype can therefore be viewed as series of physiological adaptations in various tissues and organs that give an animal the ability to survive climatic extremes. The sum of these adaptations results in a radical departure from the normal physiological homeostasis seen in most mammals.

The phylogenetic diversity of hibernating mammals is widespread, with the majority of molecular studies concentrated on various ground squirrel, hamster and marmot species.[1] Evolutionary arguments have suggested fundamental similarities in the molecular genetic basis of hibernation among all mammals,[2] including a species of hibernating primate.[3,4] Changes in metabolism, fuel selection, oxygen consumption, heart rate and body temperature are thought to be the result of environmental cues interacting with the animal's genetic hardware. Seasonal parameters of reduced daylight, cooler temperatures and a paucity of food are key environmental factors known to trigger the hibernation response. Duplication of these factors in the research setting is used to induce hibernation in captivity, with the greatest success of induction (depending on the species) occurring after the autumnal equinox in animals that have accumulated sufficient body fat.

A thorough review of the physiological characteristics of hibernating mammals can be found in Lyman et al.[5] For a small mammal like a ground squirrel, these characteristics typically include body temperatures ranging from 2 to 10°C, oxygen consumption that holds at 2–3% of the aroused condition and heart rate as low as 3–10 beats/minute, compared to 200–300 beats/minute when the animal is awake and active. Even more extreme versions of this phenotype have been seen in the Arctic ground squirrel (*Spermophilus parryii*) where core body temperatures have been measured at −2.9°C.[6] Regardless of the species, a

hallmark of these physiological extremes is metabolic rate reduction followed by whole-body hypothermia. This review will discuss our current knowledge of how the action of genes, proteins and small molecules transform a warm-blooded mammal into a seemingly lifeless entity that rebounds from this cold and motionless state of torpor on a predictable schedule.

Remodeling metabolism

Switch in fuel utilization

Formal training in the measurement of body composition and fuel selection is not required to observe that long-term (5–6 month) hibernators are big and fat immediately before they hibernate and much thinner when they emerge the following spring. These animals rely on stored lipid as their primary source of fuel as seen by the absence of feeding, a reduction in white adipose tissue mass and a respiratory quotient (RQ) of 0.7. RQ is a unit-less number representing moles of CO_2 released per moles O_2 consumed. A RQ of 0.7 indicates that fat is the major substrate for energy production; when RQ equals 1 the fuel source is carbohydrate. The switch from a carbohydrate-based metabolism to one that relies on stored fat has been the subject of intense study over the last several years[7–10] and has been covered in recent reviews.[1,11] Mechanistic control of this switch has not been fully elucidated; however, it appears to resemble aspects of metabolic regulation seen with caloric restriction,[12] starvation[13] and diabetes.[14,15] A common feature among all these conditions is the induction of the mitochondrial enzyme pyruvate dehydrogenase kinase 4 (PDK4). PDK4 inactivates pyruvate dehydrogenase and thereby blocks the conversion of pyruvate to acetyl-CoA. Inhibition of this single-step halts the flow of glycolytic intermediates into the TCA cycle giving rise to metabolic rate reduction, the conservation of carbohydrates, and assurance that lipids are the primary fuel for the production of ATP.

Metabolic oscillations

Circadian cycling of PDK4 expression in rats[16] indicates that light/dark exposure may also play a role in the activation of the PDK4 gene, where the highest levels of induction have been observed in the dark.[17] Diurnal oscillations of light and dark are mirrored on a much grander scale in hibernating mammals as oscillations of long-term torpor bouts and short-term arousals. These oscillations are seen in the form of regular interbout arousals (IBAs) where re-warming to 37°C and resumption of metabolic activity predictably interrupts several days of near freezing torpor (Fig. 1). We have recently developed a mathematical model that can be used to identify and test candidate molecules that are responsible for the oscillatory nature of the torpor–IBA cycle.[18]

It is unclear why hibernators undergo these energetically expensive arousals throughout the hibernation season; however, IBAs do offer an opportunity for the replenishment and repair of biomolecules (reviewed in Ref. 1). This interesting possibility is supported by the observation that liver proteins isolated late in a torpor bout show reduced saliency based on the blurred appearance of protein spots on two-dimensional

