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Strategies of biochemical adaptation for hibernation in a South American marsupial, *Dromiciops gliroides*: 2. Control of the Akt pathway and protein translation machinery

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ABSTRACT

When faced with harsh environmental conditions, the South American marsupial, monito del monte (*Dromiciops gliroides*), reduces its body temperature and uses either daily torpor or multiday hibernation to survive. This study used ELISA and multiplex assays to characterize the responses to hibernation by three regulatory components of protein translation machinery [p-eIF2α(S51), p-eIF4E(S209), p-4EBP(Thr37/46)] and eight targets involved in upstream signaling control of translation [p-IGF-1R(Tyr1135/1136), PTEN(S380), p-Akt(S473), p-GSK-3α(S21), p-GSK-3β(S9), p-TSC2(S939), p-mTOR(S2448), and p70S6K(T412)]. Liver, brain and kidney were analyzed comparing control and hibernation (4 days continuous torpor) conditions. In the liver, increased phosphorylation of IGF-1R, Akt, GSK-3β, TSC2, mTOR, eIF2α, and 4EBP (1.60–1.98 fold compared to control) occurred during torpor suggesting that the regulatory phosphorylation cascade and protein synthesis remained active during torpor. However, responses by brain and kidney differed; torpor resulted in increased phosphorylation of GSK-3β (2.15–4.17 fold) and TSC2 (2.03–3.65 fold), but phosphorylated Akt decreased (to 34–62% of control levels). Torpor also led to an increase in phosphorylated eIF2α (1.4 fold) content in the brain. These patterns of differential protein phosphorylation in brain and kidney were indicative of suppression of protein translation but also could suggest an increase in antioxidant and anti-apoptotic signaling during torpor. Previous studies of liver metabolism in hibernating eutherian mammals have shown that Akt kinase and its downstream signaling components play roles in facilitating hypometabolism by suppressing energy expensive anabolic processes during torpor. However, the results in this study reveal differences between eutherian and marsupial hibernators, suggesting alternative actions of liver Akt during torpor.

1. Introduction

In coping with environmental challenges (e.g. food scarcity, cold temperatures), many small mammals use reversible entry into a hypometabolic state to enhance their chances for survival. They may employ either or both of shallow/daily torpor during their inactive hours or prolonged multi-day/week torpor (i.e. winter hibernation). The small South American marsupial, monito del monte (*Dromiciops gliroides*), can use either strategy as necessary and enter multi-day torpor in response to low temperature with the length of torpor inversely proportional to ambient temperature. For example, torpor bout duration ranged from ~10 h at 20 °C to 2 days at 17.5 °C and 5 days at 12.5 °C (Bozinovic et al., 2004). It is of interest to study hibernating marsupials because they diverged from their eutherian cousins about 90 million years ago and show several characteristics that differ from eutherians such as a lack of brown fat (Oelkrug et al., 2015). The *Dromiciops* genus is of even greater interest because it is the only extant genus of the otherwise extinct Order Microbiotheria, which holds the common ancestors of the

Abbreviations: 4EBP, eukaryotic translation initiation factor 4E-binding protein; Akt, protein kinase B; eIF2α, eukaryotic translation initiation factor 2A; eIF4e, eukaryotic translation initiation factor 4E; GSK-3α, glycogen synthase kinase 3 alpha; GSK-3β, glycogen synthase kinase 3 beta; IGF-1R, insulin-like growth factor 1 receptor; mTOR, mechanistic target of rapamycin; p70S6K, ribosomal protein S6 kinase; TOP, terminal oligopyrimidine; TSC2, tuberous sclerosis complex 2

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whole clade of Australian marsupials (Mitchell et al., 2014). Indeed, *D. gliroides* was the only species within this genus until recent work reclassified it into three allopatric species, all living in the Valdivian forests of southern Chile and adjacent areas in Argentina (D'Elía et al., 2016). Hence, monito del monte is distinct from all other South and North American marsupials. Thus, in terms of exploring the basal metabolic characteristics of mammalian torpor/hibernation and tracing their development and/or modifications over evolutionary time, *D. gliroides* could provide novel input to identify common versus distinct molecular mechanisms that used by marsupial lines as compared with eutherians.

