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Strategies of biochemical adaptation for hibernation in a South American marsupial *Dromiciops gliroides*: 1. Mitogen-activated protein kinases and the cell stress response

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ABSTRACT

Hibernation is a period of torpor and heterothermy that is typically associated with a strong reduction in metabolic rate, global suppression of transcription and translation, and upregulation of various genes/proteins that are central to the cellular stress response such as protein kinases, antioxidants, and heat shock proteins. The current study examined cell signaling cascades in hibernating monito del monte, *Dromiciops gliroides*, a South American marsupial of the Order Microbiotheria. Responses to hibernation by members of the mitogen-activated protein kinase (MAPK) pathways, and their roles in coordinating hibernator metabolism were examined in liver, kidney, heart and brain of control and versus hibernating (4 days continuous torpor) *D. gliroides*. The targets evaluated included key protein kinases in their activated phosphorylated forms (*p*-ERK/MAPK 1/2, *p*-MEK1, *p*-MSK1, *p*-p38, *p*-JNK) and related target proteins (*p*-CREB 2, *p*-ATF2, *p*-c-Jun and *p*-p53). Liver exhibited a strong coordinated response by MAPK members to hibernation with significant increases in protein phosphorylation levels of *p*-MEK1, *p*-ERK/MAPK1/2, *p*-MSK1, *p*-JNK and target proteins c-Jun, and *p*-ATF2, all combining to signify a strong activation of MAPK signaling during hibernation. Kidney also showed activation of MAPK cascades with significant increases in *p*-MEK1, *p*-ERK/MAPK1/2, *p*-p38, and *p*-c-Jun levels in hibernating animals. By contrast, responses by heart and brain indicated reduced MAPK pathway function during torpor with reduced phosphorylation of targets including *p*-ERK/MAPK 1/2 in both tissues as well as lower *p*-p38 and *p*-JNK content in heart. Overall, the data indicate a vital role for MAPK signaling in regulating the cell stress response during marsupial hibernation.

1. Introduction

Daily torpor and/or seasonal hibernation have been documented in a wide variety of mammalian species including in monotremes, marsupials and eutherians (Heldmaier et al., 2004; Ruf and Geiser, 2015). Characteristics of torpor include reductions in core body temperature (T_b), basal metabolic rate, and the rates of multiple physiological parameters including heartbeat, respiration, and organ perfusion (Carey et al., 2003; Nespolo et al., 2010; Storey, 2010). Although hibernation is best known as a response to cold winter temperatures and has been best studied among obligate seasonal eutherian hibernators, extended periods of torpor in many mammalian species can also be triggered in response to food limitation, low internal body fuel supplies, heat, drought, and climatic or natural disasters (Geiser, 1988; Nowack et al., 2017).

The South American monito del monte, *Dromiciops gliroides* (Microbiotheria, Microbiotheriidae), is a small omnivorous marsupial (Greer, 1965; Marshall, 1978; Martin, 2010) that is known to hibernate during the austral winter (Greer, 1965; Grant and Temple-Smith, 1987; Bozinovic et al., 2004) but also undergoes daily or even multi-day

Abbreviations: CREB, cAMP response element binding protein; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; p38, p38 protein kinase; TNF-α, tumor necrosis factor α; MSK1, mitogen- and stress-activated protein kinase 1; MEK1, MAPK/ERK kinase 1; ATF-2, activating transcription factor 2

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torpor at other seasons, depending on ambient temperature (Bozinovic et al., 2004; Nespolo et al., 2010). Native to the Valdivian forest of southern Chile and the surrounding area of western Argentina, this species is phylogenetically grouped with the Australian marsupials, unlike all other North or South American marsupials. Long thought to be a "living fossil" as the only extant species of the otherwise extinct Order Microbiotheria, a recent reexamination concluded that *D. gliroides* should be separated into three morphologically- and geographically-distinct species with *D. gliroides* being retained as the species name in the southern half of the *Dromiciops* range (D'Elia et al., 2016).

The physiology and biochemistry of hibernating marsupials is of great interest as a means of understanding the evolution of mammalian hypometabolism and heterothermy, its progression from an ectothermic ancestry, the new mechanisms added as endothermy more fully developed, and the common versus diverse traits of torpor/hibernation in marsupial versus placental mammals. Understanding the common principles of hibernation, as well as any substantial differences, that exist in these sister groups may help us delineate those mechanisms that are most fundamental to torpor/hibernation and its use as a survival strategy (Nespolo et al., 2011). Furthermore, exploration of the mechanisms and regulation of hibernation in diverse mammalian groups can help to improve our knowledge of the crucial molecular mechanisms that could be applied to advance medical organ preservation methods.

