Comparative Biochemistry and Physiology, Part B 219–220 (2018) 1–9

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

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

A lesson from the oxidative metabolism of hibernator heart: Possible strategy for cardioprotection

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ARTICLE INFO

Keywords:
Hibernators
Heart
Mitochondria
Cardioprotection

ABSTRACT

In the present study we hypothesized that myocardial adaptive phenotype in mammalian hibernation involves rearrangement of mitochondria bioenergetic pathways providing protective pattern in states of reduced metabolism and low temperature. European ground squirrels (*Spermophilus citellus*) were exposed to low temperature (4 ± 1 °C) and then divided into two groups: (1) animals that fell into torpor (hibernating group) and (2) animals that stayed active and euthermic for 1, 3, 7, 12, or 21 days (cold-exposed group). Protein levels of selected components of the electron transport chain and ATP synthase in the heart increased after prolonged cold acclimation (mainly from day 7–21 of cold exposure) and during hibernation. Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) was also upregulated under both cold exposure and hibernating conditions. The phosphorylation state (Thr172) of 5′-AMP-activated protein kinase α increased early in cold exposure (at day 1 and 3) along with increased protein levels of phosphofructokinase and pyruvate dehydrogenase, whereas hypoxia inducible factor 1α protein levels showed no changes in response to cold exposure or hibernation. Hibernation also resulted in protein upregulation of three antioxidant defense enzymes (manganese and copper/zinc superoxide dismutases and glutathione peroxidase) and thioredoxin in the heart. Cold-exposed and hibernation-related phenotypes of the heart are characterized by improved molecular basis for mitochondrial energy-producing and antioxidant capacities that are achieved in a controlled manner. The recapitulation of such adaptive mechanisms found in hibernators could have broad application for myocardial protection from ischemia/reperfusion to improve hypothermic survival and cold preservation of hearts from non-hibernating species, including humans.

1. Introduction

Discovery of the mechanisms and triggers of cardioprotective events has been a topic of vigorous research over the last few decades. The concept of cardioprotection encompasses the regulation of adaptive processes in cardiac metabolism and function to allocate available fuel/energy/oxygen in order to preserve the viability of the heart under stress conditions. Mitochondria have been recognized as critical triggers, mediators and effectors of cardioprotective events and manipulation of the heart mitochondrial energy metabolism could help to overcome ischemia/reperfusion heart damage (Yabe et al., 1997; Crestanello et al., 2002; Novalija et al., 2003; Jovic et al., 2012). Among wide spectrum of mitochondrial derangements occurring in the ischemia-reperfusion heart, inhibition of respiratory complexes and the adenine nucleotide translocase, increased proton leak of the inner membrane and excessive generation of reactive oxygen species (ROS) contribute to impairment of energy production (Rouslin, 1983; Asimakis and Conti, 1984; Turrens et al., 1991; Borutaite et al., 1995). So, the well-established approaches for cardioprotection are mainly based on the protection from the consequences of electron transport chain inhibition during ischemia, i.e. ROS generation, Ca^{2+} overload as well as ATP depletion (Walters et al., 2012). To date, several methods including transient ischemia and anesthesia have been shown to induce energy-conserving phenotypes in the heart (Yabe et al., 1997; Crestanello et al., 2002; Novalija et al., 2003; Jovic et al., 2012).

Modern surgery utilizes mild hypothermia as one of the methods for increasing myocardial tolerance to oxygen and energy depletion. Hypothermia exerts its beneficial effects by delaying hypoxia-induced

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https://doi.org/10.1016/j.cbpb.2018.02.004
Received 21 November 2017; Received in revised form 13 February 2018; Accepted 22 February 2018

ATP decline and slowing down the subsequent injurious processes (Belzer and Southard, 1988). Underlying mechanisms of favorable actions of hypothermia is complex and involve many systemic and cellular changes that widely depend on original pathological and metabolic conditions. In species that undergo hibernation core body temperature drops by over 30 °C in a torpid state. Hence, hibernating mammals can be good models of natural myocardial protection mediated by low temperatures. Despite strong metabolic rate depression (often to < 5% of euthermic levels), the heart of the hibernators continues its vital role in maintaining blood circulation throughout the entire torpor/arousal cycle, although operating at lower body temperatures and with higher peripheral resistance than in euthermia (Wang, 1989). Several cardiac stress adaptations are known to occur in hibernators including improvement of gap junction function and Ca^{2+} handling (providing resistance to fibrillation/arrhythmias) and maintenance of Na^{+}/K^{+} ion homeostasis (Johansson, 1996; Kudej and Vatner, 2003; Yatani et al., 2004; Fedorov et al., 2008). Furthermore, Wickler et al. (1991) reported both hypertrophy of cardiac mass (for 21%) and increased citrate synthase activity. However, there are equivocal findings regarding respiration rate and oxidative capacity in heart during hibernation. Several metabolic studies performed on heart homogenates and heart slides showed increased, decreased and similar rates of substrate oxidation during hibernation and those differences were ascribed to the differences in the substrates used in the study (reviewed in Roberts and Chaffee, 1973). Also, the results from isolated heart mitochondria are not consistent; while South Jr (1960) has shown higher respiration rates from torpid animals, Brown and Staples (2014) have shown temperature-independent suppression of succinate-fueled mitochondrial respiration rates in heart during torpor. While there is no consensus of trend of changes in mitochondrial metabolism in hibernator's heart in torpor, several molecular analysis suggest mitochondrial and non-mitochondrial related ATP conserving phenotype of the heart in the preparation of the ground squirrel for winter heterothermy (Fahlman et al., 2000; Grabek et al., 2011). However, comprehensive and clear picture of molecular basis for energy-related mechanisms of cardioprotection in the physiological model of mammalian hibernation is still missing.