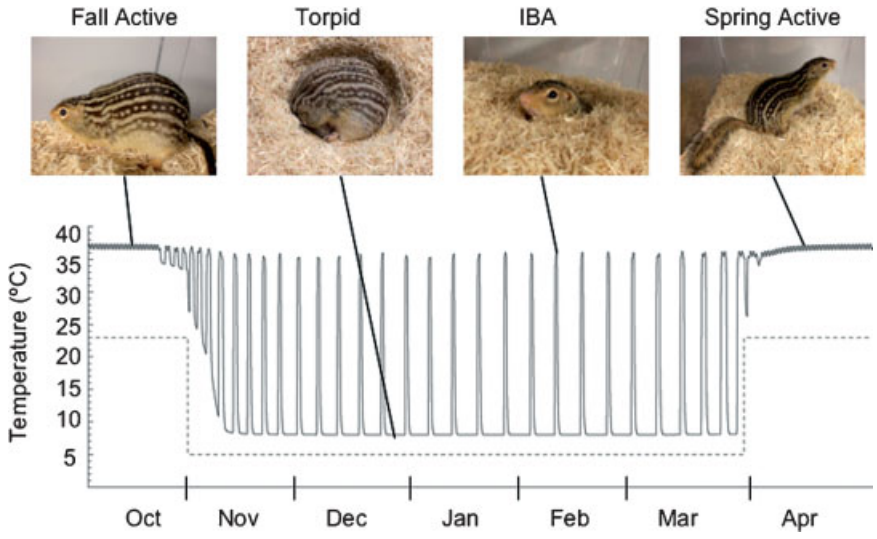


Figure 1. Diagram showing the hibernation season with respect to animal appearance and body temperature. The graph shows a simulation of body temperature tracings (solid line) of a thirteen-lined ground squirrel inside an environmental chamber beginning in October and ending in April. The dashed line represents the ambient temperature, which is lowered to 5°C on November 1 and raised back to 23°C at the end of March. Periodic interbout arousals (IBAs) are seen as regular spikes in body temperature despite constant ambient temperature. Photographs of animals at four different points during the hibernation season are indicated and shown above the graph.

gels,[19] but proteins from animals entering torpor immediately after an IBA appear intact.[20] Mechanisms controlling the regular periodicity of the torpor–IBA cycle remain a mystery but have been recently compared to biological oscillations that appear to be driven by cyclic changes in metabolism.[21]

Control of gene expression

We have proposed that an increase in circulating lipids and a decrease in serum insulin is responsible for the induction and maintenance of PDK4 expression during hibernation.[9,11] A large body of evidence has shown that synthetic agonists of peroxisome proliferator-activated receptor-alpha (PPARα) increases the levels of PDK4 mRNA in heart and skeletal muscle,[17,22,23] suggesting that specific fatty acids are a factor in the activation of the PDK4 gene. Induction of PDK4 by circulating lipids has also been shown in humans fed a high-fat diet[24] or intravenously infused with high concentrations of free fatty acids.[25] PDK4 expression has also been shown to be upregulated by peroxisome proliferator-activated receptor-gamma coactivator-1-alpha (PGC-1α)[26,27] and downregulated by insulin signaling.[14]

Mechanistic similarities of hibernation with starvation[28] and energy deprivation[29] in non-hibernating mammals, and with formation of the metabolically inert dauer larvae of the nematode *Caenorhabditis elegans* (reviewed in Ref. 1), has prompted a search for transcription factors and other regulatory proteins that may control analogous processes in hibernators. Specifically, mutants in one of the *C. elegans* dauer larvae pathways have revealed insulin-like signaling that reduces metabolism and extends lifespan (reviewed in Ref. 30). Mammalian orthologs for two of these genes, Akt/Protein kinase B[31] and FOXO1a,[32] have recently been studied in hibernating thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*). Subsequent characterization of metabolic regulators AMP-activated protein kinase (AMPK),[33] peroxisome proliferator-activated receptor-gamma (PPARγ) and PGC-1α[34] has also been reported in this same ground squirrel species. The combination of this information with the identification of *cis*-acting regulatory sequences from the recently completed thirteen-lined ground squirrel genome project (discussed later in this Review) offers a starting point for elucidating the mechanisms of differential gene expression during hibernation.

Initial analysis of the thirteen-lined ground squirrel genome has revealed an endogenous retroviral (ERV) insertion upstream of the coding region of the pancreatic triacylglycerol lipase (PTL) gene.[35] The significance of this insertion is that it may play a role in the hibernation-specific activation of the PTL gene in non-pancreatic tissues.[35] Novel expression of PTL in heart[8] and white adipose tissue[36] provides continuous low-temperature lipolysis unaffected by hormonal fluctuations that normally influence the activity of the standard mammalian lipase found in these tissues, hormone-sensitive lipase (HSL).[36] PTL has 75-fold greater activity than HSL and offers an example of a common gastrointestinal (GI) enzyme that is seasonally activated in novel locations to provide a steady stream of fatty acids at low body temperatures.[36] Enzymatic assays of recombinant human and ground squirrel PTL show nearly identical catalytic activity at temperatures as low as 0°C.[37] This low-temperature lipolytic activity therefore appears to be a common property of PTL regardless of whether the species of origin is a hibernator or non-hibernator.

