Cryobiology xxx (2017) 1–7

Contents lists available at ScienceDirect

Cryobiology

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

TonEBP/NFAT5 regulates downstream osmoregulatory proteins during freeze-thaw stress in the wood frog

Yichi Zhang, Rasha Al-attar, Kenneth B. Storey*

Institute of Biochemistry, Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, ON K1S 5B6, Canada

ARTICLE INFO

Article history:
Received 1 May 2017
Received in revised form
20 September 2017
Accepted 21 September 2017
Available online xxx

Keywords:
Freezing
Thaw
Rana sylvatica
Osmotic stress
Cryoprotection
Nuclear factor of activated T cells

ABSTRACT

Rana sylvatica, known as the wood frog, can survive extremely cold temperatures during winter by undergoing full-body freezing, where it tolerates freezing of 65–70% of its total body water. During freezing, cellular dehydration decreases damage to the cell by preventing ice crystallization. Challenged with many stresses, these animals are forced to develop physiological adaptations to osmoregulation and osmoprotection that are necessary to ensure their survival. The purpose of this study was to elucidate a potential mechanism by which the transcription factor, NFAT5, regulates the expression of three osmoregulatory proteins (aldose reductase, SMIT, and BGT-1). These three proteins control cellular concentrations of the organic osmolytes: betaine (BGT-1), myo-inositol (SMIT), and sorbitol (aldose reductase). We studied this mechanism during the freeze-thaw stress in *R. sylvatica* liver, kidney, and skeletal muscle. Protein expression of BGT-1, SMIT, aldose reductase, and NFAT5 were examined using immunoblotting. We identified that the NFAT5 pathway facilitated osmoregulation in a tissue-specific manner during freezing. In skeletal muscle, we demonstrated that NFAT5 upregulation in thawing led to increases in the protein levels of BGT-1. In liver, NFAT5 was upregulated during freezing, along with aldose reductase. Furthermore, neither of these patterns of expression were observed in kidney as none of these four proteins showed differential expression during freezing or thawing. Therefore, the NFAT5 osmoregulatory pathway appears to be tissue-specific. Our novel findings on a mechanism of osmoregulation in *R. sylvatica* highlight the importance of studying naturally stress-tolerant animals to identify novel pro-survival pathways.

1. Introduction

Many organisms that live in cold climates have developed unique ways to adapt and survive under the extreme sub-zero temperatures, as freezing can impose significant distress to these organisms. Terrestrial ectotherms employ strategies like active freeze-avoidance, while few others developed whole-body freezing as an extreme overwintering solution [47,50]. The North American wood frog, *Rana sylvatica*, is one of several amphibians that reside in northern regions where they are confronted by frigid environments. As a result, *R. sylvatica* exhibits a capacity to freeze up to 65-70% of its total body water, where water accumulates as masses of ice in the extracellular and extra-organ environments [47,48,50]. This accumulation of ice interferes with crucial physiological functions such as cardiac function, neuronal activity, muscle movement, among others [22,27,49]. To survive freezing, *R. sylvatica* have developed many different physiological and biochemical mechanisms, one of which involves a suppression of metabolic rate and organ function to conserve energy. Both transcription and translation are moderately repressed as they consume a great deal of energy [51]. This inhibition of transcriptional and translational processes leads to physiological and biochemical adaptations characteristic of freezing [50].

In preparation for freezing, water is diffused out of the cell to the extracellular matrix, resulting in intracellular dehydration that could potentially cause shrinkage and death. However, wood frogs have developed several mechanisms of cellular osmoprotection that prevent cell death caused by severe water loss. One of the most well-known osmoprotective mechanisms especially in wood frogs, is the use of glucose. In fact, glucose levels in the skeletal muscles of *R. sylvatica* during freezing can increase by as much as 20 times that of nonfreezing levels [10,43]. Alternatively, urea is another osmoregulatory molecule used by wood frogs to tolerate freezing and

* Corresponding author.
E-mail address: kenneth.storey@carleton.ca (K.B. Storey).

https://doi.org/10.1016/j.cryobiol.2017.09.003
0011-2240/

cellular dehydration stresses [8,9]. Furthermore, cryoprotective genes have also been discovered in *R. sylvatica* that aid in freeze-tolerance and possibly osmotic balance during freezing [34,35]. In this study, we were interested in another possible mechanism of osmoregulation that has not been studied in the freeze-tolerant wood frog during freeze-thaw stress. This mechanism involves the control of cellular osmotic balance using the organic osmolytes – betaine, sorbitol, and myo-inositol. These three osmolytes are uncharge and small molecules that regulate osmotic balance by replacing cellular electrolytes such as potassium, sodium, and chloride [16,56]. Although myo-inositol, betaine, and sorbitol accumulate during cell shrinkage, they are removed from cells when cells begin to swell and they are no longer needed [4,26]. Therefore, these osmolytes can control osmotic balance without disrupting cellular processes.

The cellular concentrations of these organic osmolytes are controlled by individual transporters and synthesizing enzymes. Aldose reductase is the first and rate-limiting enzyme in the polyol pathway that coverts glucose to sorbitol [7]. Myo-inositol and betaine are transported into the cell by the sodium/myo-inositol cotransporter (SMIT) and the Na^+- and Cl^--dependent betaine-GABA transporter (BGT-1), respectively [5,15]. It was demonstrated in the oocytes of another stress-tolerant amphibian, *Xenopus laevis*, that BGT-1 plays an important role in preserving cellular osmotic gradients [13]. In *R. sylvatica*, all three of these osmoregulatory proteins have been shown to be differentially regulated during anoxia-recovery and dehydration-rehydration stresses [2]. However, previous experiments from our lab have shown that blood from frozen frogs seem to lack sorbitol [44], so we are interested in determining whether the minimal concentration of sorbitol is due to decreased aldose reductase levels during freezing. Decreased expression of aldose reductase would explain not only the low concentration of sorbitol, but the accumulation of glucose in *R. sylvatica* during freezing as well [10,43].

