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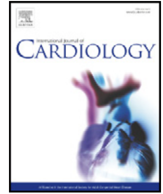

Differential activation of myocardial ER stress response: A possible role in hypoxic tolerance

Kanika Jain [1], Geetha Suryakumar *,[1], Rajendra Prasad [1], Lilly Ganju [1]

Cellular Biochemistry Division, Defence Institute of Physiology and Allied Sciences, Lucknow Road, Timarpur, Delhi 110054, India

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A B S T R A C T

Background: Low oxygen availability in the high altitude milieu causes adverse physiological and pathological consequences to the cardiopulmonary system. A key role is played by proteins in maintaining optimal cardiac function under stress. Differential response to hypoxia may be linked to the susceptibility of proteins to free radical induced modifications. The present study was designed to understand the significance of protein oxidation and ER stress in the myocardial response to hostile environments.
Methods: Sprague–Dawley rats were exposed to simulated hypoxia equivalent to 223 mm Hg pressure, screened on the basis of time taken for onset of a characteristic hyperventilatory response and categorized as susceptible (<10 min), normal (10–25 min) or tolerant (>25 min). Protein modifications and activity of cellular proteolytic enzymes were assayed in myocardial tissue extracts to identify alterations in protein homeostasis. To evaluate the ER stress response, expression of various ER marker chaperones was investigated.
Results: Susceptible animals displayed a distinct increase in protein oxidation and intracellular thiol content. They showed higher expression of ER stress hallmarks, GRP78, PDI and ERO1α, and exhibited a greater activation of the proteasome and calpain proteolytic systems, associated with elevated oxidized proteins. While a marked upregulation in the prosurvival signaling cascade PI3K/Akt/mTOR was observed in tolerant animals, the expression of pro-apoptotic caspase-3 and CHOP remained unaltered.
Conclusion: Thus, higher susceptibility to hypoxia is linked to a disruption in the proteostasis and activation of the ER stress response. Enhanced tolerance to hostile environments may be contributed by better maintenance of protein folding homeostasis.

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1. Introduction

The mammalian heart is an obligate aerobic organ, requiring a constant supply of oxygen for its optimum physiological function. Having the maximum oxygen consumption among all body organs, the cardiac tissue is highly vulnerable to the lack of oxygen. While pathophysiological conditions such as ischemia/reperfusion injury, coronary artery diseases, atherosclerosis and myocardial infarction affect the oxygen supply to the heart, hostile environmental conditions at high altitude cause high levels of physiological and metabolic demands on the heart. A fall in oxygen availability or hypobaric hypoxia induces adaptive changes in the myocardium at the cellular and systemic levels, distinctly affecting the morphology and function of the cardiopulmonary system. Prolonged high altitude exposure has been shown to increase blood pressure and cardiac output, accompanied by pulmonary arterial remodeling. Chronic hypoxia can even lead to the development of pulmonary hypertension and subsequent right ventricular hypertrophy and heart failure in some individuals [1].

An increase in free radical generation in the high altitude milieu has been well observed in a number of studies; indeed the ensuing oxidative stress has been implicated in the physiological as well as pathological consequences of hypoxic exposure [2–4]. A shift in the redox potential can cause stimulation or impairment of key biological cascades, regulated intricately by proteins. Earlier studies have brought to light the effect of reactive oxygen species on proteins such as nitrosylation of tyrosine, glutathiolation, and formation of amino acid oxidation adducts [5–7]. Evidence of elevated protein nitration and carbonylation has been shown under high altitude conditions in previous studies on both humans and animals [8–10].

Any modification to protein structure is sensed by the endoplasmic reticulum (ER), the central organelle entrusted with the essential task of protein synthesis, folding and maturation. Within the ER, the highly complex protein folding process is redox dependent and highly sensitive to the presence of reactive oxygen species [11]. Assisting at every crucial stage of the folding process in the ER, the chaperones use ingenious mechanisms to promote efficient folding while preventing protein aggregation [12,13]; the cell thus maintains a finely tuned protein

* Corresponding author. Tel.: +91 11 23883311; fax: +91 11 23914790.
E-mail address: geethasuryakumar@yahoo.com (G. Suryakumar).
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homeostasis or proteostasis [14]. Interruption of ER homeostasis by glucose or energy deprivation, enhanced protein trafficking, oxidative stress and altered calcium homeostasis, compromises the proper functioning of the ER [15]. Numerous studies have indicated the initiation of a specialized signaling pathway, the unfolded protein response (UPR), in presence of misfolded proteins in the ER. The activation of this cascade leads to an upregulation of ER chaperones and folding enzymes, along with a transient attenuation of nascent protein synthesis [15]. In addition, the degradative capacity of the cell is increased to assist in the turnover of misfolded proteins; prolonged or severe stress may even lead to activation of apoptotic cascades. Collectively, these multifaceted cytoprotective and cytodestructive responses constitute the ER stress response. Although it may act as a defense mechanism to combat against external insult, excessive ER stress often participates in a wide array of pathophysiological process. A number of diseases have shown the manifestation of this highly ubiquitous mechanism as the underlying cause [14,16,17]. Evidence has suggested a rather complex interplay between ER stress and oxidative stress in cardiac pathologies including ischemia/reperfusion injury, hypertrophy and heart failure [15,18–20]. Although ER stress may function as both a cause and a consequence of altered redox status, its role in the context of high altitude environs is only recently being explored [21,22].

