

Natural Hypothermic Preservation: The Mammalian Hibernator

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Abstract

Hibernators offer natural models of mammalian organ systems that can withstand long term hypothermia and ischemia. The biochemical mechanisms that regulate and stabilize metabolism to assure long term viability during torpor can be applied in methodologies that could improve the hypothermic preservation of human organs removed for transplant and substantially increase both the time that organs can be maintained in cold storage and the recovery of function after implantation. The present review assesses four current areas of hibernation research that have applied relevance for hypothermic preservation. These are: 1) reversible phosphorylation control of metabolism, 2) preservation of cellular protein synthesis machinery during torpor, 3) hibernation-specific gene expression, and 4) extracellular signaling and pre-conditioning. Particular emphasis is placed on the application of new techniques in genomics - the use of cDNA array screening - for identifying gene responses that underlie both hypothermic injury in non-hibernators and protective responses by hibernating mammals.

Introduction

Since 1949 when Polge et al. (1) first reported the freezing preservation of sperm in glycerol, interest and innovation in the field of biological preservation has grown explosively. Cryopreservation of cells and tissues is now an important part of modern medicine, veterinary science, and agriculture and methods for hypothermic preservation support blood banking and organ transplant technology. The long term preservation of biological materials can, I believe, be broken down into two components: physical preservation deals with structural stabilization of cells and macromolecules whereas metabolic preservation deals with the maintenance of a viable cellular metabolism during storage. Historically, the field of cryopreservation has dealt primarily with physical preservation and relied on storage at ultralow temperatures (e.g. -196°C storage in liquid nitrogen) to arrest metabolism. Problems of metabolic decay that occur at warmer temperatures during cooling or rewarming phases have typically been dealt with by minimizing these exposures through optimizing the speed and conditions of freeze/thaw for each individual cell or tissue type. However, both cryopreservation and vitrification techniques for ultralow preservation began to run into trouble when the focus of studies started to shift to intact organs and, for the present at least, hypothermic preservation at 0-5°C remains the method of choice for use with intact, transplantable organs. Problems of physical preservation of the organ are less important during hypothermic preservation, except for the regulation of cell volume. Instead, strategies for metabolic preservation must come to the forefront to deal with the negative effects of hypothermia and ischemia on the metabolism and viability of excised mammalian organs.

Methodology for cryopreservation and hypothermic preservation in medicine improves all the time, primarily drawing on empirical experimentation. However, impressive examples of both metabolic and physical preservation occur in nature from many organisms that enter arrested or frozen states naturally. In creating solutions for the preservation of biological materials, we need to remain aware that Nature, over millions of years of evolution, has already come up with many solutions for biological preservation, developed of necessity as animals and plants pushed into more and more inhospitable environments. Mechanisms for survival of subzero temperatures, extreme desiccation, and oxygen deprivation are readily found in nature (2-4), as is an excellent natural model of hypothermic preservation, the hibernating mammal.

Hibernation is a state of profound torpor where metabolic rate is typically reduced to <5% of euthermic resting rate and endothermy is abandoned. By hibernating, small mammals such as ground squirrels can save close to 90% of the energy that would otherwise be needed for winter survival (5). Animals spend days or weeks with a core body temperature (T_b) that is often near to 0°C, yet they show none of the metabolic injuries that rapidly accrue in the organs of humans and most other mammals when core T_b falls below ~25°C. Heart beat is also reduced to just a fraction of normal so that organ perfusion rates fall by ~90%, creating conditions that would constitute severe ischemia in most mammals but are non-injurious to hibernators (6). The primary reason for the lack of injury due to hypothermia or ischemia during hibernation is strong biochemical control that achieves a coordinated suppression of all metabolic processes, rebalances the rates of many cell functions to re-establish homeostasis in the torpid state, initiates hibernation-specific metabolic adaptations, and protects/stabilizes the metabolic potential of cell functions that are interrupted during torpor but must be reinstated immediately upon arousal.

The early view of hibernation was that the decrease in T_b caused the metabolic rate depression, a view supported by the fact that the change in metabolic rate (oxygen consumption) between the euthermic resting rate at ~37°C and the value in steady-state torpor at a T_b <10°C often closely approximated a Q_{10} of 2. This common effect of temperature change on chemical reactions (a 2-fold change in rate for every 10°C change in temperature) is also the underlying principle of hypothermic preservation. However, more detailed studies of the time course of changes in metabolic rate and T_b during entry into hibernation have shown that it is the active suppression of metabolic rate, coupled with a lowering of the hypothalamic set point, that reduces thermogenesis and causes the gradual decline in T_b (7,8). Reduced T_b is the result, not the cause, of metabolic rate depression. With this in mind, it is obvious that many of the mechanisms of metabolic control that are exhibited by hibernating mammals could have direct relevance for improving the viability of human organ explants during cold storage. Instead of just using Q_{10} effects to reduce the metabolic rate of excised organs, an intervention with selected hormones/drugs/chemicals that could specifically inhibit some of the energy-expensive metabolic functions of cells could substantially extend the time that a viable organ could be stored. The present article reviews four current areas of hibernation research that have applied relevance for hypothermic preservation. These are: 1) reversible phosphorylation control of metabolism, 2) preservation of cellular protein synthesis machinery during torpor, 3) hibernation-specific gene expression, and 4) extracellular signaling and pre-conditioning.

Reversible phosphorylation control of metabolism

Hibernation is a reversible state. Animals go through multiple cycles of hypothermic torpor interspersed with brief periods of arousal during which they rewarm to the euthermic T_b (5). Because of this the regulatory mechanisms that control torpor and arousal must be readily reversible as well as applicable to the control of multiple types of cellular processes in order to allow a coordinated suppression of the rates of all energy-expensive metabolic processes during hibernation and equally a rapid return to normal euthermic functions upon arousal. Studies in my lab first examined the idea that reversible protein phosphorylation was a key molecular mechanism controlling metabolic rate depression during hibernation. Our initial focus was on the inhibitory control of carbohydrate catabolism that achieved two ends: (a) to promote lipid oxidation during torpor, and (b) to contribute to the suppression of metabolic rate by curtailing the anabolic uses of carbohydrate. Research with both golden-mantled ground squirrels (*Spermophilus lateralis*) and jumping mice (*Zapus hudsonius*) showed coordinated changes in the phosphorylation state of regulatory enzymes of carbohydrate metabolism, including glycogen phosphorylase, phosphofructokinase, pyruvate kinase and pyruvate dehydrogenase (PDH) in multiple tissues during hibernation (9,10). In all cases, covalent modifications of enzymes during hibernation produced less active enzyme forms. A prime example is PDH, the enzyme that gates carbohydrate entry into the tricarboxylic acid cycle. In *Z. hudsonius*, the percentage of PDH in the dephosphorylated, active α form dropped from 15 % in heart and 29 % in kidney of euthermic mice to just 1 % in animals that had been hibernating 5-8 days (9). Similar results were found in *S. lateralis* tissues, the amount of active PDH α in hibernators being only 3 % in kidney and 4 % in heart of the corresponding euthermic values (11). PDH control by reversible phosphorylation is also a component of short-term daily torpor. In hamsters, the percentage of PDH in the active α form was 75-80% in heart, liver and brown adipose tissue (BAT) of euthermic animals but fell to 15-40% in torpid individuals (12). Not surprisingly, studies of gene expression in ground squirrel heart found a strong up-regulation of PDH kinase, the enzyme that phosphorylates and turns off PDH, during hibernation in ground squirrel tissues (13).