The Akt signal transduction pathway has been linked with hibernation in several eutherian mammals including yellow-bellied marmots, ground squirrels and bats (Abnous et al., 2008; Eddy and Storey, 2003; Hoehn et al., 2004; McMullen and Hallenbeck, 2010). The pathway is of particular interest because of its involvement in the central control of fuel metabolism as well as in regulating ATP-expensive anabolic functions such as protein synthesis. Also known as protein kinase B, Akt is a serine/threonine protein kinase that is activated by an insulin-like growth factor 1 receptor (IGF-1R)-dependent mechanism (Wan et al., 2007). Akt activation leads to phosphorylation of a variety of downstream proteins, among them glycogen synthase kinase 3 alpha (GSK-3α) and beta (GSK-3β) (Cross et al., 1995). Akt is activated by phosphorylation on its Ser473 and Thr308 residues, a process that is counteracted by protein phosphatase action, notably by the phosphatase and tensin homolog (PTEN). Studies have reported Akt suppression during hibernation in bats (brain, kidney, liver, and white adipose tissue) and ground squirrels (skeletal muscle, liver, and brain), suggesting a major role for this kinase in reducing energy-expensive cellular processes such as cell growth and proliferation during torpor (Abnous et al., 2008; Cai et al., 2004; Eddy and Storey, 2003; Wu and Storey, 2012a). Akt also plays a role during transition periods of hibernation; e.g., in the brains of bats, Akt was activated during arousal from torpor, which was hypothesized to initiate neuroprotective effects during periods of high oxygen demand (Lee et al., 2002). Akt control over protein synthesis is focused on the mechanistic (mammalian) target of rapamycin (mTOR), exerting its action by controlling the phosphorylation state of TSC2 (tuberous sclerosis complex 2) protein. Increased phosphorylation of mTOR was proposed to promote muscle mass retention through stimulation of the downstream protein synthesis machinery during arousal in hibernating bats (Lee et al., 2010). Similarly, increased mTOR phosphorylation was observed in skeletal and cardiac muscles of hibernating 13-lined ground squirrels (200% and 320% of control levels, respectively) (Wu and Storey, 2012a).

The mTOR kinase regulates protein synthesis by targeting selected eukaryotic translation initiation factors. Hyperphosphorylation of eukaryotic translation initiation factor 4E-binding protein 1 (4EBP) by mTOR prevents it from interacting with the eukaryotic translation initiation factor 4E (eIF4E), and thereby lifts 4EBP inhibition to allow eIF4E to join an assembling ribosome (Gingras et al., 1999a; Scheper and Proud, 2002). Akt/mTOR also regulates eukaryotic translation initiation factor 2 (eIF2) via the mTOR downstream target, ribosomal protein S6 kinase (p70S6K) (Bodine et al., 2001). eIF2 transfers the initiating tRNA (loaded with methionine) to the ribosome in a GTP-dependent process (Sonenberg and Hinnebusch, 2009) and phosphorylation of Ser51 on its alpha subunit (eIF2α) by p70S6K or other kinases inhibits the ability to "recharge" eIF2α-GDP back to eIF2α-GTP (Sonenberg and Hinnebusch, 2009). Additionally, eIF4E is subject to Akt/mTOR-independent regulation by reversible phosphorylation. Since eIF4E facilitates protein translation by interacting with the 5′ cap structure of mRNA transcripts (Gingras et al., 1999b), phosphorylation at Ser209 dissipates the affinity between eIF4E and mRNA (Scheper and Proud, 2002; Shveygert et al., 2010).

The present study focuses on the responses of the Akt/mTOR pathway during hibernation in *D. gliroides* with an aim to identify novel elements that may support hibernation in this evolutionary-isolated species. We sought to identify common versus diverse metabolic responses by this marsupial compared to eutherian hibernators as well as follow up on the concept that liver can play a role in thermogenesis, given the absence of brown fat in marsupials (Villarin et al., 2003; Oelkrug et al., 2015). Indeed, our data on the MAPK signaling pathway in liver compared with other organs of hibernating *D. gliroides* pointed to some responses in liver that were unlike those of eutherian hibernators but could potentially indicate a cold-triggered proliferation response by liver (Wijenayake et al., 2018). Using a Luminex-based multiplex protein assay and ELISA methods, the present study characterizes the responses by eight key targets of the Akt signaling pathway (IGF-1R, PTEN, Akt, GSK-3α, GSK-3B, TSC2, mTOR, p70S6K) and 3 ribosomal proteins (4EBP, eIF2α, and eIF4E) in the liver, brain, and kidney of monito del monte, comparing control (active) and hibernating (4 d continuous torpor) conditions. By assessing torpor-responsive changes in the phosphorylation status of the various targets, the data gathered indicate a coordinated regulation of protein synthesis and activation of pro-survival responses during torpor mediated by controls at multiple loci. Interestingly, a novel response in liver was found and suggests a more active role for this tissue in hibernating marsupials compared to their eutherian counterparts.

