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ORIGINAL PAPER

Differential hypoxic tolerance is mediated by activation of heat shock response and nitric oxide pathway

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Abstract The fall in ambient oxygen pressure in high-altitude milieu elicits a wide range of physiological responses in the myocardium, which may differ from individual to individual. This condition, known as hypobaric hypoxia, invokes the cardioprotective heat shock response. The present study focuses on the role played by this ubiquitous response in mediating a differential tolerance to acute hypoxic stress. Sprague Dawley rats were exposed to simulated hypoxia equivalent to 223 mmHg 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). The tolerant animals displayed a significant upregulation of heat shock protein (Hsp)70/HSPA, evident through immunohistochemical staining of the cardiac tissue. The increased expression of transcription factor heat shock factor-1 led to the downstream activation of other chaperones, including Hsp90/HSPC, Hsp60/HSPD1, and Hsp27/HSPB1. The higher induction of HSPs in tolerant animals contributed to higher nitric oxide synthesis mediated by both endothelial nitric oxide synthase and inducible nitric oxide synthase activation. Conversely, susceptible animals showed significantly higher expression of the proinflammatory markers tumor necrosis factor alpha and nuclear factor kappa-light-chain enhancer of activated B cells in the myocardium. Evaluation of circulatory stress markers identified increased levels of reactive oxygen species, corticosterone and endothelin-1 in the susceptible animals highlighting their vulnerability to hypoxic stress. The heat shock response, through the action of chaperones and enhanced NO generation thus contributes substantially to the ability to sustain survival under acute sub lethal hypoxia.

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Introduction

Pathophysiological stress imposed by cardiovascular diseases, neurological disorders, muscular dysfunction as well as genetic mutations, are now known to affect protein structure and folding. Several interlinked pathways are engaged in restoring the cellular homeostasis, but one of the major well-identified mechanism that acts as the first line of defense against cellular injury, is the response mediated by the heat shock family of stress proteins (heat shock proteins (HSPs)) (Barral et al. 2004). Classically termed as the heat shock response (HSR), it is a universal response, which plays a critical role in extenuating acute as well as severe challenges from internal or external environmental stimuli that pose a lethal threat to survival of the organism (Benjamin and McMillan 1998; Latchman 2001).

These chaperones modulate the response to stress through various downstream signaling pathways including the nuclear factor kappa-light-chain enhancer of activated B cells (NF-kB) and nitric oxide (NO) production pathways. In the heart, NO is a known regulator of cardiac contractility under physiological conditions (Ziolo et al. 2008). In cardiomyocytes, all three isozymes of NO synthase (NOS) are expressed–neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2), and endothelial NOS (eNOS, NOS3) (Jones and Bolli 2006). Activation of the NOS isoforms involves the HSR (Hsp90-eNOS binding) as well as the hypoxia induced upregulation (HIF 1α- iNOS) (Jung et al. 2000; Chen and Meyrick 2004). In fact, acute hypoxia has been implicated in the upregulation of all three NOS isoforms, mediating a cytoprotective effect through increased NO generation (Gess et al. 1997; Felaco et al. 2000; Jung et al. 2000; Shi et al. 2000).

The activation of the inflammatory response is known to occur under oxidative stress, a hallmark of several cardiovascular diseases (Jones et al. 2003). Under the altered redox status of the cell, the transcription factor NF-κB, constitutively inhibited by IκB, is released from its inhibitor and translocates to the nucleus. It regulates the transcription of downstream inflammatory mediators such as iNOS and inflammatory cytokines (Gordon et al. 2011). Primarily a cytoprotective mechanism, inflammatory processes can turn into apoptosis-inducing stimuli, leading eventually to cell death. Indeed while the upregulation of Hsp70 (HSPA) has been shown to prevent the nuclear translocation of NF-κB, lowering NO production, inflammation itself induces the expression of Hsp70 (Jones et al. 2003; Chen et al. 2007).