Reversible phosphorylation of enzymes is a well-known mechanism for regulating carbohydrate catabolism in response to multiple signals such as hormones or starvation. To prove that reversible phosphorylation during hibernation was linked with metabolic suppression required the demonstration that other facets of metabolism were similarly and coordinately regulated in this manner. Successful long term torpor requires a balance between the rates of ATP-production and the rates of ATP use, with both lowered in concert to a new steady-state rate of ATP turnover. Hence, it was logical to predict that the rates of major ATP-utilizing functions would also be suppressed by reversible phosphorylation during hibernation and we therefore turned our attention to some of the most energy-expensive reactions in cells, the ion-motive ATPases. These utilize a huge proportion of total cellular energy to pump ions against their concentration gradients; for example, the sodium/potassium ATPase (Na/K-ATPase) alone is responsible for 5-40% of total ATP turnover depending on cell type (14). When we assayed this enzyme in *S. lateralis* we found that the activities of Na/K-ATPase dropped sharply during hibernation; when compared at 25°C, mean activities in hibernator skeletal muscle, kidney and liver were just 40, 59 and 54% of euthermic values, respectively (15). The mechanism of Na/K-ATPase inhibition during hibernation proved to be protein phosphorylation Figure 1 shows the effects on Na/K-ATPase activity of *in vitro* incubation with added cAMP + ATP + Mg²⁺ to promote the activity of endogenous cAMP-dependent protein kinase (PKA). Stimulation of PKA action reduced Na/K-ATPase activity in muscle extracts from euthermic squirrels to just 20% of the untreated value but did not affect the enzyme in extracts from hibernator muscle. Subsequent treatment of both preparations with alkaline phosphatase fully restored enzyme activity in

euthermic extracts and also raised Na/K-ATPase activity in hibernator extracts by 3.5-fold. Other experiments showed that stimulation of protein kinases G or C also suppressed Na/K-ATPase in muscle extracts of euthermic animals (15). The data are consistent with protein phosphorylation as the main mechanism used to suppress Na/K-ATPase activity during hibernation and with dephosphorylation as the means of reactivating the enzyme during arousal when it is needed for a key role in shivering thermogenesis.

The activity of Ca-ATPase in the sarcoplasmic reticulum (SR) is also strongly suppressed during hibernation. Ca-ATPase in skeletal muscle SR of hibernating *S. undulatus* was just 46% of the value in summer active squirrels, measured per milligram of SR protein, and 55% of its specific activity in summer squirrels, measured per milligram of Ca-ATPase protein (16). These data demonstrate both a reduced total amount of Ca-ATPase among the SR proteins during hibernation squirrels and a substantial reduction of the activity of individual protein molecules. The latter decrease in specific activity is undoubtedly due to protein phosphorylation. Other proteins involved in SR calcium signaling are suppressed during hibernation. The SR calcium-release channel (ryanodine receptor) decreased by 50% in hibernation and levels of most SR calcium binding proteins (e.g. sarcalumenin, calsequestrin) were 3-4 fold lower in hibernating, compared with summer-active, animals.

Overall, then, the message is that reversible protein phosphorylation is effectively used in the tissues of hibernators to coordinate the suppression of multiple cell functions, including both ATP-generating and ATP-utilizing reactions. Treatments that can trigger the action of selected protein kinases and phosphatases to stimulate the suppression of key metabolic activities (e.g. ion motive ATPases) could offer significant improvements to the viability of organs during hypothermic storage.

Control of protein synthesis

Multiple metabolic processes are virtually shut down during hibernation yet these must be maintained in readiness so that during periodic arousals animals can return with ease to their normal euthermic state. Protein synthesis is one of the energy-expensive cell functions that is curtailed. For example, in brain of thirteen-lined ground squirrels (*S. tridecemlineatus*) the rate of ^{14}C -leucine incorporation into protein *in vivo* in torpid animals was only 0.04% of the mean value in active squirrels (17). Furthermore, when protein synthesis by brain extracts from hibernating versus euthermic animals was compared *in vitro* at 37°C, the rate was 3-fold lower in extracts from hibernators which showed that factors other than temperature contributed to the inhibition of protein synthesis in hibernating animals (17). We similarly assessed ^3H -leucine incorporation into protein at 37°C in extracts of *S. tridecemlineatus* kidney and found that the *in vitro* rate in hibernator extracts was just 15% of the euthermic value (18). However, there is little, if any, change in global mRNA levels in hibernator tissues so the suppression of protein synthesis during hibernation is not due to reduced mRNA availability (17,19). Instead, the translational machinery is largely turned off, except for some very specific cases of hibernation-specific gene up-regulation (see next section). If the turnover of most constitutive proteins is virtually halted during torpor, then, in addition to the ATP savings that would accrue, multiple potential problems could be avoided such as the adaptive/evolutionary "expenses" that would otherwise be required to adjust mammalian transcriptional and translational machinery to maintain integrated function over a 30-40°C temperature range. Protein "life extension" during torpor would also minimize the amount of NH_4^+ and urea that accumulated because proteolysis is comparably suppressed. Two mechanisms that contribute to the arrest of protein synthesis have received experimental attention recently: (1) reversible phosphorylation control of the translational machinery, and (2) the physical state of ribosome aggregation.

Reversible phosphorylation control of translation.

Frerichs et al. (17) found that both translation initiation and polypeptide elongation were inhibited in ground squirrel brain during hibernation. The inhibition of translation initiation was traced to eukaryotic initiation factor 2 (eIF2) which introduces initiator methionyl-tRNA into the translation-initiation complex. Phosphorylation of the alpha-subunit of eIF2 is well known as a mechanism for inhibiting protein synthesis and studies have shown that even small amounts of phosphorylated eIF2 α significantly inhibit global protein synthesis by blocking the initiation of nascent polypeptides (20). Western blot analysis of extracts from *S. tridecemlineatus* brain showed that the amount of phospho-eIF2 α was <2% of total eIF2 α in euthermic animals but rose markedly to ~13% in hibernating individuals. We found a similar striking increase in the amount of phospho-eIF2 α in kidneys of hibernating squirrels, again with no change in total eIF2 α protein (18). Frerichs et al. (17) also identified polypeptide elongation as another level of control showing that mean transit times for polypeptide elongation by ribosomes were 3-fold longer in post-mitochondrial supernatants from brain of hibernating versus euthermic animals. Again, the mechanism was traced to protein phosphorylation with elevated amounts of the phosphorylated form of eukaryotic elongation factor-2 (eEF-2) found in brain and liver of hibernating, compared with euthermic, ground squirrels (21). This was the result of an ~50% higher activity of eEF-2 kinase in tissues from hibernating animals and a corresponding 20-30% decrease in the activity of protein phosphatase 2A (which opposes eEF-2 kinase), as a result of a 50-60% increase in the levels of the specific inhibitor of PP2A, I₂^{PP2A}.

The involvement of a protein phosphorylation mechanism in the control of both translation initiation and peptide elongation further emphasizes the key role that reversible phosphorylation plays in multiple aspects of metabolic suppression in hibernation and links the suppression of protein synthesis with the control of multiple other metabolic functions, discussed previously. The important principle here is that multiple cell functions are controlled via a single regulatory mechanism (reversible protein phosphorylation) to achieve a coordinated suppression of metabolic rate during hibernation. This is not an unusual situation in the animal kingdom as there are numerous examples of reversible phosphorylation used to regulate stress-induced metabolic suppression in anoxia-tolerant, diapausing and estivating animals (3,4). Therefore, there is a good probability, given the correct choice of signal transduction agents, that a similar coordinated suppression of metabolism could be achieved in organ explants in order to provide "life extension" during hypothermic preservation.

