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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

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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