Nespolo et al. (2010) proposed that ambient temperature was the main determinant of torpor in *D. gliroides* but that energetics and food scarcity were also factors. Hibernation appears to be opportunistic in *D. gliroides* in response to changing biotic and abiotic factors rather than a circannual or seasonal obligation as occurs in many placental mammals. Furthermore, Cortés et al. (2009, 2014) showed that even during normothermia *D. gliroides* exhibits poor thermoregulatory ability, a relatively low body temperature, low thermal conductance, and a metabolic rate lower than expected for other South American marsupial species. However, torpid *D. gliroides* have been shown to reduce their metabolic rate by up to 92% compared with euthermic counterparts at 20 °C (Bozinovic et al., 2004; Nespolo et al., 2010). Studies of other hibernators have shown that the regulation of metabolic rate is pivotal during times of low food availability and adverse weather conditions and entails a carefully balanced suppression of many energy-expensive cellular processes including transcription, protein synthesis and degradation, transmembrane ion transport, and cell division (Wu and Storey, 2012; Storey and Storey, 2007). At the same time, however, various genes/proteins are upregulated that enhance cytoprotection including those involved in antioxidant defense, chaperone action, and anti-apoptosis, as well as a variety of tissue-specific regulatory mechanisms such as muscle remodeling (atrophy vs hypertrophy) (Eddy and Storey, 2002; Storey and Storey, 2004; Storey, 2010; Tessier and Storey, 2010; Rouble et al., 2013). All such metabolic reorganizations are triggered, regulated and coordinated via intracellular signal transduction pathways responding to environmental and/or extracellular cues. The present study explores how the mitogen activated protein kinases (MAPKs) are regulated during metabolic adaptation for hibernation in a marsupial species.

The MAPK cascades are major signaling pathways in cells. They are highly conserved (Widmann et al., 1999) and are known components of adaptive cellular responses to daily torpor and hibernation in eutherian mammals including ground squirrels, bats and lemurs (MacDonald and Storey, 2005; Zhu et al., 2005; Eddy and Storey, 2007; Biggar et al., 2015). MAPK phosphorylation of serine, threonine, or tyrosine residues on target proteins can affect their activity, function, allosteric regulation, interactions with other proteins, subcellular localization, or susceptibility to degradation (Johnson and Lapadat, 2002; Biggar et al., 2015). Three main MAPK families exist: the extracellular signal-regulated protein kinases (ERK1 and ERK2), the p38 family of kinases (p38α/β/γ/δ), and the c-Jun amino-terminal kinases (JNK1/2/3) (Chang and Karin, 2001; Johnson and Lapadat, 2002; Cargnello and Roux, 2011). MAPKs themselves are activated via phosphorylation by upstream MAPK kinases (MAPKKs) that in turn are regulated upstream by MAPKK kinases (MAPKKKs) that respond to extracellular or stress signals. These multi-tiered pathways plus extensive crosstalk between members increase the complexity and plasticity of MAPK signaling as well as provide a mechanism for rapid signal amplification (Chang and Karin, 2001; Johnson and Lapadat, 2002; Cowan and Storey, 2003). The ERK1/2 pathway is generally associated with mitogenic (pro-growth) signals to control/modulate events such as cell division, proliferation and differentiation. By contrast, the p38 and JNK families respond primarily to external stimuli such as hormones, ligands for G-protein coupled receptors, tumor necrosis factor alpha (TNF-α), inflammatory cytokines and a wide range of abiotic stresses (e.g. osmotic or heat shock, oxidative stress, ionizing radiation, DNA-damaging reagents, ischemia) (Johnson and Lapadat, 2002; Cowan and Storey, 2003; Cargnello and Roux, 2011).

Given the important role of MAPKs in the regulation of pro-growth signals and the stress response to abiotic signals, the involvement of MAPK pathways in hibernation by placental mammals (Zhu et al., 2005; MacDonald and Storey, 2005; Biggar et al., 2015), and the evolutionary position of *Dromiciops*, the present study explored responses by various members of the MAPK superfamily and selected effector protein targets to hibernation in *D. gliroides*. The data show a pattern of tissue-specific activation of MAPK pathways in liver, kidney, heart and brain of monito del monte in response to 4 days of continuous torpor. Overall, activation of MAPK signaling appears to be an efficient mechanism for activating and coordinating the cellular stress response and molecular mechanisms that support hibernation in this marsupial.

2. Materials and methods

2.1. Animal protocol

All animal capture, handling, and maintenance procedures were performed in Chile at the Instituto de Ciencias Ambientales y Evolutivas, Facultad de Ciencias, Universidad Austral de Chile and conducted in accordance with the regulations set forth by the American Society of Mammologists (Sikes et al., 2011) and authorized by the Chilean Agriculture and Livestock Bureau (SAG: Servicio Agricola Ganadero de Chile permit resolution No. 1054/2014). Adult *D. gliroides* were captured near Valdivia, Chile in January–February 2014 (39°48′S, 73°14′W). Modified tomahawk traps containing bananas and yeast were set up ~1 m above the ground in trees and shrubs. Captured animals were transported to the laboratory where they were housed in plastic cages (45 × 30 × 20 cm) with 2 cm of bedding and held in a climate controlled chamber (PiTec Instrument, Chile) at 20 ± 1 °C with a 12 h: 12 h light:dark photoperiod for two weeks. Water and food (mealworms and fruits) were available ad libitum. Animals were then randomly divided into control or torpor groups and control animals were sampled from these conditions.

The torpor group was transferred into a controlled climate chamber and exposed to a gradual decrease in ambient temperature over two to three days until a stable ambient temperature of 10 °C was reached. Although these studies were conducted during the late austral summer, *D. gliroides* readily enters torpor in response to reduced ambient temperature at any season. Furthermore, 10 °C is a regularly encountered parameter in nature, as it is the approximate mean temperature over the winter months at the capture site (with lows down to ~5 °C) (Franco et al., 2017). Incidence of torpor was monitored with direct observation and by cold skin temperature several times daily between 09:00–17:00, as previously described (Franco et al., 2013). All individuals had entered torpor within 24 h (before the time that ambient temperature had reached 15 °C) and animals remained in torpor continuously for four days until they were sampled. Food and water were available but animals did not arouse during this time. Both control and

torpid *D. gliroides* were euthanized according to protocols approved by the Ethics of Animal Experiments Committee of the Universidad Austral de Chile. Tissue samples were quickly dissected, flash frozen in liquid nitrogen, and subsequently packaged in a dry shipper and air freighted to Carleton University.