2. Materials and methods

2.1. Animals

Adult *D. gliroides* were captured near Valdivia, Chile in January–February 2014. Animals were housed in plastic cages in a climate controlled chamber at 20 ± 1 °C with a 12 h:12 h light:dark photoperiod for two weeks and fed a mixture of mealworms and fruits with water ad libitum. Subsequently, half of the animals were sampled as controls whereas others were induced to enter torpor. This was done by gradually lowering ambient temperature over 2–3 days until 10 °C was reached (all animals were confirmed as having entered torpor by ~15 °C). Continuous torpor was confirmed by cold skin temperatures and direct observation several times daily between 09:00–17:00 (Franco et al., 2013). Animals were sampled after four days of continuous torpor and tissue samples were rapidly dissected, immediately frozen in liquid nitrogen, and subsequently packed in a dry shipper for transport to Carleton University by air where they were stored at − 80 °C. Expanded details of animal studies are available in Wijenayake et al. (2018).

All animal capture, handling, and experimental procedures were performed in Chile (Instituto de Ciencias Ambientales y Evolutivas, Facultad de Ciencias, Universidad Austral de Chile) and were conducted according to regulations set forth by the American Society of Mammalogists (Gannon and Sikes, 2007) under authorization by the Chilean Agriculture and Livestock Bureau (SAG: Servicio Agricola Ganadero de Chile, permit resolution No. 1054/2014) with protocols for animal handling and euthanasia approved by the Ethics of Animal Experiments Committee of the Universidad Austral de Chile.

2.2. Protein extraction

Sample preparation was as described in Wijenayake et al. (2018). Briefly, samples of frozen tissues (~50 mg per biological replicate) were crushed in liquid nitrogen and homogenized in ice-cold lysis buffer (Millipore, Cat. No. 43-010) in a 1:5 w/v ratio with 1 mM Na_3VO_4, 10 mM NaF, 10 mM β-glycerophosphate and 1% protease inhibitor cocktail (Cat No. PIC001, BioShop) also added. Following 30 min incubation on ice, samples were centrifuged at 12,000 × *g* for 20 min at 4 °C. Supernatants were collected and soluble protein concentrations were quantified by the Bradford assay, followed by standardizing all samples to 10 μg/μL by adding small aliquots of the lysis buffer.

2.3. Multiplex analysis

Akt and its interacting targets were characterized in liver, brain, and kidney samples using a premixed Akt/mTOR multiplex panel (EMD Millipore; Cat. No. 48-611), as previously described (Tessier et al., 2015). Initial testing of a range of sample concentrations diluted in Milliplex MAP Assay Buffer 2 (Cat. No. 43-041) confirmed cross-reactivity of *D. gliroides* samples with the kit and the appropriate dilution to use for extracts of different tissues. Manufacturer-supplied positive and negative controls were also run to assure functionality and performance of the assay. These were (a) positive controls: insulin-stimulated HepG2 cells (Cat. No. 47-227) and IGF-1-stimulated MCF7 cells (Cat. No. 47-216), and (b) negative control: HeLa cell lysates treated with dual-specificity lambda phosphatase (Cat. No. 47-229).

To conduct assays, the stock premixed magnetic beads for all protein targets were thoroughly sonicated and vortexed prior to being combined into a magnetic bead cocktail and diluted to 1 × with Assay Buffer 2. In wells of the manufacturer-provided 96-well plate, 25 µL of magnetic beads were combined with an equal volume of sample (diluted to proper concentrations) for either marsupial samples or manufacturer-provided positive and negative controls. The plate was then sealed and incubated overnight at 4 °C on an orbital shaker, protected from light. Subsequently, using a magnetic plate to retain the beads, all wells were washed twice with Assay Buffer 2, before adding 25 µL of biotin-labelled detection antibody cocktail to each sample. Samples were then incubated on an orbital shaker for 1 h at room temperature, protected from light. The antibody cocktail was then discarded and 25 µL of 1 × streptavidin-phycoerythrin (25 × SAPE stock, Cat. No. 45-001D) was added to each well. Following incubation on an orbital shaker for 15 min at room temperature, 25 µL of amplification buffer (Cat. No. 43-024A) was added to each sample, with shaking for another 15 min. The SAPE/Amplification buffer was discarded and the beads were resuspended in 150 µL of Assay Buffer 2. Samples were shaken for 5 min before data acquisition was performed on a Luminex 200 instrument (Luminex, Austin, TX). Beads were analyzed using the following instrument parameters: Events: 50 beads; Sample Size: 100 µL; Gate settings: 8000 to 15,000.