HSR has been shown to play cardioprotective role against various pathological conditions including myocardial infarction, ischemia/reperfusion injury and heart failure (Willis and Patterson 2010). At higher altitudes, the fall in oxygen availability, termed as hypobaric hypoxia, elicits a wide range of physiological and metabolic responses in an organism. The heart, being an obligate aerobic organ, undergoes distinctive remodeling to cope with the change in the external environment. Numerous studies have brought to fore how prolonged hypoxic conditions lead to an increase in the blood pressure and cardiac output, followed by cardiopulmonary remodeling and right ventricular hypertrophy (reviewed by Bartsch and Gibbs 2007). While the response to any stressor differs from individual to individual, we have earlier shown a key role played by the upregulation of cellular cytoprotective mechanisms in arbitrating an enhanced hypoxic tolerance in the rodent myocardium (Jain et al. 2013a). Indeed several research trends have shown the evidence of adaptive traits in high-altitude natives, which contribute significantly to their better survival as compared with the lowlanders (reviewed by Beall 2013). In fact, recent studies have brought to light the significance of protein folding homeostasis and chaperones under acute and chronic hypoxia as well as high-altitude-induced maladies like pulmonary hypertension (Yeager et al. 2012; Dromparis et al. 2013; Jain et al. 2013b, c).

While the cardioprotective role of HSPs and NO pathways is well studied in myocardial dysfunction, the study brings to light for the first time the effects of differential HSR activation on hypoxic tolerance. Our study highlights the significance of this global response in increasing the ability of an organism to survive under acute environmental stress through a differential modulation of the NO and inflammatory signaling pathways.

Materials and methods

Animals

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 of Experiments on Animals (CPCSEA) of the Government of India.

Hypobaric hypoxia exposure

Hypoxic tolerance was determined by measuring the time taken for the onset of gasping (GT). Adult male Sprague Dawley rats were randomly selected and exposed, one at a time, to simulated hypobaric hypoxia of 9,754 m at 32 °C in an animal decompression chamber (Decibel instruments) coupled to a mercury barometer (equivalent to 223 mmHg pressure). All the decompressions and recompressions were achieved gradually at a rate of 600 m (≈40 mmHg)/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, a characteristic hyperventilatory response, was recorded using an electronic stopwatch. Based on their gasping time, animals were categorized into three groups (n= 6 animals/group): normal (10–25 min), tolerant (>25 min), and susceptible (<10 min), as described previously (Jain et al. 2013a, b). Unexposed control rats were maintained in the normoxic condition within the same laboratory conditions. Following an interval of 1 week, the animals were again exposed to the same altitude of 9,754 m to assess their tolerance or susceptibility to acute sub lethal hypoxia. In total, the animals were exposed for three consecutive weeks. Once the animals showed the onset of the characteristic hyperventilatory response, depending on their tolerance or susceptibility to hypoxic stress, they were brought down to normoxic levels at the normal rate of descent (~600 m/min) and killed immediately at the end of the third exposure. After the hypoxic exposure, the animals were anesthetized under sodium pentobarbital and tissue collected. The hearts were perfused in saline and used fresh or snap frozen in liquid nitrogen and stored at −80 °C for further use.

Immunohistochemical staining

Right ventricles of the heart were removed, fixed by 4 % formalin in PBS. Following which the tissues were cryoprotected for 24 h first with 15 % sucrose, followed by 30 % sucrose. Cryosections (8 μM) were cut and washed with 0.1 M PBS. After immersion in antigen unmasking solution, the endogenous peroxidase was inhibited by incubation with 3 % hydrogen peroxide in methanol for 30 min. Sections on slides were washed in PBS and incubated in blocking solution (10 % normal goat serum in PBS) for 1 h at room temperature. Sections were then incubated with the primary antibodies:

mouse monoclonal Hsp70 antibody at 1:100 (Sigma, USA cat. no. H5147) diluted in blocking solution, overnight at 4 °C. After washing with PBS, sections on slides were incubated with biotinylated goat anti-mouse IgG secondary antibody (2 h; 1:200; Sigma, USA cat. no. B8520), followed by the Avidin–Biotin-conjugated horseradish peroxidase complex solution for 60 min. Sections on slides were then washed and stained in 3,3-diaminobenzidine for 5 min and 0.03 % H_2O_2 for 5 min. Images were acquired using a microscope (Leica 2000).

