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Regulation of Akt during torpor in the hibernating ground squirrel, *Ictidomys tridecemlineatus*

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Abstract

The 13-lined ground squirrel (*Ictidomys tridecemlineatus*) is capable of entering into extended periods of torpor during winter hibernation. The state of torpor represents a hypometabolic shift wherein the rate of oxygen consuming processes are strongly repressed in an effort to maintain cellular homeostasis as the availability of food energy becomes limited. We are interested in studying hibernation/torpor because of the robust state of tolerance to constrained oxygen delivery, oligemia, and hypothermia achieved by the tissues of hibernating mammals. The role of the serine/threonine kinase Akt (also known as PKB) has been examined in torpor in previous studies. However, this is the first study that examines the level of Akt phosphorylation in the liver during the two transition phases of the hibernation cycle: entrance into torpor, and the subsequent arousal from torpor. Our results indicate that Akt is activated in the squirrel liver by phosphorylation of two key residues (Thr³⁰⁸ and Ser⁴⁷³) during entrance into torpor and arousal from torpor. Moreover, we observed increased phosphorylation of key substrates of Akt during the two transition stages of torpor. Finally, this study reports the novel finding that PRAS40, a component of the TORC1 multi-protein complex and a potentially important modulator of metabolism, is regulated during torpor.

Keywords

Ictidomys tridecemlineatus; Hibernation; Metabolic rate depression; Akt; PRAS40; mTOR

Introduction

The cold winter months of temperate climates can pose severe challenges for the animals inhabiting these areas. For warm-blooded animals, the defense against cold temperatures and finding enough food energy and water can be very difficult. Consequently, some small mammals and birds have adopted a strategy of regulated hypometabolism. The resulting hypometabolic shifts can range from daily light torpor bouts to winter hibernation that can last for many months (Geiser 1988; Storey and Storey 1990). The 13-lined ground squirrel, *Ictidomys tridecemlineatus* (Helgen et al. 2009), is capable of declining into a deep hibernation consisting of many torpor bouts that can last for several weeks at a time interspersed by brief periods of arousal (often less than 24 h) during which the body temperature (Tb) transiently returns to normal (Tb = 34–38°C). During torpor, the Tb of the ground squirrel can reach near ambient temperatures (0–5°C) and reduce its metabolic rate to less than 5% of its euthermic output. This strategy allows the ground squirrel to reduce its energy demands over the fall and

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winter seasons by almost 90% (Wang and Lee 1996). At temperatures that would be lethal to human cells, the ground squirrel is forced to attenuate all non-essential ATP-consuming processes in an effort to preserve cellular energy much longer than would normally be possible. Not only is hibernation interesting from a comparative biochemistry point of view, but due to the robust state of tolerance achieved by animals in torpor to profound reductions in blood flow and capacity to deliver oxygen (Frerichs and Hallenbeck 1998; Frerichs et al. 1994), it is a valuable model system for researchers aiming to improve human responses to such stresses.

Protein kinase B, also called Akt, is a serine/threonine kinase that plays a major role in many cellular processes (for reviews see Franke 2008; Huang and Manning 2009). It belongs to the AGC family of protein kinases, which also includes protein kinase A (PKA), protein kinase C (PKC), protein kinase G (PKG), phosphoinositide-dependent protein kinase-1 (PDK1), and p70S6 kinase. Akt is downstream of phosphoinositide 3-kinase (PI3K) and is activated by phosphorylation in response to insulin or insulin-like growth factor (IGF) or various cytokines that mediate the pro-growth effects of these signals. All members of the AGC protein kinase family need to be phosphorylated in the activation loop and the hydrophobic motif for maximal activity. In Akt, the phosphorylated residue located in the activation loop is Thr³⁰⁸, whereas Ser⁴⁷³ resides in the hydrophobic motif (Fig. 1). Threonine 308 is phosphorylated by PDK1 and Ser⁴⁷³ is predominantly a substrate of TORC2 ("PDK2"). TORC2 is a multi-protein complex consisting of mTOR, rictor, sin1, and mLST8 (Sarbasov et al. 2005; Jacinto et al. 2006; Reiling and Sabitini 2006). Activated Akt has many substrates playing roles in various and sometimes opposing processes such as growth/differentiation, protein synthesis (Whiteman et al. 2002), glycogen storage, lipogenesis, apoptosis (Duronio 2008), and life-extension (Paradis and Ruvkun 1998). Activated Akt can be protective in models of brain ischemia (Noshita et al. 2001).

Because of its highlighted importance in metabolism, life-extension and other functions, Akt has already been examined in selected stages of hibernation in both ground squirrel, marmots, and bats (Abnous et al. 2008; Cai et al. 2004; Fleck and Carey 2005; Hoehn et al. 2004; Eddy and Storey 2003; Lee et al. 2002). Previous results indicated that Akt activity as inferred by level of phosphorylation was generally reduced during torpor and Lee et al. (2002) found Akt activity increased in bat brain during arousal implying a decreased activity during torpor. Interestingly, Fleck and Carey (2005) found that the level of phosphorylation of Akt in the intestinal mucosa of torpid 13-lined ground squirrels was lower than in summer animals, and upon arousal levels returned to those in summer. In contrast, the total amount of Akt protein increased nearly 20-fold in this tissue during all stages of hibernation relative to summer. Hoehn et al. evaluated the seasonal regulation of Akt in the white adipose tissue of marmots from June to November. They observed a strong increase in Akt activity during the period of lipid accumulation (July and August) followed by a significant decrease in activity immediately prior to the hibernation season. To date, however, the pattern of Akt response over the entire torpor cycle from entrance to arousal has not been traced in the liver of any hibernating species. The present study examines Akt responses in liver of *I. tridecemlineatus* and shows that Akt is hyperphosphorylated during entrance into torpor and again during arousal. Moreover, this is the first study to show regulation of PRAS40 during torpor raising the possibility that TORC1 is involved in the robust state of stress (oligemia, hypothermia, constrained O₂ delivery) tolerance achieved in hibernating mammals.

