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Stress activated protein kinases, JNKs and p38 MAPK, are differentially activated in ganglia and heart of land snail *Helix lucorum* (L.) during seasonal hibernation and arousal

Basile Michaelidis *, Maria Hatzikamari, Vassiliki Antoniou, Andreas Anestis, Antigone Lazou

Laboratory of Animal Physiology, Department of Zoology, School of Biology, Faculty of Science, Aristotle University of Thessaloniki, Thessaloniki 54124, Greece

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ABSTRACT

The present work aimed to investigate the phosphorylation and hence activation of stress activated protein kinases, p38 MAPK and JNKs in the tissues of the snail *Helix lucorum* during seasonal hibernation. Snails were put in large glass boxes, which were placed outdoors so that they were exposed to natural conditions of light and temperature. Phosphorylation and hence activation of JNKs and p38 MAPK was determined in both heart and ganglia. Deep hibernation caused significant increases in the levels of the phosphorylated form of JNK and p38-MAPK in both heart and ganglia. Phosphorylation of JNK remained elevated in the ganglia or increased after a transient drop in the heart, when the snails were prepared for arousal. In addition, phosphorylation of p38-MAPK was further increased in the heart during this period. These data support the conclusion that MAPK signalling cascade might contribute in the physiological and biochemical remodelling in the tissues of land snails during hibernation and upon preparation for arousal.

1. Introduction

There is now a considerable amount of information detailing various aspects of the morphological, physiological, and biochemical changes that are associated with hibernation (Guppy and Withers, 1999; Storey, 1997; Storey and Storey, 2004). However, the cellular and molecular basis of hibernation are still poorly understood. During hibernation, vertebrate hibernators undergo a complex suite of morphological, physiological, and behavioural changes in response to seasonal periods of high-energy demand coupled with reduced energy availability in the environment (Wang and Lee, 1996; Storey, 2001; Carey et al., 2003a,b). Successful hibernation requires a variety of regulatory adaptations including those that coordinate the profound metabolic rate depression and others that reorganize cellular activities such as proliferation, differentiation and cell death (Guppy and Withers, 1999; Storey and Storey, 2004). Signal transduction cascades are a critical part of these processes and the differential regulation of various protein kinases and phosphatases has already been implicated in several aspects of metabolic control during hibernation (Carey et al., 2003a,b; Cowan and Storey, 2003; Storey and Storey, 2004; MacDonald and Storey, 2005).

The mitogen activated protein kinases (MAPKs) are known to respond to a variety of environmental stresses and are important signalling molecules involved in relaying extracellular signals to intracellular targets (Storey and Storey, 2004). The individual members of the MAPK superfamily include the extracellular signal regulated kinases (ERKs), the cJun-N-terminal kinases (JNKs) and p38 MAPK. The ERK pathway is mainly activated by mitogen or differentiation inducing agents whereas JNKs and p38 MAPK pathways are typically responsive to environmental stresses (Kyriakis and Avruch, 2001). Activated MAPKs control cellular responses by phosphorylating target proteins that can include other kinases, transcription factors and regulatory proteins. Members of MAPKs may have key actions in the induction and maintenance of hibernation because the development and maintenance of torpor requires the expression of selected hibernation-specific genes. Recent data indicates an important role of p38 MAPK signaling in heart and skeletal muscle of hibernating mammals (Eddy and Storey, 2007; MacDonald and Storey, 2005). Furthermore, our own data and other implicate the involvement of p38-MAPK and cJun-N-terminal kinases (JNKs) in the induction of heat shock proteins (Hsp) during various cell stresses (Buckley et al., 2001; Anestis et al., 2007; Anestis et al., 2008). However, the role of MAPKs in invertebrate hibernators is largely unknown.

This study aimed to investigate for the first time the phosphorylation and hence activation of stress activated protein kinases, p38 MAPK and JNKs in the tissues of *Helix lucorum* during seasonal hibernation. The land snail *H. lucorum* is found throughout the mainland of Greece especially in the northern regions where its ecology and biology have been studied (Staikou et al., 1988). In the

* Corresponding author. Tel.: +30 2310 998401; fax: +30 2310 998269.
E-mail address: michaeli@bio.auth.gr (B. Michaelidis).

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north of Greece snails of this species hibernate during winter. Hibernation starts at the beginning of November and is terminated by the end of March.

2. Materials and methods

2.1. Animals and experimental design

The snails (*H. lucorum*) used for the experiments described in this paper were collected in October 2007 from a population in the vicinity of Edessa, in northern Greece. Adult snails (35–42 mm) were transferred to the laboratory where they were put in large glass boxes filled with soil derived from the area from where snails were collected. The boxes were placed outdoors so that snails were exposed to natural conditions of light and temperature. The boxes were covered to prevent flooding and animal drowning during rainy periods. Water was added periodically to ensure that soil was kept moist and the ambient humidity was maintained above 80%. Snails were fed fresh lettuce leaves everyday until the first 5–7 days of November. Feeding stopped as soon as snails started to burrow into the ground. From the first days of November (2007) until the first 10 days of March (2008), snails were removed at regular periods and heart and ganglia of ducal mass were immediately dissected, freeze-clamped between aluminum tongs cooled in liquid nitrogen and ground under liquid nitrogen. Tissue powders were stored at −80 °C.