HSF-1 ELISA

Expression of nuclear transcription factor heat shock factor-1 (HSF-1) was quantified in the perfused rat heart tissue homogenate, using commercially available kit, rat HSF-1 ELISA (Cusabio, Wuhan, China) as per manufacturer's instructions. Nuclear extracts of the heart tissue were prepared fresh in ice-cold 20-mM Tris–HCl, 150 mM NaCl, 2 mM KCl, 2 mM EDTA, and 2 % NP-40 buffer, fortified with protease inhibitors. The homogenates were centrifuged at 15,000×*g* for 10 min. The tissue homogenates were diluted 1:5 using the sample diluent provided in the kit. The concentrations of HSF-1 were expressed as nanograms per milligram of protein.

Dot blotting

Total protein samples (5 μg each) were dotted onto nitrocellulose membranes, and dot blot analyses were performed. In brief, the membranes were blocked for 1 h at room temperature with 5 % BSA in TBS (10 mM Tris–HCl, pH 7.4, 150 mM NaCl) with 0.1 % Tween 20 and then incubated with the primary antibodies (Sigma monoclonal anti-mouse Hsp70, cat. no. H5147 and Hsp60, cat. no. H3524) in 3 % BSA in TBS/0.1 % Tween 20, overnight at 4 °C. Membranes were washed in TBS/Tween 20 (0.1 %) and then incubated with anti-mouse horseradish peroxidase-conjugated secondary antibody (Sigma cat. no. A9044) in 3 % BSA in TBS/0.1 % Tween 20 for 1 h. After washing, immunocomplexes were developed by using an enhanced 3′3′-diaminobenzidine development system (Sigma).

Hsp27 ELISA

Expression of Hsp27 was quantified in the perfused rat heart tissue homogenate, using commercially available kit, rat Hsp27 ELISA (Cusabio, Wuhan, China) as per manufacturer's instructions. The tissues were freshly homogenized in 1× PBS, and the supernatants were diluted 1:5 using the sample diluent provided in the kit. The concentrations of Hsp27 were expressed as nanograms per milligram protein.

Nitrite levels

Nitrite, a biological metabolite of NO, was measured in rat tissue homogenates using Griess reagent (Barrias et al. 2002). Briefly, tissue supernatant (100 μL) was mixed with an equal volume of Griess reagent (Sigma, USA) and incubated for 15 min in dark. The absorbance was measured at 540 nm. A standard curve was prepared by using different dilutions of sodium nitrite with each assay. The results were expressed in nanomoles per milliliter sodium nitrite.

eNOS ELISA

Activity of eNOS was quantified in the perfused rat heart tissue homogenate, using commercially available kit, rat eNOS ELISA (Cusabio, Wuhan, China) as per manufacturer's instructions. The tissues were freshly homogenized in 1× PBS, and the supernatants were diluted 1:100 using the sample diluent provided in the kit. The concentrations of eNOS were expressed as micro-international units per milliliter.

TNF-α ELISA

Proinflammatory cytokine, tumor necrosis factor alpha (TNF-α) levels, were quantified in the freshly prepared heart tissue homogenate, using commercially available kit, rat TNF-α ELISA (eBiosciences, CA, USA) as per manufacturer's instructions. The concentrations of TNF-α were expressed as picograms per milligram of protein.

ROS measurement

2′,7′-dichlorofluorescein diacetate (DCFH-DA) was used as a fluorescent probe to measure the rate of oxidant production in the fresh plasma samples according to the modified method of LeBel et al. (1999). Whole blood was collected following exposure, using heparin as anticoagulant. The samples were centrifuged at 3,500 rpm for 15 min within 30 min of collection and plasma collected. Briefly, 150 μL of rat plasma 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 flourometric units and results expressed as fold change in free radical generation.

Corticosterone ELISA

Levels of stress hormone, corticosterone were quantified in the rat plasma, using commercially available kit, rat/mouse Corticosterone ELISA (DRG, Germany) as per manufacturer's instructions. For the assay, the plasma samples were

diluted 1:5 using the sample diluent provided in the kit. The concentrations of corticosterone were expressed as nanograms per milliliter.