2.2. Protein extractions

Samples of frozen tissues (30–50 mg) were homogenized 1:5 w/v in pre-chilled 1 × lysis buffer (EMD Millipore, Cat# 43–040, provided with the Millipore multiplex panels) with protein phosphatase inhibitors (1 mM Na_3VO_4, 10 mM NaF, 10 mM β-glycerophosphate), and 10 μL/mL of protease inhibitor cocktail (104 mM AEBSF, 80 μM aprotinin, 4 mM bestatin, 1.4 mM E-64, 2 mM leupeptin, 1.5 mM pepstatin A) using a Dounce homogenizer. Homogenates were held on ice for 30 min, vortexed every 10 min, and then centrifuged at 12,000 × *g* for 20 min at 4 °C. Supernatants were removed and total soluble protein concentrations were determined using the Bradford assay (BioRad, Cat# 500-0006) with bovine serum albumin as the standard. Lysates were standardized to a final concentration of 10 μg/μL using the 1 × with added phosphatase and protease inhibitors (as described above) and stored at − 80 °C for further use. Another round of Bradford assay was conducted on the normalized samples to confirm the concentration of 10 μg/μL immediately before use.

2.3. Multiplex assays

The multiplex immunoassay kits used were (a) 10-plex MAPK/SAPK Signaling Magnetic Bead Panel (Millipore, Cat# 48-660MAG), (b) Magnetic Bead MAPmate to analyze the phosphorylated form of the cAMP response element binding protein, CREB (Ser133) (Millipore, Cat# 46-631MAG), and (c) Early Apoptosis Magnetic Bead 7-plex Kit to analyze phospho-p53 (Ser46) (Millipore, Cat# 48-6669MAG). All of these have been tested by the manufacturer and shown to cross-react with mammalian species. Assays were performed according to manufacturer's instructions. Briefly, aliquots of standardized protein lysates (10 μg/μL) were diluted with Assay Buffer 2 (Millipore, Cat# 43–041) provided in each kit to a final concentration of 1.4 μg/μL (*p*-MAPK/SAPK Kit + *p*-CREB) or 1 μg/μL (*p*-p53). Aliquots containing 35 μg of total protein were added to wells for the *p*-MAPK/SAPK and *p*-CREB assays, and 25 μg of total protein was added for the *p*-p53 assay. Manufacturer-supplied designated control samples were also run to assure functionality and performance of each assay plate. These were (a) for the MAPK/SAPK Kit: unstimulated HeLa cells (Millipore, Cat# 47-205), HS/Ars-treated HeLa cells (Millipore, Cat# 47-211), anisomycin-treated NIH/3T3 cells (Millipore, Cat# 47219), and EGF-treated A431 cells (Millipore, Cat# 47-210); (b) for the *p*-CREB MAPmate: HeLa cells treated with TNFα and calyculin A (Millipore, Cat# 47-230); and (c) for the Early Apoptosis Kit: HeLa cells treated with lambda phosphatase (Millipore, Cat# 47-229), Jurkat cells treated with anisomycin (Millipore, Cat# 47-207), A549 cells stimulated with camptothecin (Millipore, Cat# 47-218), and Jurkat cells treated with paclitaxel (Millipore, Cat# 47-220).

For the MAPK + *p*-CREB assays, a 1 × magnetic beads suspension was prepared by first sonicating a 20 × stock for 15 s and vortexing for 30 s. The beads were then diluted to 1 × by adding 150 μL of beads from the MAPK/SAPK stock and 150 μL of beads from the *p*-CREB MAPmate stock to a mixing vial and adjusting final volume to 3 mL using Assay Buffer 2. The 1 × bead suspension was further vortexed prior to use. For assays, 25 μL of Assay Buffer 2 was added to blank wells, 25 μL of sample lysates were added to test wells, and 25 μL of manufacturer controls were added to control wells. Then 25 μL of 1 × magnetic bead suspension was added to each well and the plate was incubated overnight at 4 °C on a plate shaker shielded from light. The liquid contents of wells were then removed using a handheld magnetic separation block, and each well was then washed twice with 100 μL of Assay Buffer 2. Subsequently, 25 μL of 1 × detection antibody (Millipore, Cat# 44-660MAG) was added to each well and incubated on a plate shaker for 1 h at room temperature (RT). Well contents were then decanted using the magnetic separation block and 25 μL of 1 × Streptavidin-Phycoerythrin (SAPE) (Millipore; Catalog # 45-001H) was added to each well and incubated for 15 min on a plate shaker (250 rpm) at RT. Without removing SAPE, 25 μL of amplification buffer (Millipore, Catalog # 43-024A) was added to each well and incubated for 15 min on a plate shaker at RT. Subsequently, liquid was decanted from wells using the magnetic separation block and the beads were resuspended in 150 μL of Assay Buffer 2, mixed on a plate shaker for 5 min and analyzed with a Luminex 200 instrument (Luminex, Austin, TX) using Milliplex Analyst software (Millipore, Billerica, MA). Beads were analyzed using the following parameters: Events: 50 beads; Sample Size: 100 μL; Gate settings: 8000 to 15,000.