Ribosome aggregation

The aggregation state of ribosomes is key to translation; active translation occurs on polysomes whereas monosomes are translationally silent. Not surprisingly, then, several recent studies have demonstrated that during hibernation there is a major reduction in tissue polysome content and a corresponding increase in monosomes. This has been documented for *S. tridecemlineatus* brain (17) and kidney (18) as well as in liver of Arctic ground squirrels (*S. parryii*) (19). Figure 2 shows the situation in *S. tridecemlineatus* kidney. Ribosomes separate on sucrose gradients into polysomes (fraction 1-5) versus lighter weight monosomes and messenger ribonuclear proteins (mRNP) (fractions 6 and higher). By monitoring the distribution of 18S ribosomal RNA (rRNA), the major RNA constituent of the 40S ribosomal subunit, a clear reduction in the proportion of total rRNA in polysomes can be seen in extracts from during hibernating, versus euthermic, animals. The shift is particularly striking in fraction 6 which is essentially devoid of either mRNA or rRNA in euthermic animals but contains significant signal in hibernating animals. The distribution of mRNA transcripts for a constitutive gene, cytochrome oxidase subunit 4 (*Cox4*), showed a similar shift between euthermic and hibernating states (although the total amount of *Cox4* mRNA did not change). In euthermic kidney extracts there

was a distinct distribution of *Cox4* mRNA between the heavy polyribosome and the monosome/mRNP fractions, with little or no signal present in fraction 6. In hibernator extracts, the relative amount of *Cox4* mRNA transcripts associated with the heaviest polysome fractions (#1-3) was distinctly reduced and mRNA associated with the monosome fraction was enhanced. Knight et al. (19) found a similar shift of the mRNA for another constitutive enzyme (glyceraldehyde-3-phosphate dehydrogenase) into the monosome fraction during hibernation with mRNA returning to polysome fractions during arousal.

Interestingly, the situation was very different in *S. tridecemlineatus* BAT, the thermogenic tissue that plays a key role in rewarming the hibernator during arousal. A comparison of polysome profiles in BAT from euthermic versus hibernating animals showed an increased content of 18S rRNA in the higher density sucrose fractions during hibernation, indicating an increase in polyribosome density during torpor in this tissue (18). The distribution of mRNA for the heart forms of fatty acid binding protein (H-FABP), which is up-regulated in BAT during hibernation, showed a proportional increase in the association of H-FABP mRNA with polysome fractions 1-5. By contrast, the mRNA for *Cox4*, a constitutive gene, was largely sequestered into the monosome and mRNP fractions. This suggests another level of metabolic control in hibernation which is the differential distribution of individual mRNA species between translationally active and inactive ribosomes. In this case, the heavy polyribosomes in hibernator BAT may disproportionately express those mRNAs (such as H-FABP) that are crucial to the hibernation phenotype while relegating mRNA species that are not needed during hibernation into the translationally silent monosome fraction. Via this mechanism hibernator tissues can retain mRNA transcripts that will be needed again during interbout arousals, preserving these mRNA molecules whose average life-span is normally only a few minutes.

The mechanisms by which polysome redistribution occurs during hibernation are not yet fully known although reversible phosphorylation of various factors (eIF2 α , eEF-2 and others) probably has a key role. A recent study by van Breukelen and Martin (22) suggests that a passive effect of low temperature may also occur. They monitored the distribution of polysomes versus monosomes in animals sampled at a variety of Tb values and from this concluded that translation initiation ceased when Tb fell to 18°C. Conversely, as animals rewarmed during arousal, the same 18°C Tb was associated with a large increase in initiation events and a complete restoration of the euthermic polysome distribution pattern. Whether this effect is truly a passive influence of temperature on ribosome aggregation or is a regulated effect remains to be seen. Several well-known examples of temperature-dependent metabolic switches are in fact mediated by the differential effects of temperature on the activities selected protein kinases and phosphatases. The activation of cryoprotectant synthesis at 5°C in cold-hardy insects is a prime example of this, the effect deriving from a specific low temperature inhibition of glycogen phosphorylase phosphatase (23).

Gene expression

Recent studies have begun to probe hibernation-specific gene expression. In general, overall rates of gene transcription should be strongly suppressed in hibernation, coincident with the suppression of protein synthesis discussed above. However, it is also obvious that the development and maintenance of torpor probably requires the expression of selected hibernation-specific genes. These could include genes that regulate metabolic suppression, genes that improve ischemia resistance and hypothermia tolerance, and genes that address organ-specific needs for the maintenance of long term viability. All of these have obvious applications for hypothermic organ preservation. By understanding the patterns of gene expression in mammalian

hibernation we can gain knowledge of both the key metabolic problems faced by different organs and the natural solutions that have been developed.

Studies of hibernation-specific gene expression are relatively recent and, to date, have involved several different research groups using a variety of techniques, several different model species and with interests in a number of different organs. As yet, only one consistent result has emerged and that is that entry into (and arousal from) hibernation apparently occurs with very few changes in gene expression. For example, from 4500 reverse transcription-PCR products produced from brain of *S. lateralis*, only 29 candidate cDNAs appeared to differ between euthermic and hibernating states and none of these could be confirmed as up-regulated by northern blotting (24). In our lab, screening of a cDNA library prepared from heart of hibernating *S. lateralis* retrieved only two unique clones that were confirmed as up-regulated during hibernation (25). We similarly screened a cDNA library made from skeletal muscle of hibernating little brown bats (*Myotis lucifugus*) and found ~60 candidate clones after primary screening but none of these could be confirmed as hibernation up-regulated by northern blotting (26). Use of differential display PCR to analyze gene expression in skeletal muscle of hibernating *S. lateralis* similarly failed to identify up-regulated genes (26) and low numbers of putatively up-regulated genes were also found when we applied new cDNA array screening technology to both ground squirrel and bat tissues (see section below). One conclusion that can be drawn from these data is that hibernation seems to require very few changes to the gene expression pattern of organs. This is perhaps not unexpected because (a) an energy-limited torpid state is not a time for major metabolic reorganization and (b) cells and organs must remain competent to rapidly resume normal body functions during interbout arousals. However, these results also mean that the control mechanisms governing hibernation may be vested in only a handful of gene/protein changes. This is positive news both for hibernation researchers and also for those that hope to apply the "tricks" learned from hibernators to the improvement of hypothermic preservation technology.

Hibernation-specific up-regulation of several genes has been confirmed. This includes the genes for α_2 -macroglobulin in liver (27), moesin in intestine (28), pyruvate dehydrogenase kinase isozyme 4 and pancreatic lipase in heart (13), and uncoupling protein isoforms in multiple tissues (29). Studies in our lab have identified genes coding for the ventricular isoform of myosin light chain 1 (MLC1_v) and NADH ubiquinone-oxidoreductase subunit 2 (ND2) as up-regulated in heart and skeletal muscle of *S. lateralis* (25) whereas heart and adipose isoforms of FABP were up-regulated in brown adipose tissue and heart of *S. tridecemlineatus* (30) and three genes coded on the mitochondrial genome, cytochrome c oxidase subunit 1 (COX1) and ATPase subunits 6 and 8 were up-regulated in kidney, heart and brown adipose (31). The latter finding is particularly interesting because, together with the gene for ND2 which is also mitochondrial, this suggests that a general up-regulation of the mitochondrial genome may occur during hibernation. By contrast, transcript levels of related nuclear-encoded genes (subunit 4 of cytochrome c oxidase and ATP α , a nuclear encoded subunit of the mitochondrial ATP synthase) did not change during hibernation (Figure 3) (31). Interestingly, up-regulation of the mitochondrial genome also occurs as responses to environmental stress (freezing, anoxia) in other stress-tolerant organisms (3) and this may represent a generalized stress response that requires further investigation. Other changes in gene expression during hibernation have been reported in ground squirrel brain; a 98 kDa protein with a phosphotyrosine moiety is present in membrane fractions throughout a hibernation bout but disappears within 1 h of arousal (32) whereas increased expression of "intermediate-early" genes that code for selected transcription factors (c-fos, junB, c-Jun) occurs during late torpor and peaks during arousal (24).

Selected proteins are also down-regulated during hibernation (33-35). A specific reduction in the levels of insulin-like growth factor as well as the plasma binding protein (IGFBP-3) that carries it, suggested that one facet of hibernation is a suppression of the growth-regulatory IGF axis that controls energy-expensive somatic growth in skeleto-muscular and other tissues (35). Kondo and Kondo (33) also reported the occurrence of four proteins in the blood of euthermic chipmunks (*Tamias asiaticus*) that disappeared when the animals were hibernating; one of these showed high homology with α_1 -antitrypsin. Interestingly, α_1 -antitrypsin and three other acute phase blood proteins were unaffected during hibernation in *S. richardsonii* but a fifth member of the family, α_2 -macroglobulin, increased significantly (27). Results from both species suggest modification of the acute phase response to injury and infection during hibernation.