Materials and methods

Animal treatment

Male and female 13-lined ground squirrels, *I. tridecemlineatus* ranging from 150 to 300 g (at the time of capture) were caught from the wild by USDA licensed trappers (TLS Research, Bartlett, IL, USA) annually in August. Animal housing and experimental procedures followed

the guidelines set by the NINDS animal care and use committee (ACUC). Once caught, the squirrels were brought to the NIH and kept in quarantine to be sure they contained no infectious agents. As a prophylactic measure, all incoming squirrels were treated with 0.05 ml/squirrel of Profender to kill any parasites (3 mg/kg emodepside + 12 mg/kg praziquantel). Each animal was briefly anesthetized with 5% isoflurane and injected subcutaneously in the intrascapular area with a sterile programmable temperature transponder (IPTT-300, Bio medic Data Systems) to allow daily monitoring of Tb without touching or disturbing the squirrel. While this system does not provide an exact measure of core Tb, it does provide a close estimate ($\pm 2-3^{\circ}\text{C}$) (data not shown); therefore, indicating the stage of hibernation in which the squirrels belonged. Ground squirrels were then housed in individual cages and fed water and standard rodent chow ad libitum. Animals were housed in the holding colony at 21°C under a 12 h light: 12 h dark cycle until used for experimental purposes. To enable a natural transition into torpor, some squirrels were transferred to constant darkness in an environmental chamber held at $4-5^{\circ}\text{C}$. A red safe light (3–5 lux) was used when entering the chamber so as not to disturb torpid squirrels. Also, a heavy dark curtain was used to shield the shelves containing the cages and block the light and sound resulting from opening and closing the door to the environmental chamber. Once a squirrel was observed to descent into torpor, a small amount of saw dust was dropped on the back of the animal to be sure it did not move (arouse) in between monitoring periods. Tissues were collected from animals at six different stages of the hibernation cycle based on Tb, time, and respiration rate. ACR = active in the cold room for at least 3 days ($\text{Tb} = 34-37^{\circ}\text{C}$). ACR animals were capable of entering torpor, but had not done so in the past 72 h and were chosen as the reference group to rule out environmental light and temperature effects. EN = entrance phase of torpor ($18^{\circ}\text{C} \leq \text{Tb} \leq 31^{\circ}\text{C}$) (animals were only harvested for an EN time point after at least two successive temperatures showed a declining Tb), ET = early torpor phase ($\text{Tb} = 5-8^{\circ}\text{C}$ for 1 day), LT = late torpor phase ($\text{Tb} = 5-8^{\circ}\text{C}$ for at least 5 days), E-AR = early arousal, spontaneously arousing from torpor with an increasing respiration rate $\geq 60/\text{min}$ and an increasing yet still reduced Tb ($\text{Tb} = 9-12^{\circ}\text{C}$). The final phase IA = interbout arousal. These animals had previously been in deep torpor, but had since returned to normal Tb ($\text{Tb} = 34-37^{\circ}\text{C}$).

To collect tissues at the desired time points, the squirrels were first anesthetized with 5% isoflurane and the rectal temperature was measured to verify the accuracy of the temperature transponder. The animal was then decapitated with a guillotine. The desired tissues were removed quickly, rinsed with ice-cold phosphate-buffered saline (PBS) and frozen instantly in 2-methylbutane chilled with dry ice (-50°C) and then stored in a -70°C freezer until use.

Western blots

Frozen livers were crushed on dry ice to create a fine powder which was then measured out into ice-cold RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate and 0.1% SDS.) supplemented with protease inhibitor (Sigma-Aldrich) and phosphatase (Pierce) inhibitor cocktails. The tissue was homogenized using a polytron homogenizer and subsequently sonicated on ice for 10 s. The sample was centrifuged at $15,000g$ for 10 min at 4°C . A small aliquot of the supernatant was used for protein quantification and the remainder was diluted with $2\times$ SDS sample buffer containing 5% β -mercaptoethanol and boiled for 5 min. Fifty micrograms of total protein was loaded and run on SDS-PAGE (4–20%) gels and transferred to PVDF for antibody probing. All antibodies were purchased from Cell Signaling Technologies (catalog numbers: total Akt-9272; P-Thr³⁰⁸ Akt-9275, P-Ser⁴⁷³ Akt-4051, P-Ser⁹ GSK3 β -9336, P-Thr³⁸⁹ S6K-9205, P-Thr^{37/46} 4E-BP1, and P-Ser235/236 S6-2211). (Cambridge, MA) except for phospho-Thr²⁴⁶ PRAS (catalog number 07-888) which was purchased from Upstate Biotechnology (Temecula, CA). Antibodies were used at a 1:1,000 dilution in PBS with 5% milk protein. Subsequently, membranes were incubated with HRP-linked anti-rabbit IgG secondary antibody (1:2,000 v:v dilution) in PBST

for 1 h and then blots were developed using the Immobilon chemiluminescent substrate solution from Millipore (Billerica, MA) according to the manufacturer's protocol. Bands were visualized using an Alpha Innotech fluorochem imaging system (San Leonardo, CA) and band densities were quantified using the associated AlphaEaseFC software. Single bands at the correct predicted molecular weights were observed in all cases.

