Comparative Biochemistry and Physiology, Part B xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Strategies of biochemical adaptation for hibernation in a South American marsupial, *Dromiciops gliroides*: 3. Activation of pro-survival response pathways

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ARTICLE INFO

Keywords:
Monito del monte
Hibernation
Metabolic rate depression
Stress response
Cell cycle regulation
Heat shock proteins
MDM2 ubiquitin ligase

ABSTRACT

The South American marsupial, monito del monte (*Dromiciops gliroides*) uses both daily torpor and multi-day hibernation to survive in its southern Chile native environment. The present study leverages multiplex technology to assess the contributions of key stress-inducible cell cycle regulators and heat shock proteins to hibernation in liver, heart, and brain of monito del monte in a comparison of control versus 4 day hibernating conditions. The data indicate that MDM2, a stress-responsive ubiquitin ligase, plays a crucial role in marsupial hibernation since all three tissues showed statistically significant increases in MDM2 levels during torpor (1.6–1.8 fold). MDM2 may have a cytoprotective action to deal with ischemia/reperfusion stress and is also involved in a nutrient sensing pathway where it could help regulate the metabolic switch to fatty acid oxidation during torpor. Elevated levels of stress-sensitive cell cycle regulators including ATR (2.32–3.91 fold), and the phosphorylated forms of *p*-Chk1 (Ser345) (1.92 fold), *p*-Chk2 (Thr68) (2.20 fold) and p21 (1.64 fold) were observed in heart and liver during hibernation suggesting that the cell cycle is likely suppressed to conserve energy while animals are in torpor. Upregulation of heat shock proteins also occurred as a cytoprotective strategy with increased levels of hsp27 (2.00 fold) and hsp60 (1.72–2.76 fold) during hibernation. The results suggest that cell cycle control and selective chaperone action are significant components of hibernation in *D. gliroides* and reveal common molecular responses to those seen in eutherian hibernators.

1. Introduction

All organisms must deal with changes in their environment and, when environmental stress becomes extreme, they employ adaptive mechanisms to help preserve viability for as long as possible while also working to attenuate cell death signals. Established means of dealing with unfavorable environmental conditions include the suppression of many energy-expensive anabolic and growth processes and the reprioritization of ATP use towards support for pro-survival actions (Storey and Storey, 2004). If such preservation measures fail, the balance can tip towards programmed cell death (apoptosis) when stress conditions persist (e.g. nutrient deprivation, hypoxia/ischemia, dehydration, disease, etc.). Although cell death is a natural way by which animals recycle old or damaged cells, many organisms show remarkable adaptive controls over these processes in order to endure taxing stresses without losing cell viability.

The South American marsupial, monito del monte (*Dromiciops gliroides*), has been called a "living fossil", a relic of a near-extinct marsupial lineage, the Order Microbiotheria, only distantly related to all other North and South American marsupials. It was known as the sole extant member of this group until recently when the single species was reclassified as three geographically-separate species of *Dromiciops*, all living in the temperate rainforests of southern Chile (D'Elía et al., 2016). This tiny nocturnal marsupial uses torpor to enhance survival under conditions of cold environmental temperatures or limited food availability. Both shallow daily torpor and prolonged multi-day

Abbreviations: ATR, ataxia telangiectasia and Rad3 related; Chk, checkpoint kinase; HSP, heat shock protein; H2A.X, histone 2A member X; MDM2, mouse double minute 2

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https://doi.org/10.1016/j.cbpb.2017.12.005
Received 14 August 2017; Received in revised form 6 December 2017; Accepted 7 December 2017

hibernation have been documented (Bozinovic et al., 2004). Understanding the biochemical adaptations that underlie hibernation in this ancient marsupial lineage will advance our overall understanding of mammalian torpor/hibernation and stress biology and of the conserved versus novel features utilized by marsupial versus placental mammals. For example, marsupials lack the brown adipose tissue that is the main thermogenic organ used to arouse placental (eutherian) mammals from torpor (Villarin et al., 2003).

In the previous two papers in this series on *D. gliroides* we have focused on signal transduction pathways (Wijenayake et al., 2018) that may be involved in inducing and mediating torpor and regulating protein synthesis (Luu et al., 2018), one of the most energy-expensive metabolic activities in cells (Storey and Storey, 2004). Regulated metabolic rate depression is a core feature of torpor/hibernation across the animal kingdom and, by strongly suppressing energy use by ATP-expensive functions (e.g. gene transcription, protein translation, the cell cycle), animals can prolong the time that a fixed reserve of body fuels can support survival. However, suppression of such ATP-expensive functions would predictably necessitate reduced turnover and greater stability of cell macromolecules and lead to a requirement for improved cytoprotective pro-survival measures (e.g. chaperone proteins, antioxidant defenses, anti-apoptosis mechanisms, etc.) during prolonged hibernation. Therefore, we predicted that cytoprotective mechanisms would be enhanced when *D. gliroides* transitioned into a hypometabolic state. The present paper examines this proposal by analyzing the responses to hibernation by selected cell cycle regulatory proteins and heat shock chaperone proteins in monito del monte tissues. Of further interest, regulatory proteins of the cell cycle (an energy-expensive process) and selected chaperone proteins (pro-survival) have been shown to be intimately co-regulated, in particular as responses to cold (Kühl and Rensing, 2000; Nakai and Ishikawa, 2001; Rice et al., 1986; Storey, 2004). As a result, we focused the present study on these two critical molecular hubs that play roles in balancing metabolism and survival.