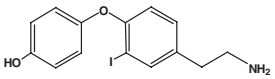
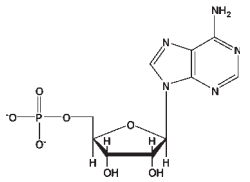
Induction of hypothermia

Thyroid hormone derivative

Hypothermia occurs as body temperature drifts downward following metabolic rate reduction.[38,39] A potential link between the reduction in metabolism and the onset of hypothermia in hibernators may lie with changes in the concentration and structure of thyroxine (T_4), the predominant form of thyroid hormone. A recently discovered derivative of thyroxine, 3-iodothyronamine (T_1AM), rapidly lowers body temperature and slows heart rate when injected into mice.[40] Since thyroid hormone is the primary determinant of the body's overall metabolic rate, the conversion of T_4 to T_1AM offers a means of coupling slower metabolism with the induction of hypothermia. T_1AM (see Table 1 for structure) is a naturally occurring substance that has been found in the brains of rats and guinea pigs, and in extracts of brain, liver and blood from adult male mice.[40] Injection of mice with T_1AM at 50 mg per kg body weight depressed body temperature from 37 to 31°C within 30 minutes; body temperatures remained depressed two hours after injection where they were measured at 29.5°C. The behavior of these animals resembles natural hibernation including inactivity, a slightly hunched back posture, drooping eyelids and skin that is cool to the touch.[40] All mice resumed normal core body temperature and behavior 6–8 hours after injection. Since these initial studies Scanlan and colleagues have produced several synthetic thyronamines that show the same or even more potent induction of hypothermia.[41]

The effect of T_1AM on mice bears a striking resemblance to the initiation of hibernation in ground squirrels and other hibernating mammals. T_1AM is a potent agonist of the orphan G-protein-coupled trace amine-associated receptor (TAAR).[40,41] Although it is not clear whether the bradycardia and temperature-lowering effects of T_1AM are mediated through an interaction with TAAR, it is clear that the effects of T_1AM are the opposite of the more-abundant thyroid hormones T_3 and T_4. T_3/T_4 treatment can lead to hyperthyroid conditions of elevated metabolism and tachycardia. In rat brain, T_3/T_4 concentrations are roughly 1–6 pmol/g,[42] whereas T_1AM concentrations are estimated to be in the subpicomole per gram range.[40] To initiate hibernation, it may be necessary to swing this ratio in the direction of T_1AM by

Table 1. Naturally occurring compounds that induce hypothermia in mice

Compound	Structure	M.W. (Daltons)	Effective conc.	Reference
3-iodothyronamine (T_1AM)		355.17	50 μg per gram injected i.p.	40,41,44
5′-adenosine monophosphate (5′-AMP)		345.21	10 μmol per gram injected i.p.	57
ghrelin	H_2N-G-S-S-F-L-S-P-E-H-Q-K-A-Q-Q-R-K-E-S-K-K-P-P-A-K-L-Q-P-R-COOH	3188.64	100 μg injected i.p.	58
hydrogen sulfide gas (H_2S)		34.08	inhalation of 80 ppm	59

Abbreviations: conc., concentration; i.p., intraperitoneal; M.W., molecular weight; ppm, parts per million.

lowering the concentration of free T_3 and T_4. Epperson and Martin[43] observed a 15-fold increase in mRNA levels of thyroxine-binding globulin (TBG) in the livers of hibernating versus summer active golden-mantled ground squirrels. TBG binds circulating T_3 and T_4 and could play a role in initiating hypothermia by reducing the ratio of T_3/T_4 to T_1AM. Upon arousal, cessation of T_1AM-mediated hypothermia could be achieved by hepatic sulfotransferases that catalyze the sulfation and inactivation of T_1AM in the liver.[44]