For the Early Apoptosis Magnetic Bead kit to analyze *p*-p53 (Ser46), a 1 × suspension of magnetic beads was prepared by sonicating 20 × stock beads (Millipore, Catalog # 42-669MAG) for 15 s and vortexing for 30 s followed by mixing 150 μL stock beads with 2.85 mL of Assay Buffer 2. The 1 × suspension was vortexed again for 15 s prior to use. Wells were loaded with 25 μL of assay buffer for blanks, 25 μL of protein lysate for samples, or 25 μL of manufacturer controls. Subsequently, 25 μL of 1 × bead suspension was added to each well and the assay plate was incubated overnight at 4 °C on a plate shaker (250 rpm) shielded from light. After this, well contents were removed using a magnetic separation block and the wells were washed twice with 100 μL of Assay Buffer 2. Wells were subsequently incubated with 25 μL of 1 × detection antibody (Millipore, Catalog # 44-669KMG) for 1 h at RT on a plate shaker. Detection antibody was decanted using the magnetic separation block and wells were then incubated with 25 μL of SAPE (45-001H) for 15 min on a plate shaker at RT. Next, 25 μL of amplification buffer (43-024A) was added to each well, followed by another 15 min incubation, as above. Finally, well contents were removed using a magnetic separation block, and the beads were reconstituted in 150 μL of Assay Buffer 2 and analyzed using on the Luminex 200.

2.4. Statistical analysis

Data were collected as median florescence intensity (MFI) of each well and then values for hibernating tissue samples were expressed relative to their corresponding control values. Data are mean ± SEM, $n = 4$ samples from different animals. Statistical analysis used the Student's *t*-test to compare control and torpor values with significance differences accepted if $p < 0.05$. Sigmaplot 11 software (Systat Software Inc., San Jose, CA) was used for statistical analysis and figure construction.

3. Results

Members of the MAPK family of protein kinases are inactive when dephosphorylated and active when phosphorylated. Hence, analysis of changes in the phosphorylation state of these proteins provides a definitive measure of changes in their activity states in response to a stimulus. The relative phosphorylation states of five protein kinases in the MAPK cascade as well as four well-known MAPK target proteins were assessed in four tissues of *D. gliroides* comparing control and hibernating (4 days of continuous torpor) conditions. Luminex 200 multiplex technology was used to measure relative changes in the phosphorylated (activated) state of *p*-MEK1 (Ser217/221), ERK/MAPK 1/2 (Thr185/Tyr187), *p*-MSK1 (Ser212), *p*-p38 (Thr180/Tyr182), *p*-JNK (Thr183/Tyr185), and selected downstream targets *p*-c-Jun (Ser73), *p*-ATF2 (Thr71), *p*-p53 (Ser46), and *p*-CREB (Ser133).

Liver exhibited the greatest number of changes in the relative phosphorylation states of these proteins compared with other tissues, with seven out of nine targets showing increased phosphorylation

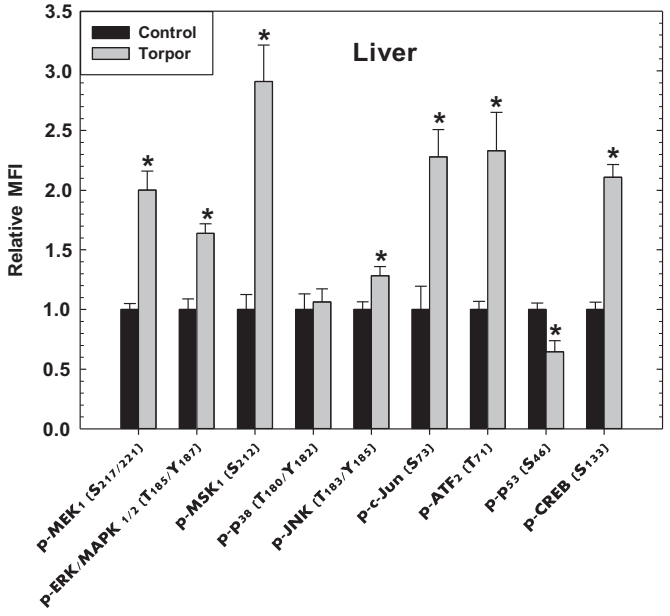

Fig. 1. Relative changes in the phosphorylation state of members of the MAPK signal transduction cascade in liver of *D. gliroides* during hibernation. Shown are relative phosphorylation levels in control versus torpid states for *p*-MEK (Ser217/221), ERK/MAPK 1/2 (Thr185/Tyr187), *p*-MSK1 (Ser212), *p*-p38 (Thr180/Tyr182), *p*-JNK (Thr183/Tyr185), *p*-c-Jun (Ser73), *p*-ATF2 (Thr71), *p*-p53 (Ser46) and *p*-CREB (S133). 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, $p < 0.05$.