2.4. ELISA

PathScan ELISA kits (New England Biolabs, Canada) were used to quantify the relative changes in the levels of phosphorylated eIF4E (p-Ser209; Cat. No. 7938S), 4EBP (p-Thr37/46; Cat. No. 7216S) and eIF2α (p-Ser51 Cat. No. 7286S) in liver, brain and kidney samples. Experiments were performed according to the manufacturer's instructions, as previously described (Zhang et al., 2015). Using 100 µL per reaction, protein samples were diluted with the assay buffer provided with each ELISA kit, and were incubated in capture antibody-coated wells at 37 °C for 2 h. All wells were then washed three times with manufacturer-provided 1 × wash buffer, before being incubated with 100 µL of detection antibody for 1 h at 37 °C. Samples were then washed again and incubated with 100 µL HRP conjugated secondary antibodies at 37 °C for 30 min. Following a wash step, colorimetric signals were developed by adding 100 µL of tetramethylbenzidine (TMB) solution to each well for 10 min. Signal development was halted after 10 min with the addition of 100 µL stop solution, and absorbance was read at 450 nm using a Multiscan spectrophotometer.

2.5. Data and statistics

Data representing relative protein levels were captured from bead-based assays as median fluorescence intensity (MFI) and from ELISA-based assays as mean absorbance, respectively. Results from both multiplex and ELISA assays were expressed as mean ± SEM for $n = 4$ biological replicates. Significance testing used the Student's *t*-test, with $P < 0.05$ accepted as a statistically significant difference. Sigmaplot 11 software (Systat Software Inc., San Jose, CA) was used for statistical analysis and figure construction.

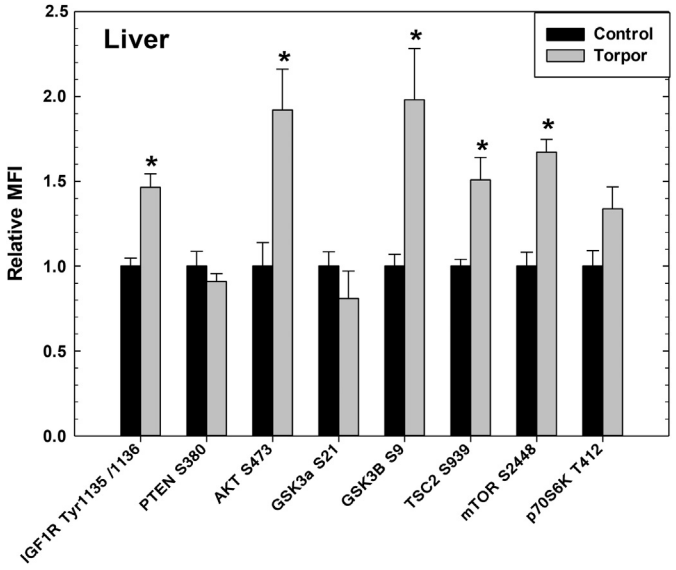

Fig. 1. Characterization of the effects of hibernation on eight targets in the Akt/mTOR pathway in liver of monito del monte comparing control (active) and torpor (4 days of continuous torpor) conditions. Relative levels of eight phosphorylated targets were assessed: p-IGF-R1 (Tyr1135/1136), p-PTEN (Ser380), p-Akt (Ser473), p-GSK-3α (Ser21), p-GSK-3β (Ser9), p-TSC2 (Ser939), p-mTOR (Ser2448), and p-p70S6K (Thr412). Data were quantified as median fluorescence intensity (MFI) for each biological replicate and are shown as mean ± SEM, $n = 4$ independent samples from different animals. For each parameter, values for the hibernating condition were expressed relative to the mean control value that was standardized to 1.0. Data were analyzed using a two-tailed Student's *t*-test. * - Significantly different from the corresponding control value, $P < 0.05$.

3. Results

Multiplex analysis was used to analyze the responses of Akt and its pathway-related targets in liver, brain, and kidney of *D. gliroides*; targets analyzed were IGF-R1, PTEN, Akt, GSK-3α, GSK-3β, TSC2, mTOR, and p70S6K. Changes in the relative phosphorylation at specific regulatory sites on these proteins were measured in control versus hibernating states: IGF-R1 (Tyr1135/1136), PTEN (Ser380), Akt (Ser473), GSK-3α (Ser21), GSK-3β (Ser9), TSC2 (Ser939), mTOR (Ser2448), and p70S6K (Thr412).