Quantification and statistics

Western blot band intensities were standardized against β -actin. Data (means $\pm$ SEM, $n = 5-6$) were plotted relative to values for the ACR group which was arbitrarily set to equal one. Significant differences between all pair-wise combinations of timepoints were assessed using a one factor ANOVA. Tukey's honest significant differences (Tukey HSD) post hoc method was used to identify those factor-level comparisons having a significant difference (set at $P < 0.05$).

Results

Figure 2 examines the total amount of the serine/threonine protein kinase Akt, as well as the level of phosphorylation of the two known Akt phosphorylation sites Thr³⁰⁸ and Ser⁴⁷³. The total amount of Akt protein does not change significantly throughout the timecourse; however, the level of phosphorylation of the two residues necessary for full activation of Akt do show a distinct pattern of regulation. The amount of phosphoSer⁴⁷³Akt in torpid squirrel liver was roughly 50% that of the ACR animals, agreeing with previously reported studies (Abnous et al. 2008). Intriguingly, the phosphorylation of Akt in the two transition phases, entrance and arousal, increased by about 2.4- and 3.7-fold, respectively as compared to the level of phosphorylation at the ACR timepoint. The level of phosphorylation of Thr³⁰⁸ mimics the pattern of Ser⁴⁷³ phosphorylation with the levels at entrance and arousal being roughly 2.0- and 3.2-fold higher than the ACR control and an overall decrease in phosphorylation during the middle of the torpor bout (E-Hib = 57%, and L-Hib = 77% as high as samples from ACR animals). The distinct pattern of Akt activation raises the possibility of Akt involvement in the transition between the active and torpid states of the ground squirrel. Also, the pattern of regulation argues against simple temperature effects as being primarily responsible for the observed results. If in fact TORC2 is the kinase responsible for Akt phosphorylation, mTOR would also necessarily be involved in this process.

Figure 3 depicts changes in the phosphorylation states of two Akt substrates, Ser⁹GSK3 β and Thr²⁴⁶PRAS40, which mirror the pattern of Akt phosphorylation with large increases seen both in entrance and arousal. Phospho-Ser⁹GSK3 β content increased by about 2.5-fold in entrance and 2.6-fold during arousal whereas phospho-Thr²⁴⁶PRAS40 increased by about 4.5-fold in entrance and 4.6-fold in arousal as compared with active control animals (ACR). Hyperphosphorylation of GSK3 β has been shown to be anti-apoptotic in several experimental systems (Rayasam et al. 2009). PRAS40 is a relatively recently discovered protein and as such is not fully characterized. However, one of the defined roles of PRAS40 is to inhibit signaling through TORC1 by interacting with raptor (Sancak et al. 2007). Only the dephosphorylated form of PRAS40 can inhibit TORC1 (see Fig. 1). Because PRAS40 is highly phosphorylated in the squirrel liver during entrance with the level of phosphorylation decreasing as the torpor bout persists and is then highly phosphorylated again during arousal, we infer that signaling through TORC1 may be important during these stages of torpor.

Figure 4 examines the activation level of p70S6K as measured by the amount of phosphorylation of Thr³⁸⁹, a direct substrate of the mTOR serine/threonine kinase activity. This kinase is located downstream of TORC1 and is often used as a marker of TORC1 activity. The pattern of activation differs from that of phospho-Ser⁹GSK3 β and phospho-Thr²⁴⁶PRAS40 in that the level of phosphorylation showed a trend towards increased

phosphorylation during torpor; however, the differences are only significant at the ET and E-AR timepoints. The same figure also shows that phosphorylation of a well described substrate of p70S6K, ribosomal protein S6, is significantly downregulated throughout torpor. The current data does not provide a ready explanation for this discrepancy. It is possible that the kinases and phosphatases that regulate this system show differential susceptibility to changes in temperature or that the system has another level of regulation that has not been identified.

Another commonly studied substrate of TORC1 is the eukaryotic initiation factor binding protein 4E (4E-BP1). The dephosphorylated form of this protein disrupts cap-dependent translation and these effects are reversed by phosphorylation. As can be seen in Fig. 4, the trend of 4E-BP1 phosphorylation is similar to that of Akt and its substrates (PRAS40 and GSK3 β), with the highest level of phosphorylation achieved during entrance and arousal and the lowest levels seen in between these time points. The only data points significantly different from ACR were those of ET and LT, showing a strong reduction in the level of phosphorylation which agreed with a previous report on translation control in the liver of golden-mantled ground squirrels (van Breukelen et al. 2004). The data presented here strongly suggests that TORC2 is activated during entrance into torpor and during arousal from torpor and there is support for TORC1 activation during these same periods. It is unlikely that the reason for activation is for stimulation of the pro-growth and differentiation pathways normally associated with mTOR activation. In fact, there are many examples of ATP-demanding processes such as transcription and translation being attenuated during torpor in the ground squirrel (Hittel and Storey 2002; Storey 2003). Our results are no exception to this generalization. Figure 4 shows that the level of phosphorylation of the ribosomal protein S6 is strongly depressed throughout the torpor cycle, decreasing by almost 85% in late torpor as compared to ACR animals. Further studies will be necessary to unravel the mechanisms through which p70S6K in its Thr³⁸⁹ phosphorylated state is prevented from phosphorylating and activating ribosomal protein S6.