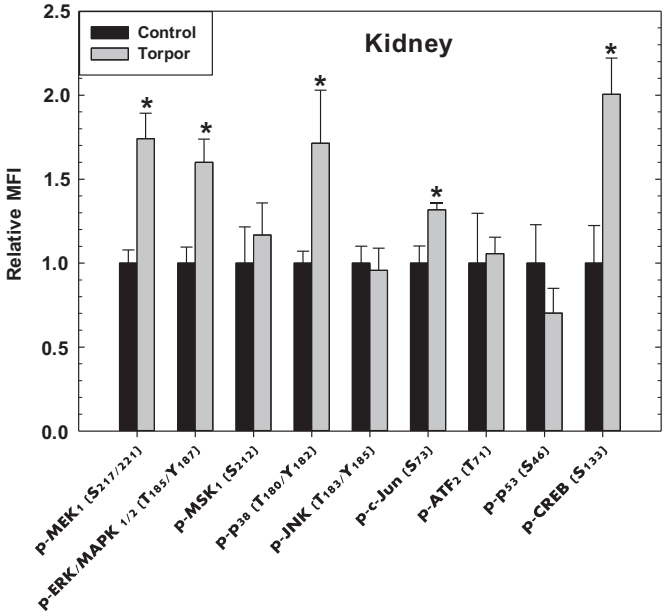

Fig. 2. Relative changes in the phosphorylation state of members of the MAPK cascade in the kidney of *D. gliroides* during hibernation. Other information as in Fig. 1.

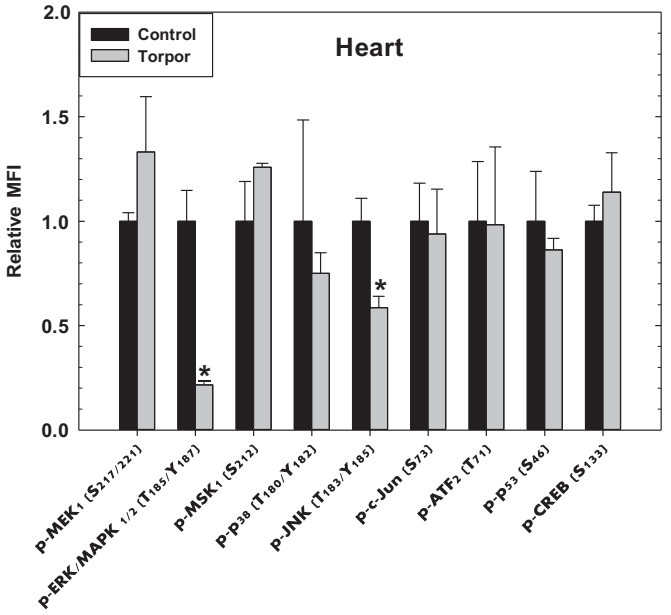

Fig. 3. Relative changes in the phosphorylation state of members of the MAPK cascade in the heart of *D. gliroides* during hibernation. Other information as in Fig. 1.

during torpor as compared to control conditions (Fig. 1). The targets that showed statistically significant increases in phosphorylation ($p < 0.05$) during torpor were *p*-MEK1 (2.0 ± 0.16 fold), *p*-ERK/MAPK1/2 (1.6 ± 0.08 fold), *p*-MSK1 (2.9 ± 0.31 fold), *p*-JNK (1.3 ± 0.08 fold), *p*-c-Jun (2.3 ± 0.23 fold), *p*-ATF2 (2.3 ± 0.32 fold), and *p*-CREB (2.1 ± 0.11 fold). By contrast, *p*-p53 content was significantly reduced to 60% of the control value during torpor and *p*-p38 was unaffected.

In kidney, five out of the nine targets showed relative increases in their phosphorylation states during torpor (Fig. 2). Statistically significant increases ($p < 0.05$) were recorded for *p*-MEK1 (1.7 ± 0.15 fold), *p*-ERK/MAPK1/2 (1.6 ± 0.14 fold), *p*-p38 (1.7 ± 0.31 fold), *p*-c-Jun (1.3 ± 0.04 fold), and *p*-CREB (2.0 ± 0.21 fold) in torpid animals, as compared with controls.

Heart showed only two significant changes between control and torpor states. Levels of *p*-ERK/MAPK 1/2 decreased strongly to just 22 ± 0.01% of the control value whereas *p*-JNK (T183/Y185) content was reduced to 59 ± 0.05% of the control (Fig. 3). In brain, the only significant change was a 39 ± 0.08% decrease in *p*-ERK/MAPK 1/2 content during torpor (Fig. 4).

4. Discussion

MAPK pathways are central to cellular signal transduction where they mediate, propagate and amplify growth and stress stimuli received at the cell surface to coordinate a wide range of output responses – metabolic, gene expression, apoptosis and cytoprotection, among others. Each of the three main MAPK cascades (ERKs, JNKs, p38s) consist of a multi-protein group of receptors, regulatory proteins, three tiers of protein kinases (each with isozymes), and downstream targets; not surprisingly, all this leads to extensive cross-talk (Widmann et al., 1999). Unphosphorylated MAPKs are essentially inactive and phosphorylation by their respective upstream kinases induces conformational changes that result in ~1000 fold increases in kinase activity (Chang and Karin, 2001; Johnson and Lapadat, 2002). Hence, the greatest insight into cascade activation can be obtained by analyzing changes to the relative amount of active phosphorylated MAPK members (Cowan and Storey, 2003). The participation of MAPK cascades in animal adaptation to environmental stress has been documented in a variety of systems including dehydration tolerance of frogs (Malik and Storey, 2009), anoxia tolerance of turtles (Cowan and Storey, 2003), hibernation in ground squirrels (Zhu et al., 2005; MacDonald and Storey, 2005) and daily torpor in gray mouse lemurs (Biggar et al., 2015). However, the role of these protein kinases in mediating stress responses during torpor/hibernation of marsupials has not previously been explored. Marsupial hibernators such as the *Dromiciops* species are of particular interest since they represent an ancient and isolated