In the liver, the relative phosphorylation of five targets increased significantly during torpor. Phosphorylated IGF-R1 (Tyr1135/1136) content rose by 1.46 ± 0.08 fold, Akt (S473) increased by 1.92 ± 0.24 fold, and GSK-3β (Ser9) rose 1.98 ± 0.30 fold (all $P < 0.05$). Phosphorylation levels of p-TSC2 (Ser939) increased by 1.51 ± 0.13 fold and p-mTOR (Ser2448) rose by 1.67 ± 0.08 fold (both $P < 0.05$) in torpor (Fig. 1). In the brain, p-GSK-3β (Ser9) and p-TSC2 (Ser939) content increased by 4.17 ± 0.54 and 3.65 ± 0.48 fold (both $P < 0.05$), respectively, during torpor, whereas p-Akt (Ser473) phosphorylation decreased to 34 ± 3.0% of control values (Fig. 2). Similar to brain, kidney showed significant changes in the same three targets during hibernation with increases in p-GSK-3β and p-TSC2 levels of 2.15 ± 0.13 and 2.03 ± 0.31 fold, respectively, whereas p-Akt decreased to 62 ± 7% of control values (all $P < 0.05$) (Fig. 3).

Changes in phosphorylation status of proteins of the translation machinery also occurred during hibernation. Relative levels of phosphorylated eIF2α (Ser51), eIF4E (Ser209), and 4EBP (Thr37/46) were measured by ELISA assays. In the liver, torpor triggered significant increases in the levels of p-eIF2α and p-4EBP by 1.6 ± 0.1 and 1.6 ± 0.1 fold ($P < 0.05$), respectively (Fig. 4). Brain also showed a significant increase in p-eIF2α of 1.4 ± 0.1 fold ($P < 0.05$) as well as

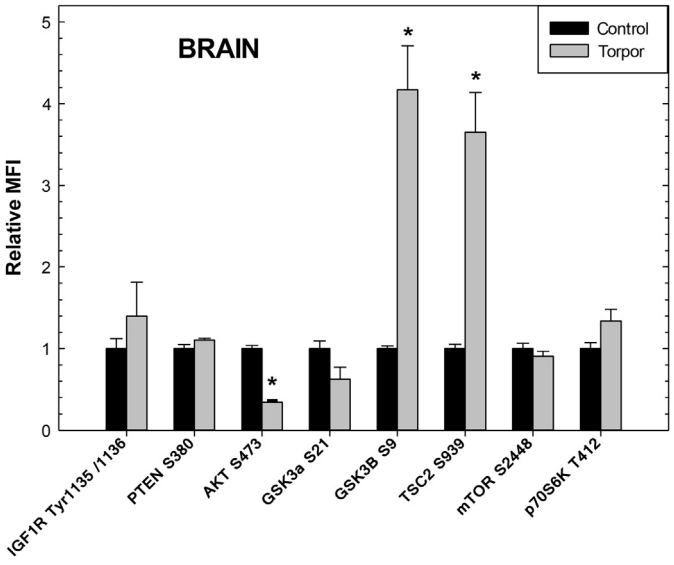

Fig. 2. Characterization of the effects of hibernation on eight targets in the Akt/mTOR pathway in brain of monito del monte comparing control and torpor conditions. Other information as in Fig. 1.

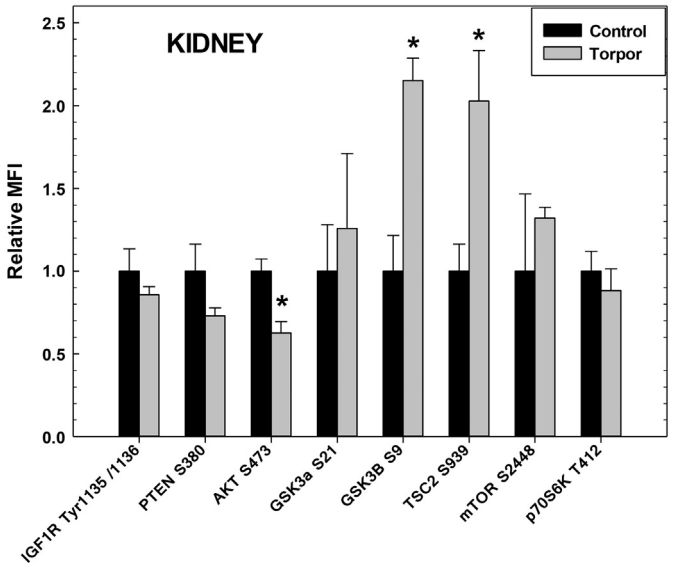

Fig. 3. Characterization of the effects of hibernation on eight targets in the Akt/mTOR pathway in kidney of monito del monte comparing control and torpor conditions. Other information as in Fig. 1.

an upward trend in p-4EBP (Fig. 5). However, no significant changes by any of the three targets were recorded in kidney (Fig. 6).