Overall, there is not yet a "big picture" of the nature and types of gene expression changes that support hibernation but rather the studies to date suggest various organ-specific requirements that need to be met in torpid organs. For example, the 2-3 fold increase in *MLC1_v* transcripts (which encode the ventricular isoform of myosin light chain subunit 1; *MLC1_v*) in *S. tridecemlineatus* heart and skeletal muscle (but not kidney and liver) during hibernation has been interpreted as an indicator of myosin restructuring for low temperature muscle function (25). Evidence of myosin restructuring during hibernation has also been found in hamster (*Cricetus cricetus*) heart (36). Cardiac function during hibernation must be regulated to deal with the increased peripheral resistance at low T_b and to do this the force of contraction actually increases. Changes in sarcoplasmic reticulum Ca^{+2} storage and release have been tested as a possible reason for enhanced contractility in hibernation (37) but a change in the composition of muscle proteins is probably another factor. An up-regulation of heart (H) and adipose (A) isoforms of FABP in hibernation in BAT and heart of ground squirrels serves the increased reliance of organs on fatty acids as metabolic fuels during torpor (30). Interestingly, the occurrence of A-FABP in ground squirrel heart appears to be unique among mammals but A-FABP also occurs in the hearts of Antarctic teleost fishes (38) which supports the idea that this isoform is important for low temperature function. It is proposed that the increased expression of heart and adipose FABPs during hibernation accelerates the rate at which fatty acids can be transported to the mitochondria for oxidization, particularly in support of the huge increase in thermogenesis by BAT and rapid increase in heart rate that are required during arousal from torpor.

New technology - cDNA array screening

To date, the identification of hibernation-specific genes has been quite spotty, involving multiple species, several tissues and diverse techniques, each with their own limitations. Recent advances in molecular biology technology are revolutionizing gene screening and will allow a broad-based and comprehensive analysis of stress-induced gene expression in multiple animal systems that is sure to produce many advances in our understanding of biochemical adaptation. New cDNA array technology offers the first chance to evaluate global changes in gene expression during hibernation. State-of-the-art arrays can have thousands of unique cDNAs bound to them and can offer one-step screening of the expression patterns of as much as 50% or more of the genome. Arrays are particularly valuable as an initial screening tool to allow assessment of the responses of broad families of genes (e.g. signal transduction systems, glycolytic enzymes, molecular transporters, transcription factors) to an imposed stress. Array screening is being applied in multiple fields in biology and medicine. A few recent examples include studies of the effects of aging, screening cancer cell lines to identify genes associated with carcinogenesis, analysis of the effects of genetically modified foods on the digestive systems of organisms, and as an aid to drug design and testing in neuropharmacology (39-42).

Two kinds of cDNA arrays are well-suited for broad screening: nylon macroarrays and glass slide microarrays. Nylon macroarrays containing hundreds of spotted cDNAs provide a good starting point for many labs because few changes need to be made to standard molecular biology protocols to use these arrays. Preparation of ^{32}P -labeled probes and array hybridization follows standard methods used for cDNA library screening and northern blotting. Nylon arrays are also reusable which makes them attractive financially. The primary limitation of nylon macroarrays is that paired arrays must be used for control and experimental samples; one array is hybridized with control probe and one with stressed. Gene expression on each array must then be normalized to the expression of one or more control or housekeeping genes on the same array before a comparison of normalized gene expression levels can be made between the two arrays. There are several potential sources of error in this process, not the least of which is the choice of housekeeping genes. Standard housekeeping genes that are constitutively expressed in euthermic mammalian cells may or may not have altered expression patterns under hypothermic conditions or during torpor.

Size limitations to the number of cDNAs that can be spotted and probed on nylon arrays has fueled the development of the newest technology: microarrays produced by spotting cDNAs on treated glass microscope slides (43). At present, microarrays can contain up to 31,500 non-redundant cDNAs (40). Most of these are identified genes but others are expressed sequence tags of as yet unknown cellular functions. Microarray technology requires some changes to standard molecular biology techniques, in particular the replacement of ^{32}P -labeled probes with probes that are labeled with Cy3 or Cy5 dyes (one for control and one for experimental samples). First strand cDNA synthesis is carried out with Cy-labeled dyes that are conjugated to dCTP. Both control and experimental probe is then hybridized to the same array (a key advantage) and binding is quantified by measuring fluorescence intensity at emission wavelengths of 570 nm for Cy3 and 670 nm for Cy5 (excitation at 550 and 649 nm, respectively) in a microarray reader (the major expense of this technology).

We have recently tried both of these technologies in our search for hibernation-responsive genes in hibernating ground squirrels and bats. Using a rat cDNA macroarray (Clontech Rat ATLASTM) containing 588 genes we first screened *S. tridecemlineatus* brown adipose tissue for genes that were up-regulated in hibernation. The analysis showed that transcript levels of most genes did not change during hibernation but expression of a small number (<1%) was elevated during torpor (30). Prominent among these were the heart (H) and adipose (A) isoforms types of FABP (discussed above). Overall, transcript levels of most of the 588 genes on the array did not change or significantly decreased during hibernation. Limited gene up-regulation during hibernation is perhaps not surprising since, intuitively, the torpid state at low T_b is not the time for major biosynthesis of proteins. From another point of view, the lack of significant change in the levels of most gene transcripts suggests that one of the keys to hibernation/torpor is bringing gene expression and protein synthesis to a halt without losing the potential (e.g. mRNA pool) for the rapid resumption of protein synthesis when animals return to euthermia. Comparable screening of *S. tridecemlineatus* muscle produced interesting data. A group of cDNAs that encode for components of the small and large ribosomal subunits were consistently down-regulated during hibernation including L19, L21, L36a, S17, S12 and S29 (44). As discussed earlier, this implicates control of the ribosomes as critical to the maintenance of long term homeostasis in the hypometabolic, hypothermic state.

Published studies to date using arrays have all used homologous probing – that is, the cDNAs immobilized on the arrays are from the same species as the samples that are being screened. For mammals, array availability is presently restricted to human, rat and mouse genomes. Clearly, the production of homologous arrays for every species that is of interest to

researchers would be a daunting task and so we wanted to know whether heterologous probing was a viable alternative. Could arrays produced with genes from one species provide useful information when used to screen samples from closely related, or even distantly related, species? Our initial results have led us to believe that heterologous probing of cDNA arrays of closely related species can provide an important first step in gene discovery. With the rat macroarrays we found that the percentage of cDNAs that cross-reacted was 93% for *S. tridecemlineatus* and 73% for *M. lucifugus*. Recently, we have also used human 19K glass slide microarrays (Ontario Cancer Institute) with both ground squirrel and bat tissues. Our initial trials produced a cross-reaction rate of only 15-20% but we were able raise this to 85-90% using a simple reduction in the post-hybridization washing temperature from 50°C, as suggested by the manufacturer's protocol, to room temperature (~21°C) (44). The success of heterologous probing depends, of course, on a high percent identity at the cDNA level between rat/human and squirrel/bat genes. With upwards of 80% sequence identity between most gene homologues in placental mammals, there is a high probability of successful cross-reaction. Indeed, in other studies, we have confirmed high sequence identity for various individual ground squirrel genes with their human or rat homologues, for example, 97.5% for MLC1_v, >93% for COX1 in *S. lateralis* (25) and >90% for FABP (30) in *S. tridecemlineatus*. With homologous probing, the glass microarrays containing 19,200 unique transcripts offer the possibility of screening a high percentage of the human genome, which is thought to contain 30,000-40,000 genes (45). With high cross-reaction rates, heterologous probing also offers an unprecedented opportunity for assessing the responses to hibernation of a high percentage of the identified genes in the ground squirrel or bat genomes.