The cell cycle is a complex biochemical process that requires significant coordination between multiple pathways to initiate or halt cell division. Cell cycle regulation is sensitive to many stresses (e.g. DNA damage, hypoxia, nutrient deficiency, etc.), allowing cell cycle arrest to be implemented as a versatile response to stress conditions. Factors that trigger cell cycle arrest often act through activation of the ataxia telangiectasia and Rad3 related (ATR) protein, a serine/threonine protein kinase that phosphorylates downstream targets such as the checkpoint kinases, Chk1 and Chk2 (Ding et al., 2013; Martin et al., 2012). Phosphorylation of Chk1 and Chk2 by ATR at Ser345 and Thr68, respectively, triggers a broader signaling cascade that spreads to encompass phosphorylation-mediated regulation of cell cycle arrest, DNA damage, and apoptosis responses (Ouchi and Ouchi, 2014; Wang et al., 2012). For example, in response to DNA damage, double-stranded breaks will trigger the immediate phosphorylation of the histone 2 variant (H2A.X) at Ser139 by ATR, and lead to the recruitment of DNA repair machinery (Rogakou et al., 1998; Singh et al., 2012). In addition to reversible protein phosphorylation, the cell cycle can also be controlled by proteins such as the mouse double minute 2 protein (MDM2) and p21, that directly interact with transcription factors and protein kinases including p53 and cyclin-dependent kinases (Moll and Petrenko, 2003; Xiong et al., 1993).

Heat shock proteins (HSPs) are a well-known family of chaperone proteins with actions that aid both folding of nascent proteins and refolding of misfolded or denatured proteins (Feder and Hofmann, 1999). Cells produce HSPs in response to numerous stresses including heat, cold, dehydration, changes in salinity, UV radiation and more, and HSPs have important roles in many diseases (Storey and Storey, 2011; Yu et al., 2015). Previous studies have determined that chaperone proteins are differentially regulated during torpor/hibernation as part of a cytoprotective response in eutherian mammals including lemurs, ground squirrels and bats (Lee et al., 2002; Mamady and Storey, 2006; Rouble et al., 2014; Wu et al., 2015). Enhanced levels of chaperones aid long-term viability during prolonged torpor since the scope for extensive repair/replacement of damaged proteins is reduced in the hypometabolic state.

The present study characterizes selected stress-responsive cell cycle regulators and HSPs to identify torpor-responsive cytoprotective pathways that aid *D. gliroides* hibernation. We used multiplex technology for analysis of three organs (liver, heart, brain), comparing aroused and hibernating (4 days of continuous torpor) conditions. The results indicate that cytoprotective mechanisms are employed during torpor in *D. gliroides*, and are differentially regulated in an organ-specific manner to manage cell cycle and chaperone pathways.

2. Materials and methods

2.1. Animals

Adult monito del monte, *D. gliroides*, was captured near Valdivia, Chile in January–February 2014. Expanded information on conditions of animal holding, acclimation and experimentation are described in Wijenayake et al. (2018). In brief, animals were acclimated at 20 ± 1 °C under a 12 h:12 h light:dark cycle with mealworms, fruits and water provided ad libitum. After two weeks, some were sampled as controls. Remaining animals were subjected to a decrease in ambient temperature over 2–3 days until 10 °C was reached; all had entered torpor by the time that temperature was lowered to ~15 °C. Experimental animals were sampled after 4 d of continuous torpor. Euthanasia followed protocols approved by the Committee on the Ethics of Animal Experiments of the Universidad Austral de Chile. Tissue samples were rapidly dissected, immediately frozen in liquid nitrogen, and air-freighted to Carleton University in a dry shipper. All animal capture, handling and maintenance followed the guidelines of the American Society of Mammalogists (Gannon and Sikes, 2007) and were authorized by the Chilean Agriculture and Livestock Bureau (SAG: Servicio Agrícola y Ganadero de Chile, permit resolution No. 1054/2014).

2.2. Protein extraction

Samples of frozen tissues (~50 mg each) were crushed under liquid nitrogen and homogenized 1:5 (w/v) using a Dounce homogenizer in pre-chilled lysis buffer (Milliplex MAP Assay Buffer 1; Cat. No. 43-010) with additions of 1 mM Na_3VO_4, 10 mM NaF, 10 mM β-glycerophosphate and 1% protease inhibitor cocktail (Cat. No. PIC001, BioShop). Samples were left to incubate on ice for 30 min with vortexing every 10 min, and were then centrifuged at 12,000 × *g* for 20 min at 4 °C. Supernatants were removed and soluble protein concentration was determined using the Bio-Rad protein assay (Cat. No. 500-0006). Samples were then standardized to 10 μg/μL with the addition of small volumes of lysis buffer and stored at − 80 °C until use.

2.3. Multiplex analysis

EMD Millipore magnetic bead kits were used to assay levels of six cell cycle markers (Milliplex DNA Damage/Genotoxicity Kit, Cat. No. 48-621MAG) and four heat shock proteins (Milliplex Heat Shock Protein Kit, Cat. No. 48-615MAG). Initial work tested dilutions of small aliquots of cell lysates to determine the limits of detection and ideal sample concentration for assays. Manufacturer-supplied negative and positive controls were run to assure functionality and performance of the assay. For cell cycle marker analysis, lambda phosphatase-treated HeLa cells were used as a negative control (Cat. No. 47-229), whereas Jurkat cells stimulated with 25 μM anisomycin for 4 h (Cat No. 47.207) and A549 cells stimulated with 5 μM camptothecin overnight (Cat. No. 47-218) were the positive controls. For heat shock protein analysis unstimulated HeLa cells were the negative control (Cat. No. 47-205) and HS/Ars-treated HeLa cells were the positive control (Cat. No. 47-