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Research paper

Myocardial ER chaperone activation and protein degradation occurs due to synergistic, not individual, cold and hypoxic stress

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ABSTRACT

Environmental stress at high altitude affects the myocardium at the physiological and molecular level. Characterized by hypobaric hypoxia and low temperatures, the cumulative impact of these stressors on the protein folding homeostasis in the heart is yet unexplored. The present study evaluates the collective effect of cold and hypoxia on the myocardial protein oxidation and activation of the endoplasmic reticulum (ER) stress response. Adult rats were exposed to either a singular acute stress of cold (10 °C; C), hypobaric hypoxia (7620 m; H) or simultaneously to both cold and hypobaric hypoxia (CH) for 6 h. Hypoxic stress amplified the free radical generation in H and CH groups, leading to enhanced HIF-1α expression. Coupled to cold stress, reduced oxygen availability caused substantial protein oxidative modifications, as well as cardiac tissue injury and matrix remodeling, evident in the histological staining. Presence of oxidized proteins caused a significant upregulation in expression of ER chaperones GRP78 and PDI in the cold hypoxia exposed animals. Enhanced proteolytic activity signaled the removal of misfolded proteins. Linked intricately to cellular stress response, cell survival kinases were expressed higher in CH group; however apoptotic CHOP (C/EBP homologous protein) expression remained unaltered. Administration of ER stress inducer, tunicamycin along with cold hypoxic stress, caused a discernible increase in protein oxidation and GRP78 expression, along with a significant elevation in proteasome and apoptotic activity. Highlighting the significance of a synergistic, rather than individual, effect of low oxygen and temperature on the protein folding machinery, our study provides evidence for the activation of ER stress response in the myocardium under acute high altitude stress.

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1. Introduction

High altitude conditions are emphasized by diminished ambient oxygen pressure, decreased temperature, lower humidity, and increased ultraviolet radiation. These hostile environmental conditions require a high degree of physiological and metabolic adaptations to preserve function and ensure long term survival; indeed prolonged exposure may have adverse medical consequences for those inhabiting high altitude regions. Hypoxia induces adaptive changes in the myocardium at systemic and cellular levels, thereby having a profound effect on the morphology and function of the cardiopulmonary system [1]. Physiologically, this leads to an increase in heart rate, myocardial contractility, and cardiac output. Chronic high altitude exposure can cause an increase in hypoxic pulmonary vasoconstriction, with the remodeling of the pulmonary arterioles, leading to pulmonary hypertension and right ventricular hypertrophy [2].

Growing evidence has shown the exacerbated production of reactive oxygen species (ROS) generation under acute hypoxic condition leading to oxidative damage to proteins, lipids and nucleic acids [3–5]. The fall in temperature in the high altitude milieu can also disrupt the physiological systems in the body. Although cold exposure decreases the metabolic rate, it also alters the microcirculation and affects the oxygen supply to the tissues. While mild hypothermia has been shown to be protective against myocardial ischemic injury and acute hypoxic stress [6–10], chronic low temperatures have been shown to have a marked impact on the cellular redox homeostasis [11,12]. Despite the evident decrease in temperatures as one ascends to high altitude, the cumulative effect of hypoxia and cold has been unexplored in most studies elaborating the impact of such environmental conditions on the mammalian physiology. A study by Templeman et al., in 2010 identified the cardiac ventricular remodeling that occurs in response to chronic hypoxia and cold [13]. They found that the cardiac muscles respond

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to the two stressors simultaneously but not additively, when exposed to environmentally realistic hypoxic cold.

Enhanced free radical generation orchestrates a shift in the redox status and alteration in the protein homeostasis in the cardiac tissue. In response, there occurs a reprogramming in the protein content of the heart, involving the upregulation of specific proteins and a general down-regulation of myocardial protein synthesis [14–16]. Stadtman in 1986 [17] proposed that partial oxygen pressure influences the rate of protein turnover and oxidative modifications of amino acids, having a considerable impact on cellular homeostasis. High altitude training increased the levels of protein carbonyl derivatives, known indicators of oxidative modifications of proteins, in skeletal muscle of rats [18]. Altered skeletal muscle protein turnover under chronic hypobaric hypoxia, accompanied by an upregulation in the ubiquitin–proteasome system and calpain activity, was recently shown by our group [19].

In the endoplasmic reticulum (ER), the site for protein folding, there are adaptive programs to detect the misfolding of protein and mediate comprehensive defense signals to remove such aberrant proteins and if needed, increase the folding capacity of the cell. Such sophisticated cellular mechanisms, known collectively as protein quality control, are highly sensitive to even minor perturbations in the redox state of the cell [20]. An accumulation of misfolded proteins in the ER lumen results in activation of the unfolded protein response (UPR) [21]. Having been implicated in myocardial ischemia, hypertrophy, cardiomyopathy and heart failure, the UPR causes simultaneous activation of both adaptive and pro-apoptotic pathways to deal with the load of improper folding in a number of cardiac pathophysiologies [22–25]. Recent evidence has indicated that this highly ubiquitous response is activated in pulmonary hypertension, associated with high altitude exposure, and its attenuation may be a novel therapeutic target for the treatment of high altitude pathologies [26,27].