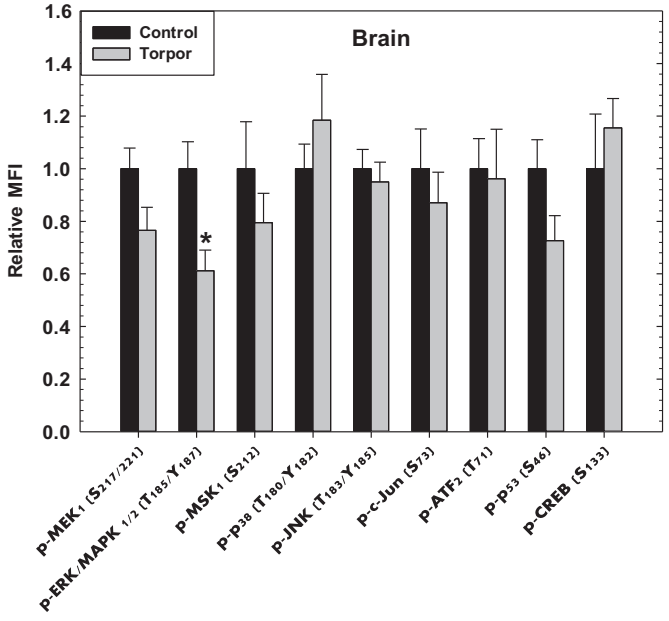

Fig. 4. Relative changes in the phosphorylation state of members of the MAPK cascade in the brain of *D. gliroides* during hibernation. Other information as in Fig. 1.

lineage that could allow us to identify unifying versus divergent principles of torpor/hibernation between marsupial and eutherian groups. The current study examined tissue-specific expression of the ERK, JNK, and p38 pathways during hibernation in *D. gliroides*.

In liver, amounts of phosphorylated kinases *p*-MEK1 (S217/221), *p*-ERK/MAPK 1/2 (T185/187), and *p*-MSK1 (S212) all increased by 1.6–2.9-fold during hibernation in *D. gliroides*, indicating activation of these kinases (Fig. 1). Correspondingly, phosphorylation of CREB (S133), a downstream transcription factor under ERK control, also rose by 2-fold. This suggests that extracellular signals, such as mitogenic stimuli, can elicit changes in gene expression in liver during hibernation by activating the ERK1/2 protein kinase cascade to phosphorylate transcription factors and potentially other downstream effector proteins. The data suggest that c-RAF (the MAPKKK of the ERK pathway) probably facilitated the 2-fold increase in the phosphorylation state of MEK1 (MAPKK) at S217/S211, which in turn phosphorylated and activated ERK1/2 (MAPK). Subsequently, the activated form of *p*-ERK/MAPK 1/2 could activate downstream cellular targets such as MSK-1 by phosphorylation at its S212 residue and this activation is directly connected to the sequential activation of CREB (Deak et al., 1998). By contrast, although ERK 1/2 activity was extremely high in liver of Richardson's ground squirrels, *Urocitellus* (*Spermophilus*) *richardsonii*, there was no change in activity between euthermic and hibernating animals (MacDonald and Storey, 2005), unlike the response seen in *D. gliroides*. Similarly, phospho-ERK 1/2 increased in *D. gliroides* kidney (Fig. 2) but ERK 1/2 activity decreased in squirrel kidney. This suggests that a substantial difference may occur in ERK signaling (and ERK-regulated responses) in marsupial versus eutherian liver and kidney during hibernation.

Although the data on ERK responses in marsupial versus eutherian liver are not fully conclusive between different studies because of different assay methods, they are intriguing and point to a functional basis. Liver is the metabolic hub that governs and maintains a variety of functions that are crucial to torpor, regulating (among others) the catabolism of carbohydrates, fatty acids and proteins, gluconeogenesis, oligonucleotide synthesis, detoxification reactions, the urea cycle, and the biosynthesis of many proteins for export (Rolfe and Brown, 1997; Fedorov et al., 2009). Liver has also been implicated as a site of thermogenesis in marsupials, first proposed for the North American opossum (*Monodelphis domestica*) (Villarin et al., 2003). Classic brown fat is missing in marsupials as is the diagnostic thermogenic response to injected noradrenaline; indeed, *D. gliroides* lacks this response (Cortés et al., 2014). However, the UCP1 gene has been found in *M. domestica* and two small Australian marsupials but a thermogenic function for marsupial UCP1 has yet to be conclusively demonstrated (Oelkrug et al., 2015). As such, the oxidative capacity of marsupial liver is of interest and has been shown to increase significantly during cold exposure. Liver of *M. domestica* showed a 48% increase in liver mass and a 20% increase in mitochondrial volume after six weeks of cold exposure indicating a significantly increased oxidative capacity (Villarin et al., 2003). *D. gliroides* liver may be undergoing a similar cold-stimulated proliferation during hibernation at 10 °C, potentially to raise its thermogenic capacity. This could account for the elevated ERK signaling is that well-known to have a mitogenic (growth or proliferation) role as well as the activation of the Akt/mTOR signaling pathway in liver of hibernating *D. gliroides*, reported by Luu et al. (2018). This latter indicated that protein translation (a major target of the Akt/mTOR axis) was upregulated during hibernation in monito del monte liver. By contrast, the amount of active phosphorylated Akt in eutherian liver was strongly suppressed in both hibernating bats and ground squirrels indicating reduced anabolic signaling by this protein kinase during eutherian hibernation (Eddy and Storey, 2003; Abnous et al., 2008). Furthermore, in support of elevated metabolic activity in liver of *D. gliroides* during hibernation, Hadj-Moussa et al. (2016) reported significant reductions in the levels of 32 torpor-sensitive microRNAs in liver of *D. gliroides* that are known to target 96 protein-coding genes in the MAPK signaling pathway. MicroRNAs are highly conserved short, non-coding RNA transcripts that bind and inhibit translation of mRNA transcripts. Decreased levels of these inhibitory microRNAs during hibernation suggest an opposite increase in the expression of the gene transcripts that they regulate. Hence, this adds further support to the current data that indicate an activation of MAPK signaling during hibernation in *D. gliroides* liver.