Data from array screening (as from cDNA library screening) still have to be confirmed using techniques such as northern blotting or quantitative RT-PCR but in our use of heterologous probing to date we have found that arrays are very reliable with positive results from array screening readily confirmed by other methods. However, one weakness of cDNA array screening is that by dealing primarily with known genes, the scope for finding novel genes (ie. unique to the hibernating condition) is low. The search for hibernation-specific genes will still probably require the use of other techniques, such as cDNA library screening, although it is possible that genes that are important to hibernation might be found among the large number of expressed sequence tags on the array. Such putative novel genes could then be pulled out of a hibernator cDNA library and characterized with the potential for identification of unique proteins that support hibernation.

cDNA array screening clearly holds unique promise as a tool for making advances in hypothermic preservation in transplant medicine. cDNA arrays are available from several commercial sources for both the human genome and major model species (mouse, rat). Using cDNA arrays, the effects of chilling, storage, rewarming, and organ recovery post-transplant on organ gene expression can be easily assessed. Array screening will allow the identification of both individual genes and functional groupings of genes that are either up- or down-regulated during hypothermic exposure. Using human cDNA arrays, for example, broad patterns of gene responses to hypothermia can be catalogued to provide an overall picture of how an individual organ responds to cold stress. Comparison can be made of the responses to cold ischemia by organs of both non-hibernator and hibernator species with reference to the responses of hibernator organs *in vivo* during torpor. These comparisons could reveal the patterns of gene expression changes that underlie both negative (cold ischemia injury) and positive (stable torpor) responses in mammalian hypothermia and open the way to investigate new strategies of metabolic intervention in organ preservation.

Extracellular Signaling and Preconditioning

The organs of summer-active ground squirrels are just as sensitive to hypothermia injury as are those of nonhibernating species so it is obvious that the metabolic mechanisms that support hibernation are not constitutively present in hibernator organs throughout the year. Metabolic adaptations must be induced, either prior to the start of the hibernating season or in direct response to entry into a torpor bout. One area of current interest is the phenomenon of "test drops"; before true hibernation begins, animals go through a number of cycles where metabolic rate drops briefly and T_b drops by several degrees. In Arctic ground squirrels, for example, test drops occur over the first 2-3 weeks of the season with T_b dropping by 15-20°C for brief periods of time (46). Only after this phase is complete do the animals enter the first true hibernation bouts where T_b drops to near-ambient. Test drops may represent a natural form of ischemia or hypothermia preconditioning. The phenomenon of ischemic preconditioning has received a lot of attention in recent years, with particular reference to heart attack and stroke. Studies have shown that the imposition of one or more short periods of ischemia results in a substantial improvement in the ability to endure a subsequent long period of ischemia (47-49). The theory is that short periods of interrupted blood flow trigger the initiation of protective responses that are then in place when the major ischemic insult is imposed. Preconditioning is also well known in the cold hardiness literature, especially among insects where brief cold shocks can trigger cellular responses that greatly improve survival of subsequent long cold exposures (50). The lesson here for hypothermic preservation of organs is that ischemic preconditioning or cold preconditioning or both may be useful treatments to apply to organs before excision from donors.

Many of the regulatory mechanisms involved in hibernation, such as reversible protein phosphorylation or changes in gene expression, are typically triggered and coordinated in mammalian cells/organs by extracellular signaling molecules. One of the remaining great gaps in hibernation research is the nature of the extracellular signaling agents involved in coordinating the metabolic adjustments that support long term torpor. Once the trigger hormone/chemical(s) is known, the door will be opened to wide-ranging studies of its effects. Clearly there is great potential for the applied use of such a chemical in medical organ preservation.

The existence of a blood-borne hibernation induction trigger (HIT) in hibernating mammals was first reported by Dawe and Spurrier (51) and was followed by various other studies that similarly showed that plasma from hibernating ground squirrels, woodchucks, black bears, or prairie dogs could induce behavioural or physiological characteristics of hibernation in active animals (51,52). However, other studies have not been able to confirm this phenomenon (53,54). Only one group, that Oeltgen and colleagues, seems to be currently active in HIT research. The HIT is believed to be opiate in nature but it has never been isolated or purified although Horton et al. (55) recently isolated and partially sequenced an 88 kDa protein from hibernating woodchucks that may be the HIT. The effects of treatment with HIT-containing serum can be mimicked by synthetic opioids, chiefly D-Ala(2),D-Leu(5)-enkephalin (DADLE), and are opposed by opiate antagonists (56,57). Both opiates and HIT-containing sera have similar protective effects on isolated organ systems of non-hibernators (e.g. ischemic dog lung, contractility of guinea pig ileum or mouse vas deferens, ischemia protection in rat heart) (55,57-60). Currently, HIT effects are primarily being pursued for their potential in extending mammalian organ preservation time. Both HIT and DADLE can extend the preservation time of lung, heart, liver and kidney preparations (61,62) and HIT, when given as a pretreatment, appeared to enhance rat heart recovery after global ischemia (63). Plasma from deeply hibernating woodchucks or DADLE increased the preservation times for canine lung preparations by 3-fold, suggesting the possibility that HIT administration to donors before organ harvest could extend the time available for transfer of the excised organ to its recipient (61).

Unfortunately, current studies HIT remain empirical in nature and focus at a physiological level, measuring parameters such as smooth muscle contractility, developed pressure in heart, oxygen consumption, and organ recovery after transplant as measures of the success of treatments with HIT-containing sera or opioids. What is clearly needed is a rigorous investigation of the molecular action of the putative HIT. Notably lacking is (a) a full purification and chemical identification of HIT, and (b) a demonstration that the HIT triggers or regulates one or more of the intracellular molecular responses associated with hibernation (e.g. reversible phosphorylation of selected target proteins). The field is wide-open at the molecular level but does not seem to have attracted much interest.

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References

1. Polge, C., Smith, A.U., Parkes, A.S. 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature* 164: 666.
2. Clegg, J.S. 2001. Cryptobiosis - a peculiar state of biological organization. *Comp. Biochem. Physiol. B* 128: 613-624.
3. Storey, K.B. 1999. Stress-induced gene expression in freeze tolerant and anoxia tolerant vertebrates. *In: Environmental Stress and Gene Regulation* (Storey, K.B., ed.) BIOS Scientific Publishers, Oxford, pp. 1-23.
4. Storey, K.B. 2001. *Molecular Mechanisms of Metabolic Arrest*. BIOS Scientific Publishers, Oxford.
5. Wang, L.C.H., Wolowyk, M.W. 1988. Torpor in mammals and birds. *Can. J. Zool.* 66: 133-137.
6. Frerichs, K.U., Kennedy, C., Sokoloff, L., Hallenbeck, J.M. 1994. Local cerebral blood flow during hibernation, a model of natural tolerance to "cerebral ischemia". *J. Cereb. Blood Flow Metab.* 14: 193-205.
7. Geiser, F. 1988. Reduction of metabolism during hibernation and daily torpor in mammals and birds: temperature effect or physiological inhibition? *J. Comp. Physiol. B* 158: 25-37.
8. Heldmaier, G., Ruf, T. 1992. Body temperature and metabolic rate during natural hypothermia in endotherms. *J. Comp. Physiol. B* 162: 696-706.
9. Storey, K.B. 1989. Integrated control of metabolic rate depression via reversible phosphorylation of enzymes in hibernating mammals. *In: Malan A., Canguilhem B., eds. Living in the Cold.* Montrouge: John Libbey Eurotext Ltd. pp. 309-319.
10. Storey, K.B. 1997. Metabolic regulation in mammalian hibernation: enzyme and protein adaptations. *Comp. Biochem. Physiol. A* 118: 1115-1124.
11. Brooks, S.P.J., Storey, K.B. 1992. Mechanisms of glycolytic control during hibernation in the ground squirrel *Spermophilus lateralis*. *J. Comp. Physiol.* 162: 23-28.
12. Heldmaier, G., Klingenspor, M., Werneker, M., Lampi, B.J., Brooks, S.P.J., Storey, K.B. (1999) Metabolic adjustments during daily torpor in the Djungarian hamster. *Am. J. Physiol.* 276: E896-E906.
13. Andrews, M.T., Squire, T.L., Bowen, C.M., Rollins, M.B. 1998. Low-temperature carbon utilization is regulated by novel gene activity in the heart of a hibernating animal. *Proc. Natl. Acad. Sci. USA* 95: 8392-8397.
14. Claussen, T. 1986. Regulation of active Na^+K^+ transport in muscle. *Physiol. Rev.* 66: 542-576.
15. MacDonald, J.A., Storey, K.B. 1999. Regulation of ground squirrel Na^+K^+ -ATPase activity by reversible phosphorylation during hibernation. *Biochem. Biophys. Res. Commun.* 254: 424-429.
16. Malysheva, A.N., Storey, K.B., Ziganshin, R.K., Lopina, O.D., Rubtsov, A.M. 2001. Characteristics of sarcoplasmic reticulum membrane preparations isolated from skeletal muscles of active and hibernating ground squirrel *Spermophilus undulatus*. *Biochemistry (Moscow)* 66: 918-925.