Discussion

Akt has been studied for many years and we now know a great deal about this critical regulator of cellular processes; however, much work remains before we will fully understand all complexities of this system. Akt activity drives many anabolic pathways including protein synthesis by activating initiation and elongation factors of the translational machinery (Shah et al. 2000). Akt is also known to oppose apoptosis by phosphorylating and inactivating several pro-apoptotic factors such as GSK3 β , BAD, Fork-head family members, and pro-caspase-9 (Seol 2008).

One of the ways Akt drives growth is by activating the serine/threonine kinase mTOR. The mammalian target of rapamycin is also a multifunctional regulator that controls cell growth, proliferation, metabolism, and autophagy in response to various growth factors, nutrient/amino acid levels and stress (Guertin and Sabatini 2007). The protein functions as part of at least two mutually exclusive complexes, TORC1 and TORC2 (see Fig. 1). Rapamycin-sensitive TORC1 promotes growth and translation. TORC2 is insensitive to the inhibitory effects of rapamycin except over prolonged periods of exposure and its major functions are actin remodeling and phosphorylation of Ser⁴⁷³ in the hydrophobic motif of Akt. Activated Akt is required for most signaling events going through TORC1, including those downstream of insulin. For example, the tuberous sclerosis complex (TSC1/2) restrains cell growth through its GTPase activity towards Rheb which needs to be bound to GTP in order to activate TORC1. TSC2 is phosphorylated and inhibited by Akt preventing GTP cleavage. Also, PRAS40 binds to the TORC1 complex through its TOR signaling (TOS) domain. In the absence of pro-growth stimuli such as insulin, PRAS40 binds to raptor in the TORC1 complex and prevents access of other well-studied TORC1 substrates such as S6K and 4E-BP1. However, phosphorylation of Thr²⁴⁶ by Akt releases PRAS40 from the TORC1 complex resulting in its sequestration by

14-3-3 proteins with as yet unknown functional effects (Kovacina et al. 2003). Release of PRAS40 allows binding of TORC1 substrates and propagation of pro-growth stimuli.

Given the pro-growth actions of Akt and mTOR, it might be expected that the activity of these two proteins would be decreased during torpor, a state in which most ATP-consuming processes are reduced. Indeed, this was initially found to be the case. The level of phosphorylation of Ser⁴⁷³ in Akt was found to be reduced by approximately 50% in the brain, liver, muscle, heart, and kidney of torpid 13-lined ground squirrels as compared to the same tissues from active animals (Cai et al. 2004). Based on these results the authors concluded that instead of Akt playing a direct active role in the stress resistance associated with torpor, down-regulation of Akt activity by inhibition of Ser⁴⁷³ phosphorylation was a component of the cellular regulation that confers stress tolerance during torpor. For example, the hypophosphorylation of Akt leads to the activation of FoxO transcription factors that transcribe genes for free radical scavengers such as catalase and Mn-superoxide dismutase in addition to upregulating Gadd45a (growth arrest and DNA damage response gene) expression (Kops et al. 2002; Tran et al. 2002). This is very plausible given the number of antioxidants known to be upregulated during torpor to fend off oxidative damage (Drew et al. 2002; Eddy et al. 2005; Morin and Storey 2007; Morin et al. 2008; Orr et al. 2009). Also, Fleck and Carey found that the amount of phosphorylated Akt was decreased in the intestinal mucosa in torpid squirrels as compared to summer animals (Fleck and Carey 2005). However, the total amount of Akt protein increased nearly 20-fold in torpor and the authors suggested that this may be to create a pool of Akt available for rapid phosphorylation during arousal. In a more recent study (Abnous et al. 2008), the level of phosphorylation and relative kinase activity of Akt was shown to be significantly reduced in both the liver and skeletal muscle of Richardson's ground squirrels during torpor as compared to euthermic controls. Hence, the data from the aforementioned studies pointed towards a limited role for Akt during the torpid state. However, there was a limitation to these studies as they only compared active versus torpid states and did not have access to tissues from animals representing the transition phases of entrance into torpor and arousal from torpor. The present study is the first to examine the state of Akt activation in the liver during these two critical phases of the hibernation cycle; it allows Akt to re-enter the ring as a potentially important transducer of key regulatory signals associated with mammalian hibernation. Figure 2 clearly shows that the level of phosphorylation of Akt fluctuates throughout the hibernation cycle. The results are in agreement with previous studies when looking solely at active versus torpid levels of Ser⁴⁷³ phosphorylation. What makes this study unique and exciting is the level of Akt activity observed in entrance and arousal. The data shows that regulation of Akt phosphorylation does not simply follow the change in Tb as might occur, for example, from differential temperature effects on the kinases and phosphatases regulating Akt. The entrance time point included samples from animals with Tbs that were still relatively high (Tb = 18–32°C), but in the process of falling. Conversely, the arousal time point included samples from animals with Tbs that were low (Tb = 10–12°C), but in the process of increasing. Therefore, the behavior of Akt is an example of regulation of an enzyme at different stages of the torpor/arousal cycle at temperatures well below the normal functional range of most enzymes in non-hibernating mammals. Also, the data shows correlative evidence for TORC2 involvement in this process.