Kidney of monito del monte showed a 1.7-fold increase in expression of *p*-MEK1 (S217/221) and 1.6-fold increase in *p*-ERK/MAPK 1/2 (T185/187) during hibernation (Fig. 2), also indicative of increased activity of the ERK/SAPK cascade. However, whereas *p*-MSK1 (S212) did not show a significant change during hibernation, the downstream transcription factor, CREB, displayed a 2-fold increase in S133 phosphorylation (Fig. 2). The *p*-CREB response and activation of the ERK pathway in the kidney could be a cell survival response to stress or oxidative damage conditions during hibernation since active *p*-CREB has been shown to regulate transcription of genes that participate in cell survival and repair such as Bcl-2 and BRCA1 (Shaywitz and Greenberg, 1999; Mayr and Montminy, 2001). Since eutherian hibernators undergo significant changes in renal function that involve increased production of concentrated urine during hibernation and restoration of regular renal function during bouts of arousal (Harlow and Braun, 1995; Zancanaro et al., 1999), the ERK cascade might also be involved in kidney cytoprotection in hibernating *D. gliroides*. Hibernating dormice, for example, have been shown to fully preserve kidney cortex structure against ischemia and hypothermia that are common over torpor-arousal cycles (Zancanaro et al., 1999). However, a discrepancy was seen in the results regarding activation of CREB in kidney. The relative level of *p*-MSK1 (S212) that is generally attributed with phosphorylation and activation of CREB did not change during hibernation in kidney relative to the control (Fig. 2). However, stimulus-dependent phosphorylation of CREB at S133 can also be mediated by a variety of other protein kinases such as cAMP-dependent protein kinase A (Montminy and Bilezikjian, 1987; Shaywitz and Greenberg, 1999), CaMK, RSK, PKC, and notably by glycogen synthase kinase 3 (GSK-3) (Shaywitz and Greenberg, 1999). Indeed, GSK-3 expression in kidney increased significantly in hibernating monito del monte (Luu et al., 2018). Therefore, CREB activation may have been mediated by GSK-3 in kidney.

Interestingly, both heart and brain showed a strong reduction in the relative phosphorylation levels of *p*-ERK/MAPK 1/2 (T185/187)

(Figs. 3, 4). This response might be due to reduced extracellular receptor kinase activity and an overall reduction in the activity of biosynthetic pathways in heart and brain of hibernating marsupials. Typically, torpor is characterized by a strong reduction in heart rate, respiratory rate, and neurological functions, which are characteristics of metabolic rate depression (Storey and Storey, 2004; Storey and Storey, 2007; Storey, 2010). As shown by Biggar et al. (2015), ERK1/2 protein levels also decreased in heart of gray mouse lemurs during torpor, and the Akt/mTOR pathway that is involved in protein translation is also suppressed during torpor in brain of hibernating *D. gliroides* (Luu et al., 2018) and hibernating 13-lined ground squirrels (Wu and Storey, 2012). Overall, these studies imply a reduction in protein translation and MAPK-mediated expression of downstream transcription factor targets in both eutherian and marsupial systems which is indicative of metabolic rate depression during torpor in heart and brain of both groups.

MAPKs in the p38 family are activated by phosphorylation of the regulatory Thr180/Tyr182 residues within their Thr-Gly-Tyr domains (Plotnikov et al., 2011) and phosphorylate a plethora of downstream targets in different cellular compartments including proteins such as ATF-2, p53, Bax, Tau, HSP 25 and 27, Stat1, Max/Myc complexes, and MEF2A/C (Cowan and Storey, 2003; Curado and Nebreda, 2010; Cargnello and Roux, 2011; Plotnikov et al., 2011). Therefore, modulation of the p38 and JNK MAPK pathways gives insight into the stress-responsive networks underlying torpor survival. Phosphorylated p38 (Thr180/Tyr182) increased 1.7-fold during hibernation in kidney (Fig. 2), yet remained unchanged in liver (Fig. 1), heart (Fig. 3) and brain (Fig. 4), suggesting a targeted organ-specific role for p38 MAPK in *D. gliroides* hibernation. However, the two downstream protein targets of p38 in kidney that were assessed, ATF-2 and p53 (that are also regulated upstream by JNK), did not show significant changes in phosphorylation levels in response to hibernation. Although multiple downstream functions of p38 kinase could be affected in hibernator kidney, Awazu et al. (2002) linked p38 in kidney with the maintenance of stromal cells that are vital for metanephros growth and nephrogenesis. Hence, enhanced expression of *p*-p38 in *D. gliroides* kidney might be indicative of proliferation and maintenance of kidney cells, perhaps in response to low temperature conditions or other factors specific to hibernation. Since hibernators restore regular renal function during bouts of arousal (Harlow and Braun, 1995; Zancanaro et al., 1999), increased *p*-p38 signaling (along with ERK activation discussed earlier) could help to maintain kidney structure and function during hibernation. Further studies exploring the role of ERK and p38 in renal function may shed light on the importance of MAPK pathway regulation in kidney adaptation to environmental stress.