Hibernation-specific protein complex

Synthesis of T_1AM is theoretically achieved in vivo by enzymatic deiodination and decarboxylation of circulating T_4, presumably at the target tissue. Identification of T_1AM in the brains of rats, mice and guinea pigs[40] raises the issue of how putative factors important for inducing and maintaining the hibernating state can be enzymatically modified and cross the blood–brain barrier with seasonal specificity. This problem of transporting effector molecules both in and out of the brain appears even more daunting at body temperatures approaching 0°C. A glimpse of how this process takes place has been provided by the work of Kondo and colleagues studying the hibernation-specific protein complex (HPc) in blood of the Siberian chipmunk, *Tamias sibiricus*. The circulating HPc comprises a complex of three distinct proteins (HP20, HP25 and HP27) referred to as the HP20c, and one larger protein (HP55) resembling a member of the serpin family of proteins, α 1-antitrypsin. Earlier studies showed the proteins of the HPc are synthesized in the liver[45–47] and that the intact HPc is found in the serum of active chipmunks, but that its level in the blood is greatly reduced during hibernation.[48,49] Recently it was shown that this near disappearance from the blood during hibernation is concomitant with its reappearance in the cerebral spinal fluid (CSF) of the central nervous system;[50] raising questions of how these liver proteins enter the brain, and what are they doing there?

In an amazing study carried out over the course of nine hibernation seasons, Kondo et al.[50] not only showed the circannual depression of HPc in the blood, but animals that were unable to hibernate did not show the same seasonal reductions in plasma levels. During hibernation, HPc is found both in the choroid plexus of the brain and in the CSF where HP55 is dissociated from the HP20c complex. In an attempt to determine the function of HPc in the central nervous system, anti-HP20c antibodies were infused into the brain ventricles of living animals in order to deplete HP20c from the CSF. These experiments showed that the concentration of HP20c in the CSF is directly proportional to the length of a hibernation bout, i.e. reduced HP20c shortened the time in hibernation. Of course, this intriguing result is the opposite of the effect of intact HPc in the blood, and implies that the permeability of the blood–brain barrier could be a key player in regulating the induction and maintenance of hibernation. It has been suggested by Hastings and Ebling[51] that transport of HPc across the blood–brain barrier may be facilitated by seasonal changes in the choroid plexus epithelium and tanycytes of the hypothalamus. The hypothalamus has been shown to be the site of seasonal transport and enzymatic deiodination of T_4 in other organisms[52,53] and could be the site of T_1AM formation and transport in hibernators.

Torpor in mice

Additional clues on the molecules involved in the induction of hypothermia have recently come from the study of torpor in mice. Torpor in mice resembles hibernation in that it is a natural phenomenon in response to environmental factors such as insufficient nutrition. This phenomenon can be induced in the laboratory by a number of different conditions including starvation and leptin deficiency.[54–56] More recently an eclectic handful of small to medium-sized molecules have been shown to induce a hibernation-like phenotype in this non-hibernator. Hyperthermia in mice has been achieved by the administration of 5′-AMP,[57] the stomach hormone ghrelin[58] and H_2S gas.[59] The lack of commonality among these very different molecules (see Table 1 for structures) makes it difficult to uncover a unifying mechanism that would lead to this state of suspended animation. However, determining the mode of action of these molecules may suggest experimental strategies for determining the induction and maintenance of torpor in natural hibernators.

Ghrelin is a 28 amino acid hormone that stimulates appetite (reviewed in Ref. 60). It is produced in both the GI tract and in the arcuate nucleus (ARC) of the hypothalamus,[61] a brain region known for regulating appetite and metabolism. Gluck et al.[58] showed that the addition of ghrelin significantly lowered body temperature beyond torpor from starvation alone, but ARC-ablated mice that could not show starvation-induced torpor were unaffected by ghrelin. A closer examination of two signaling pathways within the ARC showed that ghrelin deepened torpor in fasting mice deficient in the α-melanocyte stimulating hormone pathway by lowering body temperature ($29.1 \pm 0.6^{\circ}C$ with starvation alone versus $22.8 \pm 1.3^{\circ}C$ with ghrelin), but had no effect in neuropeptide Y double knockout (*Npy*$^{-/-}$) mice under the same fasting conditions ($29.9 \pm 1.2^{\circ}C$ versus $29.5 \pm 0.8^{\circ}C$).[58] These results suggest that ghrelin shows its temperature-lowering effects via neuropeptide Y signaling in the ARC of the hypothalamus. A similar hypothalamic mechanism may also be a component of initiating hypothermia in mammalian hibernators.