BGT-1, SMIT, and aldose reductase are all regulated at the transcriptional level by a transcription factor called the Nuclear Factors of Activated T cells 5 (NFAT5). NFAT5 is part of a family of transcription factors that contains five members in total, and these NFATs have been implicated in regulating various cellular processes such as development, apoptosis, and growth [30,38,40,41,57,58]. However, NFAT5 differs from the other NFAT members because its nuclear localization is not controlled by calcineurin, a calmodulin-dependent protein phosphatase [40]. Also, NFAT5 is the only NFAT that has been shown to be regulated by cellular hypertonicity, and it controls the expression of BGT-1, aldose reductase, and SMIT in response to changes in osmolarity [14,25,32,33,53,54,59]. This transcription factor was originally discovered because it is a regulator for two enhancer elements known as the tonicity-response enhancers (TonE) and osmotic-response elements (ORE) [24,32,37]. These two elements have been found in the regulatory domains of aldose reductase, SMIT, and BGT-1 [12,23,52]. Therefore, NFAT5 has also been named ORE-binding protein (OREBP) or TonE-binding protein (TonBP).

Due to the unique ability of freeze-tolerant wood frogs to tolerate freezing and cellular dehydration, we were interested in determining whether NFAT5/TonEBP plays a role in the regulation of osmoprotective genes during freeze-thaw stresses in *R. sylvatica*. We were particularly interested in whether the regulation of aldose reductase, SMIT, and BGT-1 by NFAT5 shows tissue-specificity. We hypothesize that this pathway will play a significant role in the kidney, if not the skeletal muscle and liver as well. A previous study from our lab demonstrated tissue specificity in osmoregulation during anoxia-recovery and dehydration-rehydration stresses in *R. sylvatica* through an NFAT5-mediated pathway [2]. Given that the role of NFAT5, BGT-1, SMIT, and aldose reductase in *R. sylvatica* has not been studied in the context of freeze tolerance, the results from this study further our knowledge about the potential mechanisms by which *R. sylvatica* tolerates whole-body freezing without incurring cellular damage.

2. Materials and methods

2.1. Animal care

All animal care and experimentation procedures were followed as described by Bansal and colleagues [3]. Freeze-tolerant wood frogs (*R. sylvatica*) were sampled from their natural habitats in the breeding ponds of wooded areas located near Oxford Mills, Ontario in Canada. The frogs were captured in the spring and delivered on ice to Carleton University, where they were washed using tetracycline. They were then placed in containers and the temperature was maintained at 5 °C. These conditions were maintained for two weeks before experimentation on the frogs began. The Carleton University Animal Care Committee approved all procedures followed during animal experimentation, animal care, and euthanasia, and this is covered under animal protocol no. 13,683.

2.2. Freezing treatment

Freezing experiments were performed as described by Aguilar et al. [1], where plastic containers held at -4 °C were used to hold 8-12 frogs for 45 min. This method facilitates the gradual decrease in frog body temperatures (T_b) and assists in ice nucleation. After the 45 min incubation, temperature was subsequently raised to -2.5 °C, and maintained for 24 h for the 24 h freezing experimental condition. Animals in the 24 h freezing group were quickly sampled at this time and temperature. Frogs in the other experimental group, the 8 h thaw group, were transferred from their containers at -2.5 °C to 5 °C, where they were allowed to defrost for 8 h. Pithing was used to euthanize all animals before tissue harvesting. Tissues from hindlimb thigh muscle, kidney, and liver were dissected and quickly frozen in liquid nitrogen before they were stored at -80 °C.

2.3. Total protein extraction

The preparation of total protein extracts for the 8 h thaw, 24 h freezing, and control skeletal muscle, liver, and kidney samples were carried out as described in [1]. Frozen samples from four different biological replicates were grinded under liquid nitrogen to attain approximately 0.5 g per sample, and were then combined with homogenization buffer (10 mM β-glycerophosphate, 0.1 mM EDTA, 20 mM Hepes, 10 mM NaF, 200 mM NaCl, 1 mM PMSF, 1 μL of protease inhibitor, and 1 mM Na_3VO_4 at pH 7.5) at a ratio of 1:2 w:v. Homogenization was then performed using a Polytron PT10 homogenizer for approximately 15–20 s. Soluble proteins were then separated via centrifugation for 10 min at 10,000 × g and 4 °C, the supernatants were collected. Supernatants were mixed with Bio-Rad reagent, and soluble protein concentrations were subsequently quantified using a MR5000 microplate reader at a wavelength of 595 nm. The control skeletal muscle samples, in addition to all the liver and kidney samples were standardized to 10 μg/μl by adding homogenization buffer. Freeze-thaw skeletal muscle samples on the other hand were standardized to 5 μg/μl. Protein homogenates were prepared for SDS-PAGE through a 1× dilution with 2× SDS loading buffer (10% v/v 2-mercaptoethanol, 0.2% w/v bromophenol blue, 20% v/v glycerol, 4% w/v SDS, 100 mM Tris-base, pH 6.8). The samples were subsequently boiled and stored in a -20 °C freezer until use.

Please cite this article in press as: Y. Zhang, et al., TonEBP/NFAT5 regulates downstream osmoregulatory proteins during freeze-thaw stress in the wood frog, Cryobiology (2017), https://doi.org/10.1016/j.cryobiol.2017.09.003