Regarding the final MAPK pathway assessed, the JNK cascade is activated by phosphorylation at Thr183/Tyr185 residues in the activation loop by the c-Jun amino terminal kinase kinases, JNKK1 and JNKK2, the latter also known as MKK4 (mitogen activated protein kinase kinase 4) (Tournier et al., 1999; Cargnello and Roux, 2011). JNK activation is responsive to multiple triggers including cytokines, ultraviolet irradiation, heat and osmotic shock, and apoptosis. Upon activation, JNK phosphorylates both cytoplasmic and nuclear protein targets such as c-Jun, ATF-2, p53, Elk-1/3 (Ets-domain containing protein 1), JunD, Myc, and transcription factors including HSF-1 (heat shock factor 1), NFAT4 (nuclear factor of activated t-cells), and SMAD4 (Cowan and Storey, 2003; Plotnikov et al., 2011). Phosphorylation levels of *p*-JNK (T183/Y185) significantly increased in monito del monte liver during hibernation (Fig. 1) indicating activation of JNK. Correlated with this, phosphorylation of two JNK downstream targets, *p*-c-Jun (S73) and *p*-ATF-2 (T71), also rose significantly by 2.3-fold (Fig. 1). Comparable results were seen in liver of hibernating Richardson's ground squirrels (*U. richardsonii*); JNK activity increased by ~2.5 fold compared with euthermic controls and levels of c-Jun protein rose by ~1.8 fold (MacDonald and Storey, 2005). This suggests that both eutherian and marsupial hibernators rely on JNK signaling to induce or control selected metabolic responses to torpor. JNK activation is known to occur in response to a variety of cell stress conditions (Macdonald and Storey, 2005) and its phosphorylation of c-Jun enhances the ability of c-Jun to bind to the AP-1 (activator protein-1) transcription factor (Shaulian and Karin, 2002; Cowan and Storey, 2003), and initiate transcription of a variety of stress-responsive genes. In addition, phosphorylated c-Jun can form heterodimers with ATF-2 and enhance cAMP-responsive element dependent transcription (Gupta et al., 1995) that could also have benefits in hibernation. Phosphorylation of p53 at S46 significantly decreased in liver (Fig. 1), and according to Schreiber et al. (1999), increased levels of c-Jun prevent the expression of this pro-apoptotic protein by binding to a variant AP-1 site in the p53 promoter. Furthermore, c-Jun can function as a pro-cell survival factor by associating with NF-κB and prevent apoptosis induced by TNF-α (Wisdom et al., 1999). Therefore, in liver of monito del monte as well as eutherian hibernators, JNK signaling and enhanced levels of phosphorylated c-Jun could have conserved roles in regulating multiple cytoprotective mechanisms during hibernation.

However, in kidney, heart, and brain of *D. gliroides*, there was inconclusive evidence for *p*-JNK mediated regulation during hibernation. In particular, kidney showed no change in *p*-JNK and *p*-ATF-2 levels during hibernation compared to controls although phosphorylated c-Jun levels increased slightly (Fig. 2). By contrast, heart showed a significant decrease in *p*-JNK during hibernation whereas phospho-c-Jun and *p*-ATF-2 levels remained unchanged (Fig. 3). This contrasts with 7- and 2-fold increases in JNK activity kidney and heart of hibernating *U. richardsonii* (MacDonald and Storey, 2005) but both monito and ground squirrel showed little evidence for JNK cascade activation in brain during hibernation (Fig. 4). Future proteomic studies focusing on protein-protein interactions and novel post-translational modifications are perhaps required to fully define the regulation of p38 and JNK pathways in *D. gliroides* tissues in response to torpor.

5. Conclusions

Tissue-specific expression patterns were observed for members of the *p*-ERK 1/2, *p*-p38, and *p*-JNK signaling cascades in hibernating *D. gliroides*, suggesting an involvement of MAPK cascades in regulating important cellular functions such as apoptosis, cell proliferation and differentiation, and metabolism in the torpid state. This study identified liver as the most torpor-responsive tissue with respect to MAPK signaling in the hibernating marsupial. Liver is central to the regulation and coordination of metabolic functions in hibernators, being a primary source of metabolic fuels as well as a site of detoxification pathways that would be important for long term health of the body during torpor. Control of such pathways is typically regulated by reversible protein phosphorylation of key enzymes, under the control of signal transduction cascades. Therefore, it is not surprising that the most robust changes in phosphorylation status of MAPK pathway enzymes were seen in liver. Furthermore, some distinct differences in the ERK signaling pathway in liver were noted between *D. gliroides* and eutherian hibernators that may be linked with a potential thermogenic role for marsupial liver. Substantial MAPK involvement in kidney metabolism of *D. gliroides* was also indicated, potentially regulating cytoprotective functions but the ERK pathway was suppressed in heart and brain, possibly as a component of hypometabolism.

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