In a study aimed at determining the role of constant darkness (DD) as a circadian metabolic signal, Zhang et al.[57] found that circulating levels of 5′-AMP were elevated in DD mice and that fasting-induced torpor increased this level another three-fold. Intriguingly, they found that injection of 5′-AMP into wild-type mice under a 12 h light:12 h dark (LD) leads to torpor in a dose-specific manner.[57] An injection of 10 μmol 5′-AMP per gram lowered body temperature from 37°C to approximately 27–25°C for over 3 hours and activated the expression of a lipolytic gene in the liver. Interestingly, two genes encoding lipolytic activity in DD mice are closely related to the PTL gene product expressed in various tissues during hibernation in thirteen-lined ground squirrels.[35,37] Genes for both the PTL cofactor colipase and pancreatic lipase-related protein 2 (normally expressed in GI tissues) are activated in the liver of DD animals. Moreover, liver extracts showed enhanced lipolysis, and a survey of non-GI tissues from mice injected with 5′-AMP showed colipase expression in seven of the eight organs tested, with brain as the only exception.[57] Expression of colipase and pancreatic lipase-related protein 2 in torpid mice is remarkably similar to the multi-tissue expression of cold-adapted PTL in the natural hibernator.[8,36,37]

Maintaining organ function

Neuronal plasticity

Hibernating mammals show tremendous plasticity in their rapid recovery from multi-day bouts of near freezing body temperatures. This plasticity can be seen at the cellular level in the brain as neurons of hibernators shrink during hypothermia, but rapidly grow back to their original size in 2–3 hours during arousals.[62–64] This neuronal contraction and expansion repeats itself throughout the hibernation season in various regions of the brain in a temperature-dependent manner.[62] Figure 2 shows a representation of these remarkable changes in neuronal morphology that includes a 55–60% reduction of synapses and dispersal of synaptic proteins.[63] Monitoring the location of four proteins (MAP2, Piccolo, PSD95 and synaptophysin) in golden-mantled ground squirrels (*Spermophilus lateralis*), Heller and colleagues showed the synaptic clustering of these proteins decreases at lower body temperatures without protein loss, and recovers within 2 hours during the rewarming that accompanies arousal.[63] This rapid reversibility was also seen with the dispersed small clear synaptic vesicle population in mossy fiber terminals of CA3 pyramidal neurons in hibernating European hamsters (*Cricetus cricetus*).[64] It was proposed that this neuronal plasticity could maintain brain function and provide

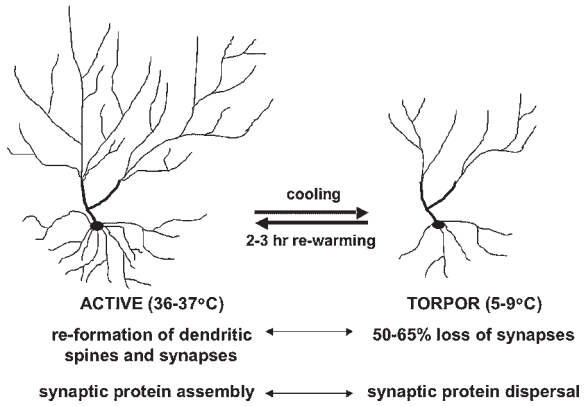


Figure 2. Two drawings of the same brain neuron representing neuronal plasticity during hibernation based on recent findings of von der Ohe et al.[62,63] in golden-mantled ground squirrels and Magarinos et al.[64] in European hamsters.

neuroprotection by reducing glutamatergic excitotoxicity and avoiding reperfusion injury upon arousal.

Discovery driven projects

Conditions of hypothermia, reduced blood flow, little or no food consumption and the potential of reperfusion injury would be expected to place severe constraints on even basal organ function in non-hibernators. To combat these physiological extremes, it has been postulated that hibernators use adaptive mechanisms that are controlled by the differential expression of genes common to most mammals.[2] Operating under that premise, several discovery-driven projects have been undertaken with the goal of identifying differentially expressed gene products in various organs during hibernation. These projects include transcriptome analysis in the heart of thirteen-lined ground squirrels,[65] in the heart, liver and brain of the golden-mantled ground squirrel,[66] and in brown adipose tissue of Arctic ground squirrels.[67] Studies of the proteome during hibernation include an analysis of the liver in golden-mantled ground squirrels[20] and heart and skeletal muscle in thirteen-lined ground squirrels.[68] The latter study also involved an evaluation of software used to identify proteins by mass spectrometry of short peptides from non-model organisms such as natural hibernators.[68]