In addition to simply measuring the level of phosphorylation of Akt, the levels of phosphorylation of two known direct substrates of Akt were also examined. GSK3 β is a multifunctional kinase most commonly known for its roles in glycogen storage/utilization and apoptosis. Phosphorylation of GSK3 β at Ser⁹ by Akt results in its inhibition. Under basal conditions, GSK3 β inhibits glycogen synthesis and this inhibition is attenuated upon phosphorylation. However, it is unlikely that the reason for phosphorylation of this enzyme during entrance and arousal is for glycogen storage as the squirrels stop eating prior to entering torpor and, except for the brain, tissues switch over to a primary dependence on fatty acid

metabolism to meet energy demands during torpor. Several studies have concluded that Akt inactivation of GSK3 β is cytoprotective by blocking apoptosis. For example, both in primary microglial cell cultures and in an EOC 2 microglial cell line, Akt activity was necessary to prevent apoptosis when these cells were exposed to oxygen-glucose deprivation and virtually all of the protection conferred by Akt was lost when GSK3 β was inhibited (Chong et al. 2007). The same results were found in a human hepatoma cell line where drug-induced apoptosis could be diminished by GSK3 β blockade (Alexia et al. 2006).

This is the first study to report regulation of PRAS40 during torpor in any species. Due to its recent discovery, it is likely that a more varied role for PRAS40 will emerge in the years to come. As for now, PRAS40 is known to interact with the components of the TORC1 complex. Debate continues as to whether PRAS40's primary function is to inhibit TORC1 signaling (Sancak et al. 2007; Wang et al. 2007), act as a substrate for TORC1 (Oshiro et al. 2007), or both (Thedieck et al. 2007). There is evidence for all three scenarios. What is accepted is that PRAS40 is phosphorylated at Thr²⁴⁶ by Akt and this is necessary for insulin-mediated activation of mTOR (Vander Haar et al. 2007). The situation has been further complicated by recent studies showing that PIM1, another Ser/Thr kinase implicated in several forms of cancer, can also phosphorylate PRAS40 at the Thr²⁴⁶ residue (Zhang et al. 2009). However, it should be noted that this occurred when PIM1 was overexpressed above physiological levels and this phenomenon has not been observed under in vivo conditions. It has been difficult to define a clear role for PRAS40 because of the wide variety of techniques and models used in the published studies. Results from this study represent the natural physiology of a living system that has not been manipulated. This study suggests that PRAS40 phosphorylation may be a direct result of Akt activation and that this, in turn, de-represses mTOR activity as demonstrated by increased phosphorylation of the well-characterized mTOR substrate, S6 kinase.

Because mTOR activity is normally associated with pro-growth and differentiation pathways, it may be surprising that increased mTOR activity was observed in a hypometabolic state such as hibernation. However, recent studies have linked mTOR signaling to stress and therefore may point to a pro-survival role for this enzyme. For example, hypoxia generally results in an overall decrease in mTOR activity in an effort to maintain energy homeostasis. The decrease in mTOR activity coincides with an increase in HIF1 α activity that can be blocked by the mTOR inhibitor, rapamycin (Toschi et al. 2008). This indicates that mTOR is not simply "on or off", but that mTOR-driven processes are selectively regulated. So even though the majority of mTOR related activities are decreased in stresses such as hypoxia, selected substrate(s) (such as HIF1 α) may be activated. Also, in a human prostate cancer cell line (PC-3), it was found that increased mTOR activity resulting from inactivating mutations in two endogenous inhibitors of mTOR signaling (PTEN and TSC2) rendered the cells more resistant to the deleterious effects of hypoxia (Kaper et al. 2006). It is possible that the pro-survival pathways activated in this study are similar or the same as those activated in the ground squirrel during torpor leading to the robust tolerance to stress.

The most clearly defined role of mTOR signaling is activation of protein synthesis. It is well established that during torpor most energy-consuming processes such as transcription and translation are strongly reduced (Frerichs et al. 1998; Van Breukelen and Martin 2001, and 2002; Storey and Storey 2004; Morin and Storey 2006); however, some genes and their protein products are selectively upregulated during torpor (Eddy et al. 2005; Morin and Storey 2007; Mamady and Storey 2006). The increase in mTOR activity during entrance and arousal suggested by the data in the present study may be to support efficient translation during these phases so that upregulation of a few necessary proteins can be achieved. However, one should not discount other functions of these proteins that have yet to be uncovered. Hibernating animals provide an interesting model system for studying this intricate and complex pathway without having to manipulate signal pathways pharmacologically or by knocking down or over-

expressing proteins at non-physiological levels. Because of the strong metabolic shifts that accompany the lifecycle of hibernators, this model system is ideally suited to elucidate the role of proteins linked to metabolism such as Akt, PRAS40, and mTOR. More work is needed to fully elucidate the signaling events required during the transition into and out of torpor; however, the work presented here provides insight for future studies. Also, the data presented here adds support to a hypothesis presented by Hoehn et al. that hibernators have the ability to uncouple Akt activity from circulating insulin levels in certain tissues (Hoehn et al. 2004). Finally, the results from this paper combined with those of Hoehn et al. as well as Fleck and Carey demonstrate that there are striking tissue-specific differences in the activity of Akt during the hibernation cycle and a better understanding of these systems employed by hibernators would be very valuable.