4. Discussion

The present study investigated the phosphorylation status of key factors connected to the Akt pathway and protein translation machinery in tissues of control versus hibernating monito del monte. Overall, the results indicated that torpor-induced modulation of Akt and its related targets is tissue-specific and potentially plays multiple roles in facilitating hibernation in this species. Liver showed concerted responses by most targets during hibernation. The increase in relative phosphorylation of IGF-1R (Tyr1135/1136) in liver, compared to controls, suggests that the insulin-signaling cascade is modulated upstream of Akt during torpor to potentially influence a wide range of downstream targets (Fig. 1). Interaction of IGF-1R with its ligand IGF-1 results in autophosphorylation and activation of the receptor (Li and Miller, 2006).

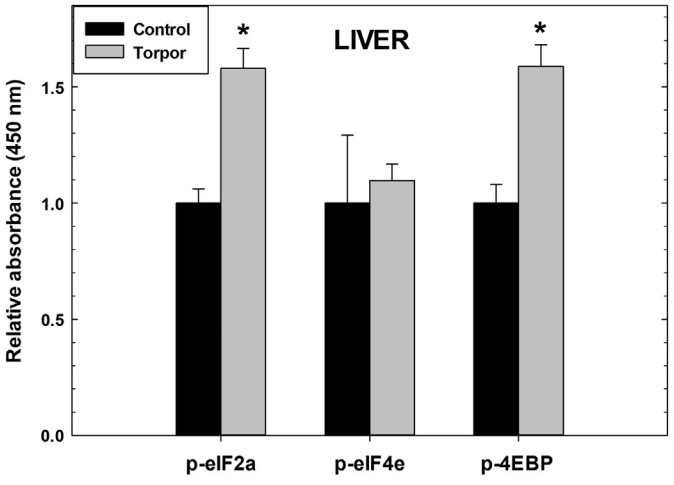

Fig. 4. Effects of hibernation on three targets of the protein translation machinery in monito del monte liver comparing control (active) and torpor conditions. Protein levels of p-eIF2α (Ser51), p-eIF4E (Ser209), and p-4EBP (Thr37/46) were assessed. Antibody-based ELISAs quantified the relative abundance of target proteins via spectrophotometric readings of sample absorbance at 450 nm. Data for hibernator samples were then expressed relative to their respective control values that were standardized to 1.0. Data are mean ± SEM, $n = 4$ independent biological replicates. Asterisks (*) denote a statistically significant difference between control and torpor conditions as determined with the Student's two-tailed *t*-test, $P < 0.05$.

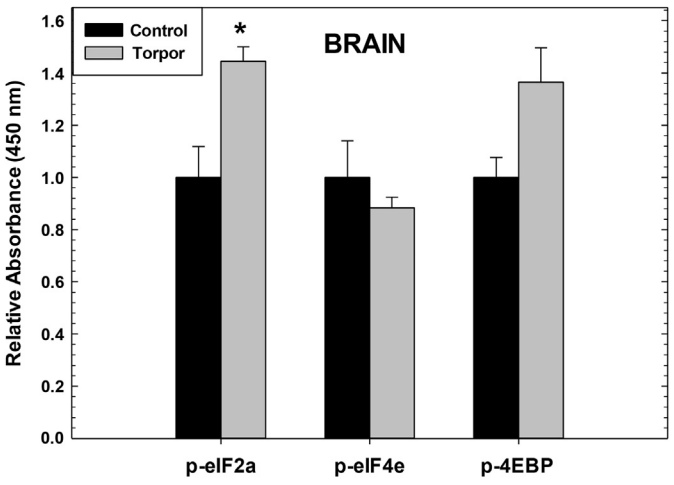

Fig. 5. Effects of hibernation on three targets of protein translation machinery in monito del monte brain comparing control and torpor conditions. Other information as in Fig. 4.

Active p-IGF-1R then initiates a signal transduction response that includes phosphoinositide 3-kinase (PI3-K), that in turn promotes phosphorylation and activation of Akt.

Importantly, IGF-1/IGF-1R and downstream targets (PI3-K/Akt) are known to be involved in the phosphorylation of GSK-3β at serine 9 which acts to inhibit its protein kinase activity (Fang et al., 2000; Fukumoto et al., 2001). Our results showed that the p-GSK-3β (Ser9) levels increased substantially in all three organs analyzed (Figs. 1–3), thereby providing good confirmation of an operational IGF-1/IGF-1R → PI3-K/Akt → GSK-3β signal transduction pathway during hibernation. GSK-3β not only regulates glycogen synthase activity but controls a wide range of other downstream proteins including transcription factors, metabolic enzymes, and receptor proteins that are linked to various cellular events (Plyte, 1992). Hence, the inhibitory phosphorylation of GSK-3β may facilitate (alone or in partnership with other regulatory proteins) broad, coordinated responses by multiple facets of metabolism during torpor that includes changes in gene expression, cell proliferation, and metabolic targets including glycogen