Low temperature cardiac function

The heart is an example of a contractile organ that must continue to work despite conditions of depressed O_2 consumption and single-digit body temperatures. At body temperatures below 20°C, the vast majority of mammals experience cardiac arrest due to severe arrhythmias and ventricular fibrillation; however, hibernating mammals remain in sinus rhythm at body temperatures approaching 0°C.[69] Several mechanisms have been proposed to account for continued heart function at low body temperatures including an increase in gap junctions to provide low-resistance intracellular channels to facilitate synchronous contraction[70] and enhancements in Ca^{2+} handling within the cardiomyocytes.[71] Upregulation of gap junction protein connexin 43 has been reported in the hearts of hibernating golden hamsters (*Mesocricetus auratus*)[70] and connexins 43 and 45 are upregulated during hibernation in Siberian ground squirrels (*Citellus undulatas*).[72] Differential expression of genes resulting in improved Ca^{2+} handling under hypothermic conditions has been reported in hibernating woodchucks (*Marmota monax*)[73] and as part of a larger expressed sequence tag (EST) screen of hearts from active and hibernating thirteen-lined ground squirrels.[65]

Ca^{2+} handling in the cold

Calcium entry into cardiomyocytes is regulated in part by L-type calcium channels (LTCCs). This influx of calcium provides the trigger to release sarcoplasmic reticulum (SR) calcium stores into the cytoplasm of the cardiomyocyte via opening of the ryanodine receptor, thus inducing heart contraction.[74] Based on the hibernation EST screen,[65] sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a; a muscle-specific splice variant of the SERCA2 gene) was found to be upregulated during hibernation and the SERCA2a inhibitor phospholamban (PLB)[75] was found to be downregulated. Increased protein expression of SERCA2a and decreased expression of PLB was also seen in hibernating versus active woodchucks.[73] These observations provide an explanation for the increased rate of calcium reuptake and larger calcium stores originally observed in the SR of hibernating Richardson's ground squirrels (*Spermophilus richardsonii*).[76] Enhanced cytoplasmic clearance of Ca^{2+} in hibernating ground squirrels versus non-hibernating rats also explains, in part, why the hibernator heart continues to function at low temperatures where the heart of a non-hibernator fails.[77] A higher density of SERCA2a in the SR membrane not only increases Ca^{2+}-uptake, but the concomitant hydrolysis of ATP also provides the potential for regional endothermy[78] within cardiomyocytes despite core body temperatures of 5–6°C during hibernation. Similarly, SERCA1 in billfishes such as the blue marlin is enriched in the SR of the "heater organ", which warms the brain and eyes up to 14°C above ambient water temperature.[79]

Figure 3 shows a model where the torpid animal could potentially decrease its likelihood for arrhythmia by decreasing LTCC calcium influx, tightly regulating increased SR calcium stores, and decreasing its dependence on the excitation–contraction coupling regulator calcium-calmodulin protein kinase II (CaMKII) during periods of low energy expenditure. Messenger RNAs encoding CAMKII and its activator calmodulin (CaM) were found at high levels prior to hibernation based on the EST screen.[65] Increased positive thermal modulation of protein kinase A (PKA) at low temperatures in Richardson's ground squirrels compared to rabbit PKA[80] may afford the hibernator needed β-adrenergic stimulation to increase both cardiac output and heart rate during arousal from torpor. PKA has been shown to inactivate PLB,[81] and increase the open channel probabilities of both LTCCs[82,83] and ryanodine receptors (reviewed in Ref. 84), all via phosphorylation. Post-translational modifications throughout the animal, such as the phosphorylations catalyzed by PKA and PDK4, provide a rapid physiological response at low body temperatures compared to de novo protein synthesis, which requires a minimum body temperature of 18°C during hibernation.[85]

Sequenced genomes, suspended animation and beyond

Hibernator genome projects

Genome sequencing has begun on three distantly related hibernating mammals, the little brown bat (*Myotis lucifugus*),