Although each stress paradigm at high altitude affects the myocardium, low oxygen tension and temperature, singularly and in a synergistic manner, may cause a significant disruption in the myocardial proteostasis. The present study was performed to identify the effect of such simulated stress conditions of cold and hypobaric hypoxia, individually and in combination, on the protein oxidation within the heart and the subsequent activation of the cytoprotective responses to alleviate the misfolded protein load. We show that the protein homeostasis is most substantially altered on exposure to simultaneous cold hypoxic stress and the interference in ER function leads to the activation of ER stress response in the heart under such conditions.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals, including tunicamycin, were obtained from Sigma (St. Louis, MO, USA) and were at least analytical grade. All antibodies were from Sigma (St. Louis, MO, USA) and Santa Cruz Biotechnology (Santa Cruz, Ca, USA). Nitrocellulose membrane was from Millipore (Millipore, Billerica, USA). X-ray films were purchased from Kodak (Kodak, Rochester, NY, USA).

2.2. Study design

2.2.1. Ethical clearance for animal study

Male Sprague–Dawley rats (150 ± 15 g) were used for all experiments. Animals were maintained under a 12-h light–dark cycle at temperature 24 ± 2 °C in the Institute's animal house facility. The study was approved by the Animal Ethical Committee of the institute in accordance with Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) of the Government of India.

2.2.2. Experimental design

The rats were randomly divided into four groups with $n = 8$ in each group – Group 1: unexposed normoxic (N), Group II: exposed to cold (C, 10 °C), Group III: exposed to hypobaric hypoxia (H, 7,620 m) and Group IV: exposed to cold & hypobaric hypoxia (CH, 10 °C & 7,620 m), each for 6 h duration. Simulated high altitude exposure was performed in an animal decompression chamber maintained at pressure of 282 torr (equivalent to an altitude of 7620 m, 8% oxygen), coupled to mercury barometer, at 25 °C for hypoxic group and at 10 °C for cold hypoxic group (Decibel Instruments, India). The airflow in the chamber was 2 L/min with relative humidity maintained at 50–55%. Control group rats were maintained in the normoxic condition within the same laboratory.

To assess the contribution of ER stress response to simultaneous cold hypoxic stress in the myocardium, we administered tunicamycin (Tm, 0.3 mg/kg bw) intraperitoneally, freshly prepared as a 0.05 mg/ml suspension in 150 mM dextrose [28]. Animals were categorized randomly as control, only tunicamycin (Tm only), cold hypoxia and exposure to cold hypoxic stress after tunicamycin administration (Tm + cold hypoxia), with $n = 6$ animals per group.

2.3. Oxidative stress markers

After exposure, animals were anaesthetized using sodium pentobarbital. Hearts from control and exposed animals were rapidly excised and used fresh or snap frozen in liquid nitrogen and stored at −80 °C for further use. For biochemical estimations the tissue were homogenized in 0.154 M KCl–EDTA buffer.

2.3.1. ROS measurement

ROS levels were measured with a nonfluorescent lipophilic dye, dichlorofluorescein diacetate (DCFH-DA). DCFH-DA passively diffuses through cellular membranes where it is cleaved into 2, 7-dichlorofluorescein (DCF) by the action of intracellular esterases and thus fluoresces in the presence of ROS. The production of free radicals was determined as described earlier [29]. Briefly, 150 μl of heart homogenate was incubated with 10 μl of 100 μM DCFH-DA for 30 min in dark. Fluorescence was read using a fluorimeter (Perkin Elmer, UK) with excitation at 485 nm and emission at 535 nm. Readings were obtained in arbitrary fluorometric units and results expressed as fold change in free radical generation.

2.3.2. Lipid peroxidation

Lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form more complex and reactive compounds such as Malondialdehyde (MDA). Lipid peroxidation was measured by direct estimation of MDA using the modified method of Buege and Aust [30]. In brief, 100 mg tissue was homogenized in 15% (w/v) TCA. 0.355% (w/v) TBA was added to supernatant and incubated in boiling water bath for 30 min. Following which, the absorbance was read at 535 nm using UV–vis spectrophotometer (BioRad, USA). The malondialdehyde levels were expressed as μmol/ml.

2.4. Histopathological studies

For histological studies, after the hypoxia and cold exposure, heart was excised and immediately fixed in 10% buffered neutral formalin solution. The fixed tissues were embedded in paraffin and serial sections (4 μm thick) were cut using microtome (Leica RM 2125, Germany). Each section was stained with hematoxylin and eosin (H and E). The sections were examined under the light microscope (Nikon Tokyo, Japan) and photomicrographs were taken.

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