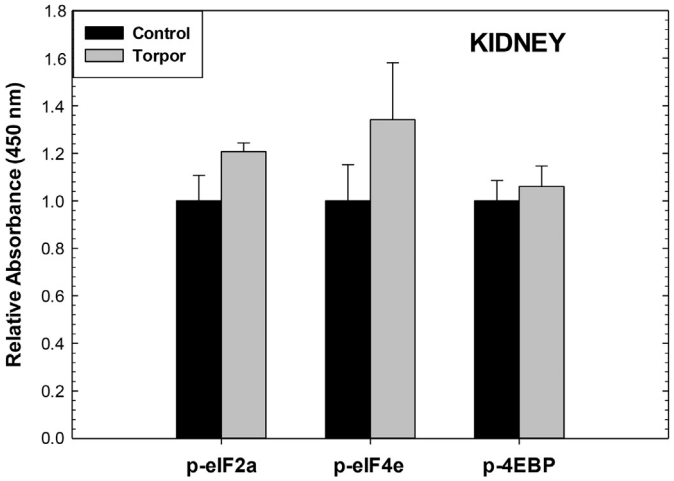

Fig. 6. Effects of hibernation on three targets of protein translation machinery in monito del monte kidney comparing control and torpor conditions. Other information as in Fig. 4.

metabolism. The strongest torpor-induced suppression of GSK-3β occurred in the brain of hibernating monito del monte (Fig. 2). Studies with rats found that inhibition of GSK-3β provided a neuroprotective effect against ischemia/reperfusion by reducing oxidative stress, apoptosis, and inflammation (Collino et al., 2008). Other studies using ischemic stroke models demonstrated that inhibition of GSK-3β attenuated infarction and improved recovery, likely by stimulating anti-apoptotic pathways and neuroprotective genes such as brain-derived neurotrophic factor and heat shock protein 70 (Chuang et al., 2011). The substantial decrease of GSK-3β activity that would result from increased phosphorylation of this protein kinase at Ser9 may be an indication of similar roles for the enzyme during torpor in marsupials. Other studies have also associated the inhibition of GSK-3β with increased antioxidant signaling in tissues other than brain, including in liver and kidney (Li et al., 2015; Wang et al., 2016). Hence, our results show an activation of the IGF-1R/Akt/GSK-3β signaling cascade during torpor in the marsupial liver, as well as a multi-tissue role for GSK-3β in the brain and kidney that may facilitate antioxidant responses. An up-regulation of antioxidant potential could also assist monito del monte in dealing with oxidative stress, particularly in association with the arousal phase from hibernation. In eutherian hibernators, arousal is well known as a time of high oxidative stress and high consumption of antioxidants associated with the huge increase in oxygen consumption needed for nonshivering thermogenesis (Storey, 2010). The same may be true of the liver in this study which is thought to be the organ that facilitates thermogenesis in these animals in the absence of brown fat (Villarin et al., 2003; Oelkrug et al., 2015).

Along the Akt signaling pathway is TSC2, which together with GSK-3β, were the only two proteins that showed consistent increases in phosphorylation during hibernation in all three organs tested. TSC2 mediates the influence of multiple signals (e.g. low energy, low amino acids, growth, cytokine and stress signals, etc.) onto mTORC1 that in turn regulates the protein translation machinery (Huang and Manning, 2008). TSC2 also mediates the translational repression of TOP (terminal oligopyrimidine) mRNAs that encode proteins of the ribosome translation machinery (Miloslavski et al., 2014). For example, knockout studies showed that TSC2 plays a crucial role in hypoxia-induced inhibition of TOP mRNA translation (Miloslavski et al., 2014). This shows that the control of protein synthesis is not just at the level of regulating the translation process but also encompasses the production of the ribosomal components. It is unlikely that hibernating animals are under hypoxic stress. However, a general suppression of anabolic processes is expected, which includes the inhibition of protein synthesis, a response that is an integral part of metabolic rate depression in response to environmental stress by many species (Storey and Storey, 2004). Hence, it is probable that TSC2 has a major regulatory role to play in mediating a reduction in energy-expensive protein synthesis (and thereby various growth & proliferation actions, etc.) in order to conserve fuel reserves for vital processes during torpor. Not surprisingly, then, in all three marsupial tissues, the phosphorylation state of p-TSC2 (Ser939) increased significantly during hibernation (Figs. 1–3).