So, we aimed here to examine expression profiles of selected proteins involved in the electron transport chain and oxidative phosphorylation and key regulators of energy metabolism in the heart of the European ground squirrel (*Spermophilus citellus*) during hibernation. Furthermore, in the context of an adaptive preconditioning phenomenon the protein expression patterns of antioxidant enzymes were examined. In addition to hibernation, we aimed to reveal molecular adaptations during prolonged cold challenging as a state similar to transition period to hibernation (prehibernation).

2. Materials and methods

2.1. Animals

The experimental protocol was approved by the ethical committee for the treatment of experimental animals of the Institute for Biological Research, Belgrade, Serbia. Adult male European ground squirrels (*Spermophilus citellus*) were trapped during mid-July in Deliblatska peščara (southeastern part of Vojvodina, Serbia) and transported to the animal facility at the Institute for Biological Research, Belgrade, Serbia. Ground squirrels were housed in individual plastic cages at room temperature (22 ± 1 °C) and fed rodent chow, fresh carrots, and apples ad libitum until early September when one group of six animals continued to be maintained under these conditions (control group) and another animals were moved to a cold chamber, set to an ambient temperature of 4 ± 1 °C, with food and water ad libitum and on photoperiod of 12 h light:12 h dark. The hibernation state was monitored by daily rectal temperature measuring at approximately 10 a.m. Also the animals were checked visually every afternoon (approximately 6 p.m.) to determine if they were hibernating. Those animals showing body temperature of ~4 °C were considered torpid and six of these (hibernation group) were euthanized and heart sampled after each individual had been hibernating for 2–5 days. Control animals were sampled on the same day as the hibernating ones, in the middle of September. The squirrels kept at low temperature but that not enter into hibernation (stay active, euthermic) (~60%) were further separated in several cold exposed groups (each of six animals) that were euthanized and heart sampled at different time points: 1, 3, 7, 12, or 21 days. The last cold-exposed group was scheduled in the second half of September. All animals were euthanized by decapitation between 8 and 10 a.m. to avoid any cyclic daily variation in examined parameters. Hearts were removed within 3 min and the right part of the tissue, atrium and ventricle, was used for Western blot and spectrophotometric analysis. Tissue was quickly minced and washed with saline to remove all traces of blood. Samples were then snap-frozen in liquid nitrogen and stored at −80 °C until use.

2.2. Western blotting

Preparation of soluble protein extracts of tissues, SDS-polyacrylamide gel electrophoresis and Western blotting were conducted as described previously (Vasilijevic et al., 2010). The antibodies used were as follows: the Ndufa9 subunit of complex I (2.5 μg ml^{-1}; ab55521), succinate dehydrogenase of complex II (0.1 μg ml^{-1}, ab14715), ubiquinol cytochrome *c* reductase of complex III (1 μg ml^{-1}; ab14745), subunit IV of cytochrome *c* oxidase (COX IV, 1 μg ml^{-1}; ab14744), cytochrome *c* (2 μg ml^{-1}; ab18738), ATP synthase (0.8 μg ml^{-1}; ab14730), peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α, 1:1000; ab54481), hypoxia inducible factor-1α (HIF-1α, 1:2000; ab51608), phosphofructokinase (1:1000; ab119796), medium chain fatty acid acyl-CoA dehydrogenase (1:10000; ab92461), pyruvate dehydrogenase (1 μg ml^{-1}; ab84588), copper-zinc superoxide dismutase (CuZnSOD, 0.2 μg ml^{-1}; ab13498), manganese superoxide dismutase (MnSOD, 1:5000, ab1353), glutathione peroxidase (GSH-Px, 1:2000; ab16798), catalase (1:1000, ab1877), thioredoxin (Trx, 1 μg ml^{-1}; ab26320) and β-actin (1:1000; ab8226) (all purchased from Abcam, Cambridge, UK) and AMPKα phospho-Thr172 (2 μg ml^{-1}; 07–681) (Millipore International, Billerica, MA, USA). Quantitative analysis of immunoreactive bands was conducted with ImageQuant software (Uppsala, Sweden). Total band density was calculated as the sum of pixel intensities within a band (one pixel = 0.007744 mm^2). We averaged the ratio of dots per band for β-actin (the gel loading standard) from three independent experiments and then expressed changes in target protein expression as a percentage of euthermic control, which was standardized as 100%. Data were then statistically analyzed.

2.3. Measuring of enzymes activity

The part of the heart tissue was homogenized (Ultra/Turrax homogenizer, Janke and Kunkel Ka/Werke, Staufen, Germany, 0–4-C) in 0.25 M sucrose, 0.1 mM EDTA and 50 mM Tris–HCl buffer, pH 7.4, and the homogenates were sonicated (Takada et al., 1982).

Total superoxide dismutase (SOD) activity was examined by a modified method of Misra and Fridovich (1972). For determination of MnSOD activity, the assay was performed after preincubation with 4 mM KCN. The CuZnSOD activity was calculated as the difference between total SOD and MnSOD activities. Enzymatic activity was expressed in U mg^{-1} protein. SOD units were defined as the amount of the enzyme inhibiting epinephrine autooxidation under the appropriate reaction conditions. Catalase was assayed as suggested by the supplier (Sigma-Aldrich, St. Louis, MO, USA) and the activity is expressed in μM H_2O_2 min^{-1} mg^{-1} protein. GSH-Px was determined with t-butylhydroperoxide as a substrate (Paglia and Valentine, 1967) and the activity is expressed in nM NADPH min^{-1} mg^{-1} protein. Glutathione reductase (GR) activity was assayed as suggested by Glatzle et al.