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References

- Abnous K, Dieni CA, Storey KB. Regulation of Akt during hibernation in Richardson's ground squirrels. *Biochim Biophys Acta* 2008;1780(2):185–193. [PubMed: 17983605]
- Alexia C, Bras M, Fallot G, Vadrot N, Daniel F, Lasfer M, Tamouza H, Grover A. Pleiotropic effects of PI-3' kinase/Akt signaling in human hepatoma cell proliferation and drug-induced apoptosis. *Ann N Y Acad Sci* 2006;1090:1–17. [PubMed: 17384242]
- Cai D, McCarron RM, Yu EZ, Li Y, Hallenbeck J. Akt phosphorylation and kinase activity are down-regulated during hibernation in the 13-lined ground squirrel. *Brain Res* 2004;1014(1–2):14–21. [PubMed: 15212987]
- Chong ZZ, Li F, Maiese K. The pro-survival pathways of mTOR and protein kinase B target glycogen synthase kinase-3 β and nuclear factor- κ B to foster endogenous microglial cell protection. *Int J Mol Med* 2007;19(2):263–272. [PubMed: 17203200]
- Drew KL, Tórien Ó, Rivera PM, Smith MA, Perry G, Rice ME. Role of the antioxidant ascorbate in hibernation and warming from hibernation. *Comp Biochem Physiol C Toxicol Pharmacol* 2002;133(4):483–492. [PubMed: 12458177]
- Duronio V. The life of a cell: apoptosis regulation by the PI3K/PKB pathway. *Biochem J* 2008;415(3):333–344. [PubMed: 18842113]
- Eddy SF, Storey KB. Differential expression of Akt, PPAR γ , and PGC-1 during hibernation in bats. *Biochem Cell Biol* 2003;81(4):269–274. [PubMed: 14569299]
- Eddy SF, McNally JD, Storey KB. Up-regulation of a thioredoxin peroxidase-like protein, proliferation-associated gene, in hibernating bats. *Arch Biochem Biophys* 2005;435(1):103–111. [PubMed: 15680912]
- Fleck CC, Carey HV. Modulation of apoptotic pathways in intestinal mucosa during hibernation. *Am J Physiol Regul Integr Comp Physiol* 2005;289(2):586–595.
- Franke TF. Intracellular signaling by Akt: bound to be specific. *Sci Signal* 2008;1(24):pe29. [PubMed: 18560018]
- Frerichs KU, Hallenbeck JM. Hibernation in ground squirrels induces state and species-specific tolerance to hypoxia and aglycemia: an in vitro study in hippocampal slices. *J Cereb Blood Flow Metab* 1998;18(2):168–175. [PubMed: 9469159]
- Frerichs KU, Kennedy C, Sokoloff L, Hallenbeck JM. Local cerebral blood flow during hibernation, a model of natural tolerance to "cerebral ischemia". *J Cereb Blood Flow Metab* 1994;14(2):193–205. [PubMed: 8113316]
- Frerichs KU, Smith CB, Brenner M, DeGracia DJ, Krause GS, Marrone L, Dever TE, Hallenbeck JM. Suppression of protein synthesis in brain during hibernation involves inhibition of protein initiation and elongation. *Proc Natl Acad Sci USA* 1998;95(24):14511–14516. [PubMed: 9826731]