Our results also show that reversible phosphorylation regulation of Akt and mTOR in monito del monte is tissue-specific during torpor. In the brain and kidney, a decrease in Akt phosphorylation (Ser473) suggests that Akt kinase activity is suppressed during torpor (Figs. 2, 3), consistent with previous observations for the same tissues of eutherian hibernators (Cai et al., 2004; Eddy and Storey, 2003). Akt is also known to directly phosphorylate the cell cycle inhibitors p21 and p27 (Xu et al., 2012) and cell cycle machinery is known to be suppressed during torpor in other hibernators (Wu and Storey, 2012b). Hence, reduced Akt phosphorylation in the brain and kidney of monito del monte may activate p21 and p27 by relieving inhibitory phosphorylation and thereby contribute to cell cycle suppression in the hibernating state. Interestingly, whereas phosphorylated active Akt content is reduced in liver of eutherian hibernators (bats, ground squirrels) (Eddy and Storey, 2003; Abnous et al., 2008), it rose strongly in liver of torpid monito del monte together with key upstream and downstream targets of the Akt pathway such as IGF-1R, GSK-3β, and mTOR (Fig. 1). The full consequences of enhanced liver Akt activity remain to be determined but two intriguing possibilities exist. The first relates to thermogenesis. The classic thermogenic tissue, brown fat, is largely absent from marsupial species, including monito del monte (Cortés et al., 2014; Oelkrug et al., 2015), thereby raising a question about how marsupials rewarm themselves from torpor. Although passive rewarming as ambient temperature rises or muscle shivering could contribute, studies of *Monodelphis domestica* (the North American opossum) pointed to liver as a possible thermogenic organ. Sustained cold exposure elevated opossum liver oxidative capacity; liver mass increased by ~50% and liver mitochondrial volume rose ~20% (Villarin et al., 2003). Notably, Akt was uniquely activated in brown adipose tissue of bats during hibernation but not in six other tissues (Eddy and Storey, 2003), suggesting its link to thermogenesis and supporting an idea that Akt activation in monito del monte liver may have the same function. The second possible consequence for elevated Akt in *D. gliroides* liver could be an anti-apoptotic action. Akt phosphorylation of the pro-apoptotic protein BAD suppresses apoptosis and promotes cell survival (Datta et al., 1997). Furthermore, although mTOR phosphorylation was unchanged in monito del monte brain and kidney, it was elevated in the liver, suggesting activation of mTOR function during hibernation (Fig. 1). Taken together, both Akt and mTOR activation in liver could be associated with proliferation of liver mass and oxidative capacity (as in opossums) as well as with inhibiting apoptosis in a thermogenic organ.

Although the present brain and kidney data failed to show any p-mTOR or p-4EBP-associated consequences to the elevated levels of p-TSC2, such a trend was present for the liver. An increase in both p-mTOR and p-4EBP levels indicated that liver has an active role during torpor (Fig. 4). mTOR phosphorylates 4EBP in both simple and complex eukaryotes (Gingras et al., 1999a, 1999b) and since phosphorylation of 4EBP at Thr37/46 suppresses its ability to bind eIF4E, this promotes translation initiation by allowing eIF4E to bring mRNA into the forming ribosome. Furthermore, the level of Ser51 phosphorylated eIF2α was augmented in both brain and liver during torpor (Figs. 4 and 5). Although phosphorylation of this residue could suggest a repression of protein translation in these tissues, other studies have suggested additional functions in cellular stress survival. For example, enhanced phosphorylation of this residue reportedly resulted in a higher tolerance to oxidative stress (Rajesh et al., 2013), whereas another study demonstrated that this modification of eIF2α was key in overriding apoptosis in prolonged glucose-deficient environments by triggering the expression of proteins and activation of anti-apoptotic pathways (Muaddi et al., 2010). Our results, then, may be evidence of such eIF2α

functional adaptations during hibernation in marsupials. Due to their pivotal roles in regulating protein synthesis and cell survival pathways, the adjusted phosphorylation status of 4EBP and eIF2α are likely crucial to survival for monito del monte during torpor.

5. Conclusions

Studies of eutherian hibernators have demonstrated that reversible protein phosphorylation is a crucial mechanism by which cellular processes are differentially controlled when animals cycle through bouts of torpor and arousal. The present study characterized the phosphorylation state of key targets in the Akt pathway in liver, brain and kidney of *D. gliroides* producing results that were indicative of changes in kinase signaling pathways, and suppression of TOP mRNA translation in the brain and kidney during torpor. In addition, the data indicated active functions in the liver that could promote enhanced protein synthesis during marsupial hibernation as indicated by increased levels of phosphorylated proteins involved in mRNA translation. This response is contrary to that seen in liver of eutherian hibernators. The results also showed potential regulation of neuroprotection, antioxidant pathways, autophagy and anti-apoptosis responses to the challenges involved in descending into and arising out of torpor, and could also be involved in a cold-induced elevation of liver thermogenic capacity. For the first time, our study demonstrated that Akt and related downstream pathways participate in facilitating molecular and cellular adaptations in marsupial tissues during torpor.

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