2.2. Preparation of tissue samples

Tissue powders were homogenized in 3 ml/g of cold lysis buffer (20 mM β-glycerophosphate, 50 mM NaF, 2 mM EDTA, 20 mM Hepes, 0.2 mM Na_3VO_4, 10 mM benzamidine, pH 7, supplemented with 200 μM leupeptin, 10 μM trans-epoxy succinyl-L-leucylamido-(4-guanidino) butane, 5 mM dithiothreitol, 300 μM phenyl methyl sulfonyl fluoride (PMSF), 120 μM pepstatin and 1% v/v Triton X-100) and extracted on ice for 30 min. Samples were centrifuged (10,000 *g*, 10 min, 4 °C) and the supernatants were boiled with 0.33 volumes of SDS/PAGE sample buffer (330 mM Tris–HCl, pH 6.8, 13% v/v glycerol, 133 mM DTT, 10% w/v SDS, 0.2% w/v bromophenol blue). Protein concentration was determined using the BioRad protein assay (BioRad, Hercules, CA).

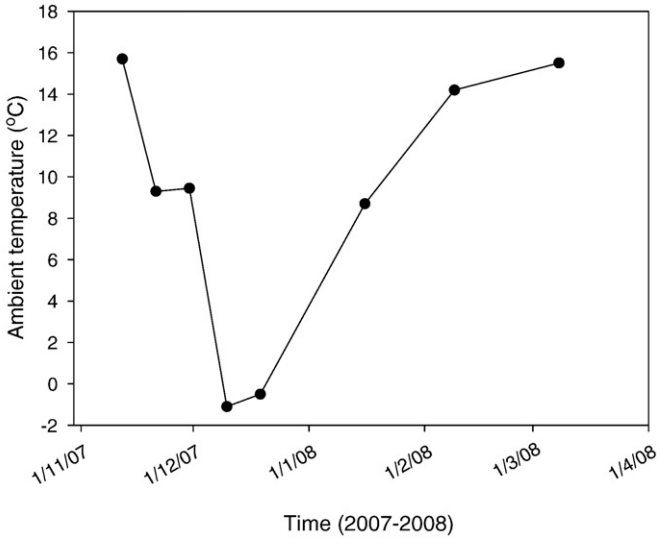

Fig. 1. Changes in the ambient temperature where land snails enter seasonal hibernation.

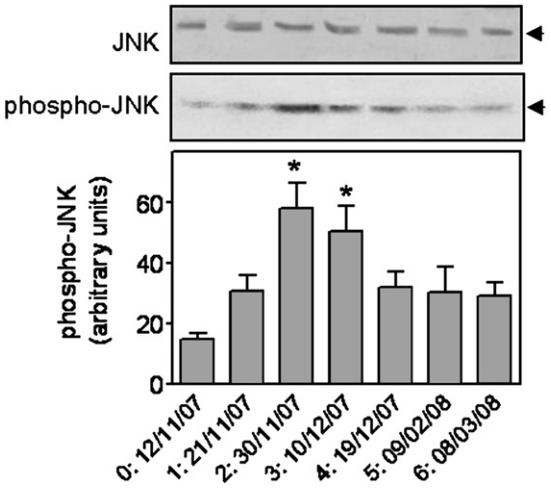

Fig. 2. Phosphorylation levels of cJun-N-terminal kinase (JNK) in the heart of *H. lucorum* during hibernation. Tissue extracts were subjected to SDS-PAGE and immunoblotted for the total (upper panel) and phosphorylated (middle panel) form of JNKs. Representative immunoblots are shown. Blots were quantified by laser-scanning densitometry (lower panel). Values are means ± SE; $n = 6$ preparations from different animals. Asterisks indicate the values, which are significantly different from the control value (prehibernation animals sampled at the beginning of November 2007); $^*p < 0.05$.

2.3. SDS/PAGE and immunoblotting

Equal amounts of proteins (100 μg) were separated on 10% (w/v) acrylamide, 0.275% (w/v) bisacrylamide slab gels and transferred electrophoretically onto nitrocellulose membranes (0.45 μm, Schleicher & Schuell, Keene, NH., USA). Non-specific binding sites on the membranes were blocked with 5% (w/v) nonfat milk in TBST (20 mM Tris–HCl, pH 7.5, 137 mM NaCl, 0.1% (v/v) Tween 20) for 30 min at room temperature. Subsequently, the membranes were incubated overnight with the appropriate primary antibodies. Antibodies used were: rabbit anti-SAPK-JNK, mouse anti-phospho-SAPK-JNK (Thr183/Tyr185), rabbit anti-p38 MAPK and rabbit anti-phospho-p38 MAP kinase (Thr180/Tyr182) (Cell Signaling, Beverly, MA, USA). After washing in TBST (3 × 5 min) the blots were incubated with horseradish peroxidase-linked secondary antibodies, washed again in TBST (3 × 5 min) and the bands were detected using enhanced chemiluminescence (Chemicon International, Inc) with exposure to Fuji Medical X-ray films. Films were quantified by laser scanning densitometry (GelPro Analyzer Software, Graphpad).

2.4. Statistics

Changes over time were tested for significance at the 5% level by using one-way analysis of variance (ANOVA) and by performing Bonferroni *post-hoc* tests for group comparisons. Values are presented as means ± s.e.m.

3. Results

Snails entered the hibernated state from the first 20 days of November of 2007 and almost all the individuals burrowed into the soil (about 10–15 cm) by the end of November. As in the field, at the end of October, land snails formed a thick epiphragm sealing the shell aperture. The mean ambient temperature decreased from 15.8 °C to −1 °C by the middle of December of 2007 (Fig. 1). Thereafter ambient temperature was increased gradually and it reached 15 °C by the middle of March 2008.

Expression and phosphorylation of JNKs and p38 MAPK were determined in both heart and ganglia of *H. lucorum* during seasonal hibernation. One form of JNKs was detected, which corresponds to the 46 kDa isoform of the mammalian enzyme. Expression of JNK protein