- Geiser F. Reduction of metabolism during hibernation and daily torpor in mammals and birds: temperature effect or physiological inhibition? *J Comp Physiol B* 1988;158(1):25–37. [PubMed: 3385059]
- Guertin DA, Sabatini DM. Defining the role of mTOR in cancer. *Cancer Cell* 2007;12(1):9–22. [PubMed: 17613433]
- Helgen KM, Cole FR, Helgen LE, Wilson DE. Genetic revision of in the holarctic ground squirrel genus *Spermophilus*. *J Mammal* 2009;90:270–305.
- Hittel D, Storey KB. The translation state of differentially expressed mRNAs in the hibernating 13-lined ground squirrel (*Ictidomys tridecemlineatus*). *Arch Biochem Biophys* 2002;401(2):244–254. [PubMed: 12054475]
- Hoehn KL, Hudachek SF, Summers SA, Florant GL. Seasonal, tissue-specific regulation of Akt/protein kinase B and glycogen synthase in hibernators. *Am J Physiol Regul Integr Comp Physiol* 2004;286:R498–R504. [PubMed: 14656767]
- Huang J, Manning BD. A complex interplay between Akt, TSC2 and the two mTOR complexes. *Biochem Soc Trans* 2009;37(Pt 1):217–222. [PubMed: 19143635]
- Jacinto E, Facchinetti V, Liu D, Soto N, Wei S, Jung SY, Huang Q, Qin J, Su B. SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell* 2006;127(1):125–137. [PubMed: 16962653]
- Kaper F, Dornhoefer N, Giaccia AJ. Mutations in the PI3K/PTEN/TSC2 pathway contribute to mammalian target of rapamycin activity and increased translation under hypoxic conditions. *Cancer Res* 2006;66(3):1561–1569. [PubMed: 16452213]
- Kops GJ, Dansen TB, Polderman PE, Sarloos I, Wertz KW, Coffey PJ, Huang TT, Bos JL, Medema RH, Bergering BM. Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. *Nature* 2002;419(6904):316–321. [PubMed: 12239572]
- Kovacina KS, Park GY, Bae SS, Guzzetta EW, Schaeffer E, Birnbaum MJ, Roth RA. Identification of a proline-rich Akt substrate as a 14-3-3 binding partner. *J Biol Chem* 2003;278(12):10189–10194. [PubMed: 12524439]
- Lee M, Choi I, Park K. Activation of stress signaling molecules in bat brain during arousal from hibernation. *J Neurochem* 2002;82(4):867–873. [PubMed: 12358792]
- Mamady H, Storey KB. Up-regulation of the endoplasmic reticulum molecular chaperone GRP78 during hibernation in thirteen-lined ground squirrels. *Mol Cell Biochem* 2006;292(1–2):89–98. [PubMed: 16788740]
- Morin P Jr, Storey KB. Evidence for a reduced transcriptional state during hibernation in ground squirrels. *Cryobiology* 2006;53(3):310–318. [PubMed: 16979617]
- Morin P Jr, Storey KB. Antioxidant defense in hibernation: cloning and expression of peroxiredoxins from hibernating ground squirrels, *Spermophilus tridecemlineatus*. *Arch Biochem Biophys* 2007;461(1):59–65. [PubMed: 17362873]
- Morin P Jr, Ni J, McMullen DC, Storey KB. Expression of Nrf2 and its downstream gene targets in hibernating 13-lined ground squirrels, *Spermophilus tridecemlineatus*. *Mol Cell Biochem* 2008;312(1–2):121–129. [PubMed: 18327701]
- Noshita N, Lewén A, Sugawara T, Chan PH. Evidence of phosphorylation of Akt and neuronal survival after transient focal cerebral ischemia in mice. *J Cereb Blood Flow Metab* 2001;21:1442–1450. [PubMed: 11740206]
- Orr AL, Lohse LA, Drew KL, Hermes-Lima M. Physiological oxidative stress after arousal from hibernation in Arctic ground squirrel. *Comp Biochem Physiol A Mol Integr Physiol* 2009;153(2):213–221. [PubMed: 19233307]
- Oshiro N, Takahashi R, Yoshino K, Tanimura K, Nakashima A, Egushi S, Miyamoto T, Hara K, Takehana K, Avruch J, Kikkawa U, Yonezawa K. The proline-rich Akt substrate of 40 kDa (PRAS40) is a physiological substrate of mammalian target of rapamycin complex 1. *J Biol Chem* 2007;282(28):20329–20339. [PubMed: 17517883]
- Paradis S, Ruvkun G. *Caenorhabditis elegans* Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor. *Genes Dev* 1998;12(16):2488–2498. [PubMed: 9716402]