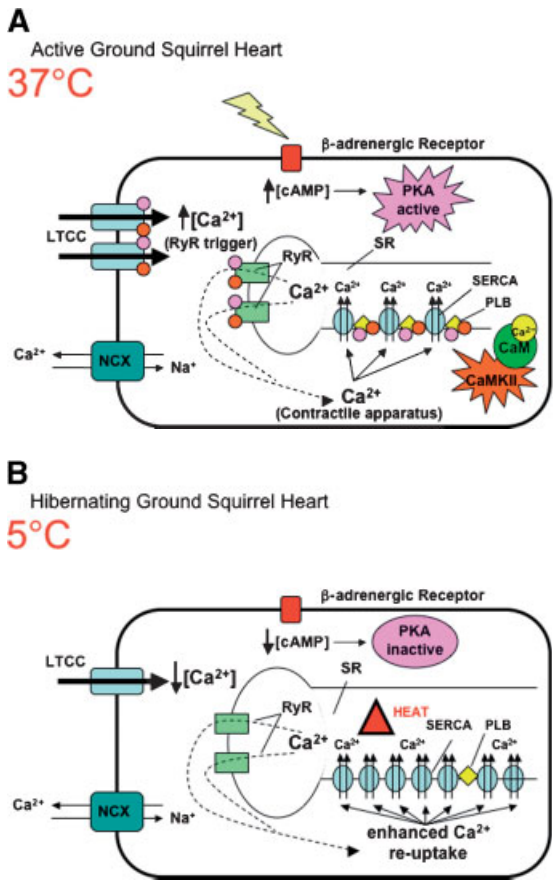


Figure 3. Model showing Ca^{2+} handling in cardiomyocytes of (***A***) summer active ($T_b = 37°C$) and (***B***) hibernating ($T_b = 5°C$) ground squirrels. Calcium-calmodulin protein kinase II (CaMKII) is activated by calcium-dependent calmodulin (CaM). **A:** In summer active animals CaMKII phosphorlyates (orange circles) L-type calcium channels (LTCC), increasing probability of open channels and the amount of calcium entering the myocyte during depolarization. This entering calcium opens the ryanodine receptors (RyR), which can also be phosphorylated by CaMKII, releasing sarcoplasmic reticulum (SR) calcium stores into the cytosol to bind to the contractile apparatus. Phospholamban (PLB) is also phosphorylated by CaMKII, which releases inhibition of sarco(endo)plasmic reticulum calcium-ATPase 2a (SERCA). This release of SERCA inhibition allows increased re-uptake of SR calcium after myocyte contraction. β-adrenergic stimulation (lightning bolt) of the heart during the fight-or-flight response, and/or arousal from torpor, activates protein kinase A (PKA) which phosphorylates PLB, LTCC and RyR (pink circles). **B:** During hibernation decreased or non-existent β-adrenergic stimulation of the heart results in inactive PKA. Increased SERCA and decreased PLB expression provide enhanced re-uptake of calcium into the SR after myocyte contraction. An increase in SERCA activity results in regional endothermy (heat) at the SR. Decreased amounts of calcium entering the myocyte via the LTCC may also decrease the likelihood of delayed afterdepolarizations and arrhythmias in hibernators because the Na^+/Ca^{2+} exchanger (NCX) would have less cytosolic calcium to clear during relaxation.

European hedgehog (*Erinaceus europaeus*), and thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*). Gene expression in the hedgehog has not been studied in detail, and an examination of gene expression in the hibernating bat has just begun,[86–88] but the large and growing number of thirteen-lined ground squirrel ESTs can serve as genomic markers that will rapidly increase annotation of the *S. tridecemlineatus* genome. The decision to choose *S. tridecemlineatus* for a genome project over other ground squirrel species was based largely on its commercial and geographic availability along with its capacity for captive breeding.[89]

The bat, hedgehog and ground squirrel genomes have been shotgun sequenced at 2× coverage as part of the National Human Genome Research Institute (NHGRI) funded Mammalian Genome Project at the Broad Institute (Cambridge, MA). In this approach, the genome was randomly broken into relatively small pieces that can be easily sequenced and reassembled as contigs. Because many of these pieces overlap, the total number of bases sequenced must exceed the length of the genome. 2× coverage means that the total number of bases sequenced is approximately twice the number in the genome. This roughly approximates to 86% of the genome being sequenced,[90] although this percentage is highly dependent on the organism and details of the sequencing methodology. In order to obtain higher quality sequence data, it was recently announced by the Broad Institute that sequencing of the little brown bat genome will be increased to 6–7× coverage. By comparison, the human genome project draft announced in 2000 had about 5× coverage, and the first "finished" version in 2003 had about 8–9× coverage (www.ornl.gov/sci/techresources/Human_Genome/faq/seqfacts.shtml).

Initial sequencing of the *S. tridecemlineatus* genome has been completed and, as of January 2007, the NCBI Nucleotide database contained a total of approximately 2 billion nucleotides that are available in the form of 797,481 contigs. This whole genome shotgun sequence (WGS) is accessible through the NCBI nucleotide database as project accession AAQQ00000000 and consists of sequences AAQQ01000001-AAQQ01797481. A cursory examination of the currently available sequence using BLAST shows extensive coverage with 93% of the 3362 thirteen-lined ground squirrel ESTs in GenBank having one or more hits to the genomic sequence with an e-value of 10^{-5} or better.