17. Frerichs, K.U., Smith, C.B., Brenner, M., DeGracia, D.J., Krause, G.S., Marrone, L., Dever, T.E., Hallenbeck, J.M. 1998. Suppression of protein synthesis in brain during hibernation involves inhibition of protein initiation and elongation. *Proc. Natl. Acad. Sci. USA* 95: 14511-14516.
18. Hittel, D. and Storey, K.B. 2002. The translation status of differentially expressed mRNAs in the hibernating thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*). *Arch. Biochem. Biophys.* 401: 244-254.
19. Knight, J.E., Narus, E.N., Martin, S.L., Jacobson, A., Barnes, B.M., Boyer, B.B. 2000. mRNA stability and polysome loss in hibernating Arctic ground squirrels (*Spermophilus parryii*). *Mol. Cell. Biol.* 20: 6374-6379.
20. Mikulits, W., Pradet-Balade, B., Habermann, B., Beug, H., Garcia-Sanz, J.A., Mullner, E.W. 2000. Isolation of translationally controlled mRNAs by differential screening. *FASEB J.* 14: 1641-1652.
21. Chen, Y., Matsushita, M., Nairn, A.C., Damuni, Z., Cai, D., Frerichs, K.U., Hallenbeck, J.M. 2001. Mechanisms for increased levels of phosphorylation of elongation factor-2 during hibernation in ground squirrels. *Biochemistry* 40: 11565-11570.
22. Van Breukelen, F., Martin, S.L. 2001. Translational initiation is uncoupled from elongation at 18°C during mammalian hibernation. *Am. J. Physiol.* 281: R1374-R1379.
23. Storey, K.B., Storey, J.M. 1991. Biochemistry of cryoprotectants. In: Denlinger, D., Lee, R.E., eds. *Insects at Low Temperature*. New York: Chapman and Hall, pp. 64-93.
24. O'Hara, B.F., Watson, F.L., Srere, H.K., Kumar, H., Wiler, S.W., Welch, S.K., Bitting, L., Heller, H.C., Kilduff, T.S. 1999. Gene expression in the brain across the hibernation cycle. *J. Neurosci.* 19: 3781-3790.
25. Fahlman, A., Storey, J.M., Storey, K.B. 2000. Gene up-regulation in heart during mammalian hibernation. *Cryobiology* 40: 332-342.
26. Eddy, S.F., Storey, K.B. 2001. Gene expression in hibernation: testing skeletal muscle of little brown bats, *Myotis lucifugus*, using commercially available cDNA microarrays. Proceedings of the Virtual Conference in Genomics and Bioinformatics, October 15 -16, 2001. <http://www.ndsu.nodak.edu/virtual-genomics/meetings.htm>
27. Srere, H.K., Belke, D., Wang, L.C.H., Martin, S.L. 1995. α_2 -Macroglobulin gene expression during hibernation in ground squirrels is independent of acute phase response. *Am. J. Physiol.* 268: R1507-R1512.
28. Gorham, D.A., Bretscher, A., Carey, H.V. 1998. Hibernation induces expression of moesin in intestinal epithelia cells. *Cryobiology* 37: 146-154.
29. Boyer, B.B., Barnes, B.M., Lowell, B.B., Grujic, D. 1998. Differential regulation of uncoupling protein gene homologues in multiple tissues of hibernating ground squirrels. *Am. J. Physiol.* 275: R1232-R1238.
30. Hittel, D., Storey, K.B. 2001. Differential expression of adipose and heart type fatty acid binding proteins in hibernating ground squirrels. *Biochim. Biophys. Acta* 1522: 238-243.
31. Hittel, D., Storey, K.B. 2002. The differential expression of a mitochondrial-encoded subunit of cytochrome c oxidase in the kidney of a hibernating mammal. *J. Exp. Biol.* 205: 1625-1631.
32. Ohtsuki, T., Jaffe, H., Brenner, M., Azzam, N., Azzam, R., Frerichs, K.U., Hallenbeck, J.M. 1998. Stimulation of tyrosine phosphorylation of a brain protein by hibernation. *J. Cereb. Blood Flow Metab.* 18: 1040-1045.
33. Kondo, N., Kondo, J. 1992. Identification of novel blood proteins specific for mammalian hibernation. *J. Biol. Chem.* 267: 473-478.
34. Soukri, A., Valverde, F., Hafid, N., Elkebbaj, M.S., Serrano, A. 1996. Occurrence of a differential expression of the glyceraldehyde-3-phosphate dehydrogenase gene in muscle and liver from euthermic and induced hibernating jerboa (*Jaculus orientalis*). *Gene* 181: 139-145.
35. Schmidt, K.E., Kelley, K.M. 2001. Down-regulation in the insulin-like growth factor (IGF) axis during hibernation in the golden-mantled ground squirrel, *Spermophilus lateralis*: IGF-I and the IGF-binding proteins (IGFBPs). *J. Exp. Zool.* 289: 66-73.
36. Morano, I., Adler, K., Agostini, B., and Hasselbach, W. 1992. Expression of myosin heavy and light chains and phosphorylation of the phosphorylatable myosin light chain in the heart ventricle of the European hamster during hibernation and in summer. *J. Muscle Res. Cell Motil.* 13: 64-70.
37. Wang, L.C.H., and Lee, T.F. 1996. Torpor and hibernation in mammals: metabolic, physiological, and biochemical adaptations. In: Fregley, M.J., Blatteis, C.M., eds. *Handbook of Physiology, Environmental Physiology*. Oxford: Oxford University Press. Section 4, Vol. 1., pp. 507-532.
38. Vayada, M.E., Londraville, R.L., Cashion, R.E., Costello, L., Sidell, B. 1998. Two distinct types of fatty acid-binding protein are expressed in heart ventricle of Antarctic teleost fishes. *Biochem. J.* 330: 375-382.
39. Helmberg, A. 2001. DNA-microarrays: novel techniques to study aging and guide gerontologic medicine. *Exp. Gerontol.* 36: 1189-1198.
40. Boer, J.M., Huber, W.K., Sultmann, H., Wilmer, F., von Heydebreck, A., Haas, S., Korn, B., Gunawan, B., Vente, A., Fuzesi, L., Vingron, M. and Poustka, A. 2001. Identification and classification of differentially