- Rayasam GV, Tulasi VK, Soghi R, Davis JA, Ray A. Glycogen Synthase Kinase 3: more than a namesake. *Br J Pharmacol* 2009;156:885–898. [PubMed: 19366350]
- Reiling JH, Sabatini DM. Stress and mTORC1 signaling. *Oncogene* 2006;25(48):6373–6383. [PubMed: 17041623]
- Sancak Y, Thoreen CC, Peterson TR, Lindquist RA, Kang SA, Spooner E, Carr SA, Sabatini DM. PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. *Mol Cell* 2007;25(6):903–915. [PubMed: 17386266]
- Sarbassov DD, Ali SM, Sabatini DM. Growing roles for the mTOR pathway. *Curr Opin Cell Biol* 2005;17(6):596–603. [PubMed: 16226444]
- Seol DW. Up-regulation of IAPs by PI-3K: a cell survival signal-mediated anti-apoptotic mechanism. *Biochem Biophys Res Commun* 2008;377(2):508–511. [PubMed: 18929542]
- Shah OJ, Kimball SR, Jefferson LS. Among translational effectors, p70S6k is uniquely sensitive to inhibition by gluco-corticoids. *Biochem J* 2000;347(Pt 2):389–397. [PubMed: 10749668]
- Storey KB. Mammalian hibernation transcriptional and translational controls. *Adv Exp Med Biol* 2003;543:21–38. [PubMed: 14713112]
- Storey KB, Storey JM. Metabolic rate depression and biochemical adaptation in anaerobiosis, hibernation and estivation. *Q Rev Biol* 1990;65(2):145–174. [PubMed: 2201054]
- Storey KB, Storey JM. Metabolic rate depression in animals: transcriptional and translational controls. *Biol Rev Camb Philos Soc* 2004;79(1):207–233. [PubMed: 15005178]
- Thedieck K, Polak P, Kim ML, Molle KD, Cohen A, Jeno P, Arrieumerlou C, Hall MN. PRAS40 and PRR5-like protein are new mTOR interactors that regulate apoptosis. *PLoS ONE* 2007;2(11):e1217. [PubMed: 18030348]
- Toschi A, Lee E, Gadir N, Ohh M, Foster DA. Differential dependence of hypoxia-inducible factors 1 alpha and 2 alpha on mTORC1 and mTORC2. *J Biol Chem* 2008;283(50):34495–34499. [PubMed: 18945681]
- Tran H, Brunet A, Grenier JM, Datta SR, Fornace AJ Jr, DiStefano PS, Chiang LW, Greenberg ME. DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. *Science* 2002;296(5567):530–534. [PubMed: 11964479]
- Van Breukelen F, Martin SL. Translational initiation is uncoupled from elongation at 18 degrees C during mammalian hibernation. *Am J Physiol Regul Integr Comp Physiol* 2001;281:R1374–R1379. [PubMed: 11641105]
- Van Breukelen F, Martin SL. Reversible depression of transcription during hibernation. *J Comp Physiol B* 2002;172:355–361. [PubMed: 12122451]
- Van Breukelen F, Sonenberg N, Martin SL. Seasonal and state-dependent changes of eIF4E and 4E-BP1 during mammalian hibernation: implications for the control of translation during torpor. *Am J Physiol Regul Integr Comp Physiol* 2004;287(2):R349–R353. [PubMed: 15059792]
- Vander Haar E, Lee SE, Bandhakavi S, Griffin TJ, Kim DH. Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. *Nat Cell Biol* 2007;9(3):316–323. [PubMed: 17277771]
- Wang, LCH.; Lee, TF. Torpor and hibernation in mammals: metabolic, physiological, and biochemical adaptations. In: Fregley, MJ.; Blatteis, CM., editors. *Handbook of physiology: environmental physiology*. Vol. 1. Oxford University Press; New York: 1996. p. 507-532.
- Wang L, Harris TE, Roth RA, Laurence JC Jr. PRAS40 regulates mTORC1 kinase activity by functioning as a direct inhibitor of substrate binding. *J Biol Chem* 2007;282(27):20036–20044. [PubMed: 17510057]
- Whiteman EL, Cho H, Birnbaum MJ. Role of Akt/protein kinase B in metabolism. *Trends Endocrinol Metab* 2002;13(10):444–451. [PubMed: 12431841]
- Zhang F, Beharry ZM, Harris TE, Lilly MB, Smith CD, Mahajan S, Kraft AS. PIM1 protein kinase regulates PRAS40 phosphorylation and mTOR activity in FDCP1 cells. *Cancer Biol Ther* 2009;8(9):846–853. [PubMed: 19276681]

Fig. 1.

A schematic presentation of the Akt/mTOR signaling network. Note, there are many other proteins and interactions involved in this system; however, the diagram has been simplified to contain only the interaction discussed in the contexts of this manuscript

Fig. 2.

Total Akt, phospho-Thr³⁰⁸, and phospho-Ser⁴⁷³ protein levels in the liver of the 13-lined ground squirrel. **a** Representative Western blots showing prominent bands at 60 kDa. The molecular weights correspond with those observed in other mammals. **b** Histogram showing relative phospho-protein expression in squirrels held in a cold room (4°C) and sampled at different stages of the hibernation cycle (see “Materials and methods” for more details). *ACR* active in room, *EN* entrance into torpor, *ET* early torpor, *LT* late torpor, *E-Ar* arousal from torpor, and *IA* interbout arousal. Band intensities are standardized to the ACR value for each trial and normalized to β -actin. Data are means $\pm$ SEM, $n = 5$ –6 animals per group. *Value is significantly different from corresponding ACR value, $P < 0.05$

Fig. 3.

Phospho-Ser⁹GSK3 β , and phospho-Thr²⁴⁶PRAS40 protein levels in the liver of the 13-lined ground squirrel. **a** Representative Western blots showing prominent bands at 46, and 40 kDa, respectively. The molecular weights correspond with those observed in other mammals. **b** Histogram showing relative phospho-protein expression in squirrels held in a cold room (4°C) and sampled at different stages of the hibernation cycle (see “Materials and methods” for more details). *ACR* active in room, *EN* entrance into torpor, *ET* early torpor, *LT* late torpor, *E-Ar* arousal from torpor, and *IA* interbout arousal. Band intensities are standardized to the *ACR* value for each trial and normalized to β -actin. Data are means $\pm$ SEM, $n = 5-6$ animals per group. *Value is significantly different from corresponding *ACR* value, $P < 0.05$

Fig. 4. Phospho-Thr³⁸⁹, phospho-thr^{37/46}-4E-BP1, and phospho-Ser^{235/236}-S6 protein levels in the liver of the 13-lined ground squirrel. **a** Representative Western blots showing prominent bands at 70, 18, and 32 kDa, respectively. The molecular weights correspond with those observed in other mammals. **b** Histogram showing relative phospho-protein expression in squirrels held in a cold room (4°C) and sampled at different stages of the hibernation cycle (see “Materials and methods” for more details). *ACR* active in room, *EN* entrance into torpor, *ET* early torpor, *LT* late torpor, *E-Ar* arousal from torpor, and *IA* interbout arousal. Band intensities are standardized to the ACR value for each trial and normalized to β-actin. Data are means ± SEM, *n* = 5–6 animals per group. *Value is significantly different from corresponding ACR value, *P* < 0.05