Both the thirteen-lined ground squirrel and the little brown bat are also participants in the ENCyclopedia Of DNA Elements (ENCODE) project led by the NHGRI. ENCODE is a bacterial artificial chromosome (BAC) based sequencing project that will compare about one percent of the human genome to matching regions from a wide variety of other vertebrate species, including *S. tridecemlineatus* and *M. lucifugus*. A target-by-target, species-by-species sequencing summary of the ENCODE project can be found

at www.nisc.nih.gov/projects/encode/index.cgi?all_grid=1. It is likely that a comparison of the human genome with the genome of hibernating mammals will reveal a high level of conservation within the protein-coding regions, as the vast majority of the identifiable ground squirrel ESTs in GenBank are orthologs of human genes.[91] However, the key to hibernation-specific gene expression may well reside in the non-coding regulatory regions that surround those differentially expressed genes that give rise to the hibernation phenotype.

Biomedical applications

If humans and natural hibernators indeed possess the same sets of genes, then there is tremendous potential for applying hibernation strategies to improve the human condition. Identification of the effector molecules shown in Table 1 suggests a variety of biomedical applications and has recently fueled speculation of inducing suspended animation and/or hypothermia in humans.[92,93] Placing a human in a state of suspended animation has long been proposed as a means of conserving resources during long-term space travel, however more immediate applications could be in the arena of emergency medicine such as improved outcomes from traumatic injury and hemorrhagic shock.

Other potential benefits derived from the molecular biology of hibernation in mammals are extensive. The almost exclusive combustion of fat during hibernation points to possible therapies for obesity. Resistance to ischemia and reperfusion injury suggests strategies for neuroprotection and reducing damage from stroke. Understanding the apparent lack of bone and muscle disuse atrophy could be beneficial to patients that are immobilized and/or confined to their beds for extended periods. Improved preservation of organs used for transplantation could lengthen viable storage times and lead to the establishment of organ banks. Understanding how the heart works under the physiological extremes of hibernation can lead to prevention and treatment of cardiovascular diseases that are a primary cause of death in humans. For example, myocardial infarction produces necrosis when the need for oxygenated blood in heart tissue exceeds the oxygen being supplied by the blood resulting in a number of other heart problems, including arrhythmias, decreased contractility and ultimately, heart failure. Analysis of the genes, proteins and small molecules responsible for heart function and cardioprotection during hibernation is therefore of important biomedical interest.

Finally, it has been known for some time that hibernation prolongs lifespan.[94] The more we learn about the molecular biology of hibernation, the more we are seeing striking similarities to the anti-aging mechanisms associated with caloric restriction in mammals and the long-lived dauer larvae mutants in *Caenorhabditis elegans* (reviewed in Ref. 1). The unifying theme that hibernation shares with these other paradigms is metabolic rate reduction. Changes in the concentrations of often ignored metabolites such as pyruvate, β-hydroxybutyrate, acetyl-CoA and many others, not only play a prominent role in hibernation, but are likely to be important in increasing longevity and quality of life. Toward this end, an in vivo NMR study recently measured metabolite levels in the brain of active and hibernating thirteen-lined ground squirrels.[95] Beyond gene expression, beyond the translation and modification of proteins, it is the small molecules whizzing through our cells that make us tick. A better understanding of metabolic flux and the dynamics of metabolites in hibernators may help us all keep on ticking a little bit longer.

Conclusion

In conclusion, the field of hibernation molecular biology is only in its infancy. At this point, there are still many unanswered questions of how hibernating mammals shut-down their physiology on a seasonal basis, but still maintain a flicker of cellular and molecular activity that allows them to resume an active lifestyle when climatic conditions improve. The convergence of modern physiology with molecular biology, newly sequenced genomes, and model genetic organisms, such as mice and *Caenorhabditis elegans*, has presented the field of hibernation with a newfound opportunity to make advances in determining mechanism. Because hibernation is a whole body phenomenon, the field is wide open for new investigators and approaches to study various organs and systemic aspects of a biological adaptation that has persisted throughout mammalian evolution. Exploitation of mammalian hibernation for biomedical purposes is now being considered on many fronts, and a thorough understanding of the molecular biology will likely accelerate the application of hibernation strategies in the clinic.

Acknowledgments

The author would like to thank members of his laboratory for critical review of this paper. Thanks to Katharine Brauch for contributions to Figure 3 and for her insights on heart function during hibernation. Thanks to Marshall Hampton for contributing to Figure 1 and for providing details on genome sequence projects, and thanks to Kevin Russeth for his help with assembly of this paper.

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