- expressed genes in renal cell carcinoma by expression profiling on a global human 31,500-element cDNA array. *Genome Res.* 11: 1861-1870.
41. Hal, N.L.H. van, Vorst, O., van Houwelingen, A.M.M.L., Kok, E.J., Peijnenburg, A., Ahoroni, A., van Tunen, A.J., Keijer, J. 2000. The application of DNA microarrays in gene expression analysis. *J. Biotech.* 78: 271-280.
 42. Marcotte, E.R., Srivastava, L.K., Quirion, R. 2001. DNA microarrays in neuropsychopharmacology. *Trends Pharm. Sci.* 22: 426-436.
 43. Schena, M., Shalon, D., Davis R.W., Brown P.O. 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270: 467-470.
 44. Eddy, S.F. and Storey, K.B. 2002. Dynamic use of cDNA arrays: heterologous probing for gene discovery and exploration of animal adaptations in stressful environments. *In: Cell and Molecular Responses to Stress* (Storey, K.B. and Storey, J.M., eds.) Elsevier Press, Amsterdam, Vol. 3, 315-325.
 45. Venter, J.C., Adams, M.D., Myers, E.W., et al. 2001. The sequence of the human genome. *Science* 291: 1304-1351.
 46. Boyer, B.B., Barnes, B.M. 1999. Molecular and metabolic aspects of mammalian hibernation. *Bioscience* 49: 713-724.
 47. Liu, J., Ginis, I., Spatz, M., Hallenbeck, J.M. 2000. Hypoxic preconditioning protects cultured neurons against hypoxic stress via TNF-alpha and ceramide. *Am. J. Physiol.* 278: C144-C153.
 48. Cohen, M.V., Baines, C.P., Downey, J.M. 2000. Ischemic preconditioning: from adenosine receptor to K_{ATP} channel. *Annu. Rev. Physiol.* 62: 79-109.47.
 49. Bonventre, J.V. 2002. Kidney ischemic preconditioning. *Curr. Opin. Nephrol. Hypertens.* 11: 43-48.
 50. Kelty, J.D., Lee, R.E. 2001. Rapid cold-hardening of *Drosophila melanogaster* (Diptera: Drosophilidae) during ecologically based thermoperiodic cycles. *J. Exp. Biol.* 204: 1659-1666.
 51. Dawe, A.R., Spurrier, W.A. 1969. Hibernation induced in ground squirrels by blood transfusion. *Science* 163: 298-299.
 52. Oeltgen, P.R., Bergman, L.C., Spurrier, W.A., Jones, S.B. 1978. Isolation of a hibernation inducing trigger(s) from the plasma of hibernating woodchucks. *Prep. Biochem.* 8: 171-188.
 53. Wang, L.C., Belke, D., Jourdan, M.L., Lee, T.F., Westly, J., Nurnberger, F. 1988. The "hibernation induction trigger": specificity and validity of bioassay using the 13-lined ground squirrel. *Cryobiology* 25: 355-362.
 54. Vybiral, S., Jansky, L. 1997. Hibernation triggers and cryogens: do they play a role in hibernation? *Comp. Biochem. Physiol. A* 118: 1125-1133.
 55. Horton, N.D., Kaftani, D.J., Bruce, D.S., Bailey, E.C., Krober, A.S., Jones, J.R., Turker, M., Khattar, N., Su, T.P., Bolling, S.F., Oeltgen, P.R. 1998. Isolation and partial characterization of an opioid-like 88 kDa hibernation-related protein. *Comp. Biochem. Physiol. B* 119: 787-805.
 56. Oeltgen, P.R., Welborn, J.R., Nuchols, P.A., Spurrier, W.A., Bruce, D.S., Su, T.P. 1987. Opioids and hibernation. II. Effects of kappa opioid u69593 on induction of hibernation in summer-active ground squirrels by "hibernation induction trigger" (HIT). *Life Sci* 41: 2115-2120.
 57. Oeltgen, P.R., Nilekani, S.P., Nuchols, P.A., Spurrier, W.A., Su, T.P. 1988. Further studies on opioids and hibernation: delta opioid receptor ligand selectively induced hibernation in summer-active ground squirrels. *Life Sci.* 43: 1565-1574
 58. Bruce, D.S., Bailey, E.C., Crane, S.K., Oeltgen, P.R., Horton, N.D., Harlow, H.J. 1997. Hibernation-induction trigger. I. Opioid-like effects of prairie dog plasma albumin on induced contractility of guinea pig ileum. *Pharmacol. Biochem. Behav.* 58: 621-625.
 59. Bruce, D.S., Bailey, E.C., Setran, D.P., Tramell, M.S., Jacobson, D., Oeltgen, P.R., Horton, N.D., Hellgren, E.C. 1996. Circannual variations in bear plasma albumin and its opioid-like effects on guinea pig ileum. *Pharmacol. Biochem. Behav.* 53: 885-889.
 60. Bolling, S.F., Tramontini, N.L., Kilgore, K.S., Su, T.P., Oeltgen, P.R. Harlow, H.H., 1997. Use of "natural" hibernation induction triggers for myocardial protection. *Ann. Thorac. Surg.* 64: 623-627.
 61. Oeltgen, P.R., Horton, N.D., Bolling, S.F., Su, T.P. 1996. Extended lung preservation with the use of hibernation trigger factors. *Ann. Thorac. Surg.* 61: 1488-1493.
 62. Su, T.P. 2000. Delta opioid peptide[D- Ala(2),D-Leu(5)]enkephalin promotes cell survival. *J. Biomed. Sci.* 7:195-199.
 63. Bolling, S.F., Benedict, M.B., Tramontini, N.L., Kilgore, K.S., Harlow, H.H., Su, T.P., Oeltgen, P.R. 1998. Hibernation triggers and myocardial protection. *Circulation* 98 (19 Suppl): II220-II224.

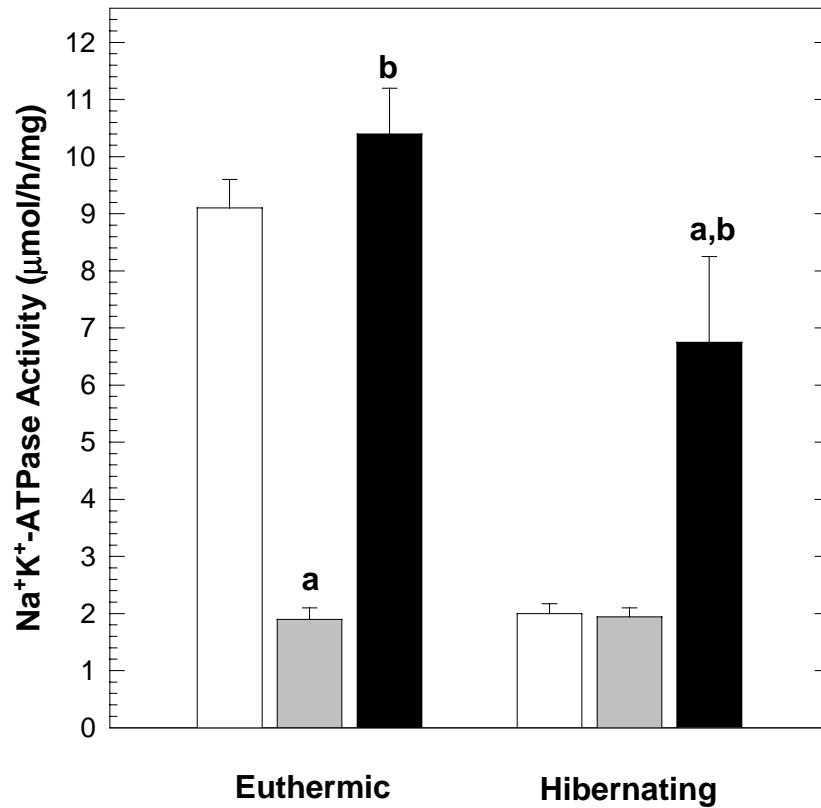


Figure 1, Storey

Figure 1. Effect of stimulating endogenous protein kinase A (incubation with 10 mM ATP, 10 mM MgCl₂, 0.3 mM cAMP) and subsequent alkaline phosphatase treatment (10 units) on Na/K-ATPase activity in skeletal muscle extracts of hibernating and euthermic ground squirrels. Bar fills are: untreated extracts (white), after protein kinase treatment (grey), after phosphatase treatment (black). Data are means \pm SEM, n=4. a - significantly different from untreated; b- from protein kinase treated, P<0.05. From MacDonald and Storey (15).