Individuals differ in their ability to respond to environmental stressors. Along with the genetic adaptations likely playing a role in shaping the morphological and physiological differences [23]; each individual has an inherent threshold of stress which it can deal with. We have recently shown that although animals with better endurance for low oxygen tensions displayed enhanced antioxidant and cytoprotective responses, significantly higher levels of oxidized proteins were present in organisms with greater vulnerability to hypoxic stress [24]. The enhanced free radical generation in such animals may play a part in disrupting the folding homeostasis in the ER. The present study was designed to understand the contribution of myocardial oxidative protein modifications and the physiological ER stress response to the differential ability of organisms to endure acute sub lethal hypoxic stress. An intricate interplay between the activation of pro-survival pathways and maintenance of protein folding homeostasis may be central to the susceptibility or tolerance to adverse environmental milieu.

2. Materials & methods

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 °C ± 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 of Experiments on Animals (CPCSEA) of the Government of India.

2.2. Hypobaric hypoxia exposure

Hypoxic tolerance was determined by measuring the time taken for the onset of gasping (GT). Adult male Sprague–Dawley rats were exposed, one at a time, to simulated hypobaric hypoxia of 9754 m at 32 °C in an animal decompression chamber (Decibel Instruments) coupled to a mercury barometer (223 mm Hg pressure) at 32 °C [25,26]. All the decompressions and recompressions were achieved gradually at a rate of 600 m (≈40 mm Hg)/min to prevent any tissue injury to the organism as a result of a sudden fall or rise in ambient pressure. The airflow in the chamber was 2 l/min, while the relative humidity was maintained at 40% to 50%. The time taken for appearance of the first sign of gasping was recorded using an electronic stopwatch. Onset of gasping was identified as a hyperventilatory response, accompanied by a visible difficulty in breathing and enhanced breathing rate, beyond which time, if exposed, there occurred a cessation of breathing in the animal. Based on their gasping time after three consecutive exposures to identify their tolerance to hypoxic stress, animals were categorized into three groups ($n = 7$ animals per group): normal (10–25 min, N), tolerant (>25 min, T) and susceptible (<10 min, S), as described previously [24]. Unexposed control group rats were maintained in the normoxic condition within the same laboratory conditions. After the hypoxic exposure, the animals were anesthetized under sodium pentobarbital and tissues collected. The hearts were perfused in saline and used fresh or snap frozen in liquid nitrogen and stored at −80 °C for further use.

2.3. Fluorescence measurement of protein oxidation

Isolated frozen hearts were thawed and homogenized in 0.3 M sucrose and 10 mM HEPES, pH 7.4. Homogenate was centrifuged (1200 ×g for 10 min) to remove the debris (total membrane fraction). Protein concentration was determined by the Bradford method [27]. Fluorescence measurements were performed in solution containing 50 μg of homogenate protein per ml, 10 mM HEPES, and 100 mM KCl, pH 7.0 at 25 °C on a Perkin-Elmer fluorimeter.

Fluorescence emission spectra (300 to 450 nm, slit width 2 nm) of tryptophan were measured with excitation at 295 nm (slit width 1 nm) [28]. Fluorescence emission spectra of bityrosine, product of tyrosine oxidation, were recorded in range of 380 to 440 nm, at excitation wavelength of 325 nm [29]. Emission spectra of Lys conjugates with lipid peroxidation products (425 to 480 nm) were recorded at 365 nm and excitation spectra of conjugates of Lys (325 to 380 nm) were measured with emission at 440 nm [28].