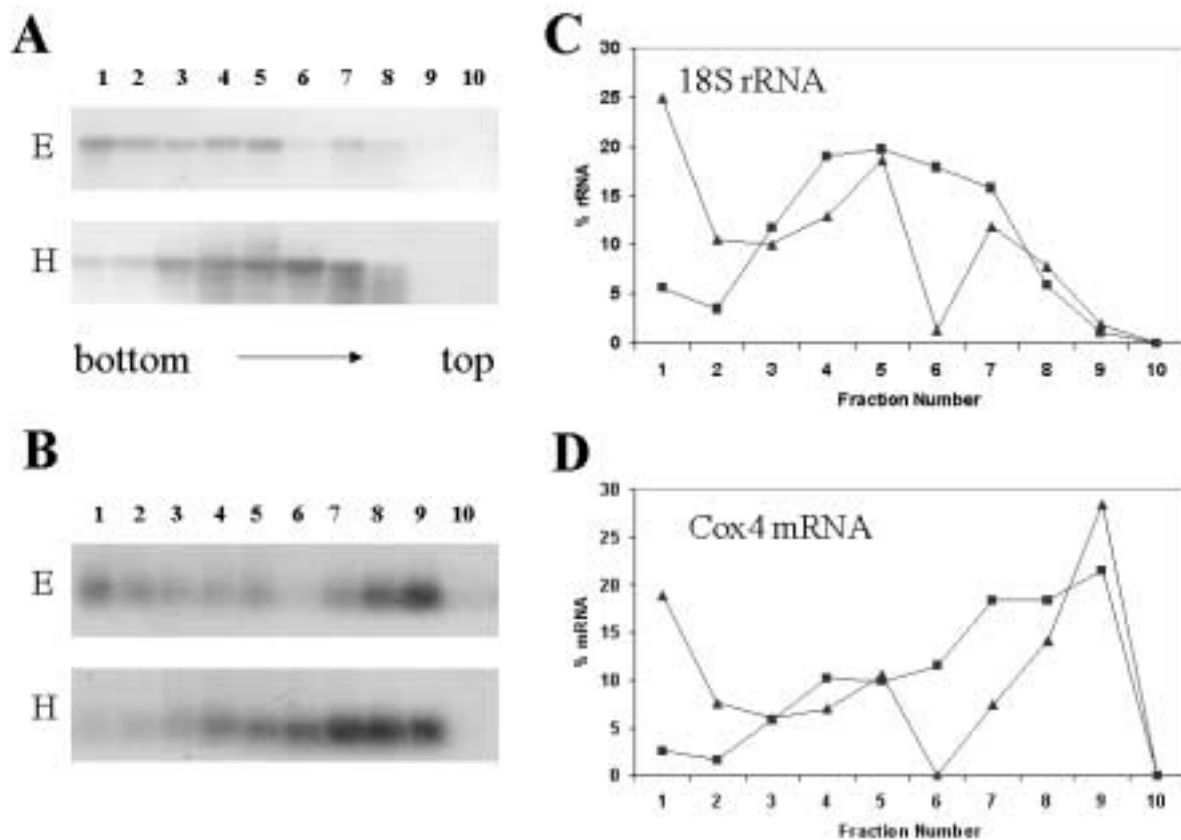


Figure 2. Effect of hibernation on the distribution of ribosomes between polysomes and monosomes and the associated distribution of *Cox4* mRNA. Soluble extracts of kidneys from euthermic (E) versus hibernating (H) ground squirrels (*S. tridecemlineatus*) were separated on sucrose gradients, followed by RNA extraction from each of 10 fractions, RNA separation on agarose gels and then blotting onto nylon membranes. Blots were hybridized with (A) 32 P-dCTP-labeled 18S rRNA to track the position of the 40S ribosomal subunit and (B) *Cox4* cDNA probe to show the distribution of *Cox4* mRNA. Blots were scanned and mRNA intensity in each lane was expressed as a percentage of the total mRNA signal in the whole gradient. Fraction numbers increase with decreasing density. Panels A and B show representative blots; panels C and D show the mean signal in each fraction for n=3 trials using tissue from different animals. Triangles show signal in euthermic kidney; squares are hibernating kidney. Data from (18).

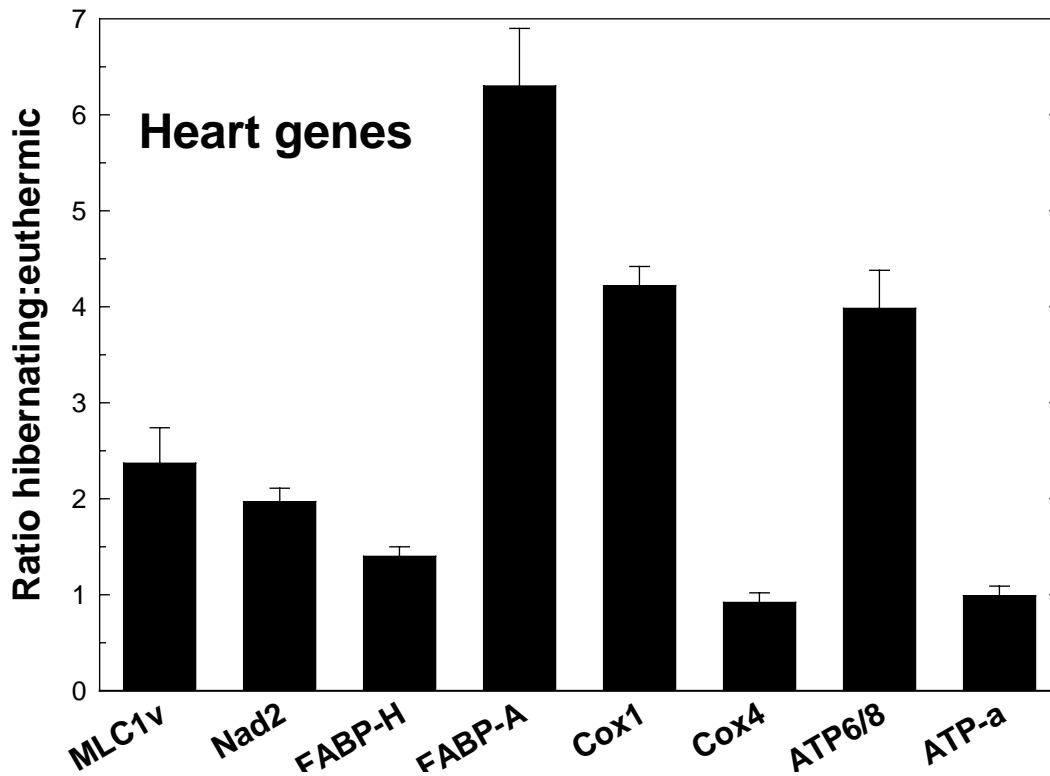


Figure 3. Histograms showing effect of hibernation on gene up-regulation in ground squirrel heart. Relative transcript levels (determined from northern blots) in hibernating versus euthermic heart are shown for eight genes: *MLC1_v*, myosin light chain 1 ventricular isoform; *Nad2*, subunit 2 of NADH-ubiquinone oxidoreductase; *FABP-H* and *FABP-A*, heart and adipose isoforms of fatty acid binding protein; *Cox1* and *Cox4*, subunits 1 and 4 of cytochrome c oxidase; *ATP6/8*, ATPase 6/8 bicistronic mRNA; and *ATP α* , alpha subunit of the mitochondrial ATP synthase. *Nad2*, *Cox1* and *ATP6/8* are mitochondria-encoded subunits and *Cox4* and *ATP α* are nuclear-encoded subunits of mitochondrial proteins. Data for *MLC1_v* and *Nad2* transcripts are from *S. lateralis* (25); all others are from *S. tridecemlineatus* (30,31). Data are means \pm S.E.M., n=3.