2.4. Protein sulfahydryl

The concentrations of total SH-groups (T-SH), protein SH-groups (P-SH) and non protein SH-groups (Np-SH) were estimated based on their reactivity with 5,5′-dithiobis (2-nitrobenzoate) (DTNB) by the method of Sedlak and Lindsay [30]. For determination of T-SH, a 0.125 ml aliquot of homogenate was added to 0.375 ml of standard incubation medium (40 mM Tris, 2 mM EDTA, 100 mM KCl, pH 8.0). After addition of 25 μl of DTNB (10 mM in methanol) and 2.5 ml methanol, the mixture was incubated for 30 min and centrifuged at 2500 rpm for 10 min. The concentration of Np-SH was determined after addition of 0.5 ml homogenate to 1.75 ml H_2O and 0.25 ml trichloroacetic acid (50%). Following centrifugation, 25 μl DTNB and 1 ml 0.4 M Tris (pH 8.9) were added to 0.5 ml supernatant and incubated for 5 min. The absorbance of the 2-nitro-5-mercaptobenzoic acid formed was measured at 412 nm. GSH served as a standard. The concentration of P-SH was calculated by subtracting values for Np-SH from that of T-SH.

2.5. Protein degradation

2.5.1. 20S proteasome activity

Degradation via the proteasome pathway was studied by assaying the chymotrypsin-like enzyme activity of 20S proteasome, as described earlier [31]. The fluorigenic peptide Succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-AMC), obtained from Sigma (St. Louis, MO, USA), served as substrate for the chymotrypsin-like activity. Small portion (~50 mg) of tissue was homogenized in buffer containing 50 mM Tris–HCl, 1 mM DTT, 5 mM $MgCl_2$, 5% glycerol and 5 mM ATP. The homogenates were incubated for 30 min at 37 °C in 50 μl of a buffer containing 100 mM Tris–HCl (pH 8.0), 1 mM DTT, 5 mM $MgCl_2$, 1 mM Suc-LLVY-AMC, 2 mg/ml ovalbumin, and 0.07% SDS. The reaction was terminated by 25 μl of 10% SDS and diluted by 2 ml of 0.1 M Tris–HCl (pH 9.0). Fluorescence of the liberated amido methylcoumarin was monitored in a Perkin-Elmer fluorimeter at excitation at 380 nm and emission at 460 nm. Chymotrypsin-like enzyme activity was expressed as arbitrary units per minute per milligram of protein.

2.5.2. Degradation of protein tryptophan and tyrosine residues

Frozen tissues were homogenized in ice-cold phosphate buffer (0.1 M, pH 7.4) containing 140 mM KCl, 1 mM EDTA, and 1 mM PMSF. The homogenate was centrifuged at 960 *g* for 10 min and the supernatant was used. All steps were carried out at 4 °C. Sodium dodecyl sulfate was added to sample aliquots (final concentration 0.1%). The tryptophan content within solubilized proteins was determined fluorimetrically at excitation and emission wavelengths of 280 and 345 nm, respectively [32]. The tyrosine content within solubilized proteins was determined fluorimetrically at excitation and emission wavelengths of 277 and 320 nm, respectively [33].

2.5.3. Calpain assay

Activity of calpains, calcium activated proteases, was measured in the homogenate using N-succinyl-Leu-Tyr-7-amido-4-methylcoumarin (SLY-AMC) as a substrate [34]. A stock solution of 50 mM SLY-AMC (Sigma, St. Louis, MO, USA) was prepared in dimethyl sulfoxide and stored at −20 °C. Hearts were homogenized in buffer having 50 mM Tris–HCl, 150 mM NaCl, 10 mM NaH_2PO_4, 1% Nonidet P-40, and 0.4 mM sodium orthovanadate. Homogenate was then centrifuged at 13,000 *g* for 15 min at 4 °C. The following procedure was used for measuring calpain activity in tissue extracts: 30 μl heart tissue extract was incubated for 60 min at 37 °C in a buffer solution (pH 7.4) containing 25 mM HEPES, 0.1% CHAPS, 10% sucrose, 10 mM DTT, and 0.1 mg/ml bovine serum albumin. After addition of 5 μl of the substrate solution, buffer was added to adjust the volume of the assay to 2 ml. Fluorescence of the liberated AMC was monitored in a Perkin-Elmer fluorimeter (LS-45) at excitation at 380 nm and emission at 460 nm. Calpain activity, Ca^{+2} dependent cleavage of SLY-AMC, was expressed as arbitrary units of AMC released per milligram of protein.

2.6. Caspase-3 substrate cleavage assay

Caspase-3 is an enzyme activated during the induction of apoptosis. To measure its activity in the cardiac tissue, a colorimetric substrate Ac-Asp-Glu-Val-Asp p-Nitroaniline, Ac-DEVD-pNA (Sigma, St. Louis, MO, USA) was used, as previously described [35]. Heart tissue was homogenized in 10 volume of ice-cold lysis buffer (50 mM HEPES, pH 7.4, 0.1% CHAPS, 5 mM DTT, 2 mM EDTA, 2 mM EGTA, 0.1% Triton X-100) containing 1 mM PMSF and protease inhibitors. Homogenates were centrifuged at 14,000 *g* for 10 min

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