Endothelin-1 content

Expression of endothelin-1, a potent vasoconstrictor released from the endothelium in hypoxic stress, was quantified in the rat plasma, using commercially available kit, rat endothelin-1 ELISA (Cusabio, China) as per manufacturer's instructions. The tissue homogenates were diluted 1:20 using the sample diluent provided in the kit. The concentrations of endothelin-1 were expressed as picograms per milliliter.

Erythropoietin content

Erythropoietin was quantified in the rat plasma using commercially available kits, rat/Mouse Erythropoietin Immunoassay (R&D Systems, UK) as per manufacturer's instructions. The concentrations of the hormone in plasma were expressed as picograms per milliliter.

Immunoblotting

i. Preparation of nuclear and cytosolic extract
   For nuclear and cytosolic fractionation, frozen heart tissue was homogenized in an ice-cold buffer (0.5 M sucrose, 10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 10 % glycerol, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF with protease inhibitors). Homogenates were kept on ice for 15 min, 0.6 % Nonidet P-40 added, and then centrifuged for 10 min at 5,000×g at 4 °C. The supernatant with cytosolic fraction was collected while the remaining pellet was dissolved in ice-cold nuclear extraction buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM NaCl, 0.2 mM EDTA, 20 % glycerol, 0.5 mM DTT, 0.5 mM PMSF, and protease inhibitors) for nuclear fraction. Following incubation for 30 min on ice and centrifugation at 20,000×g at 4 °C for 15 min, the supernatant containing the nuclear fraction was aliquoted and stored at −80 °C for further analysis. Total protein concentrations were determined using the Bradford method (Bradford 1976).
ii. Western blotting
   Protein (50 μg) was separated by 8, 10, or 12 % SDS-polyacrylamide gel electrophoresis, based on the molecular weight of the target protein and transferred onto a nitrocellulose membrane (Millipore, Billerica, USA). The membranes were blocked with 3 % bovine serum albumin in PBS containing 0.1 % Tween 20 (Sigma), washed and probed with respective mouse/rabbit monoclonal antibodies. Primary antibodies Hsp90 (cat. no. H1775), iNOS (cat. no. N9657), NF-kB (cat. no. SAB4502610), and β-actin (cat. no. A5441) were obtained from Sigma (MO, USA) while antibodies against eNOS (cat. no. 9572), Hsp27 (cat. no. 2442), and phosphorylated IκB (cat. no. 9246) were obtained from Cell Signaling Technology (MA, USA). The membranes were then incubated with anti-mouse/rabbit-IgG HRP conjugate (Sigma cat. no. A9044 and A9169, respectively). The membrane was washed and incubated with chemiluminescent substrate (Sigma) and the bands were developed using X-ray films (Kodak, NY, USA). Quantification was performed by densitometry using Image J software.

Statistical analysis

All the experiments were performed on a minimum of three different occasions, and data are presented as mean±SEM. One-way analysis of variance with post-hoc Bonferroni analysis was used to determine statistical significance between groups. All analysis was conducted using GraphPad Prism Ver 6.00 software (Graph Pad, CA, USA). The p value of <0.05, with a 95 % confidence interval, was considered significant.

Results

Acute hypoxic stress elicits the protective HSR in the heart

Using the onset of gasping as an efficient indicator for the ability of animals to tolerate the stress, we have previously shown that rodents respond differentially on exposure to extreme altitudes, equivalent to 32,000 ft. or 9,754 m (Jain et al. 2013a, b). HSR forms a universal response to cell injury, mediated by a family of chaperone proteins with Hsp70 as the central player (Christians et al. 2002). In our study, we identified the myocardial tissue levels of Hsp70 through immunohistochemical analysis. We observed that compared with control, a significantly higher tissue localization of Hsp70 was observed in the myocardial sections from animals with a gasping time of more than 10 min, i.e., the normal (10–25 min) and tolerant animals (more than 25 min) (Fig. 1a–d). In the present study, we find a marked elevation in the levels of HSF-1 expression as quantified by ELISA in cardiac nuclear extracts in animals with better tolerance for hypoxic stress. Compared with control (475.20±30.35 ng/mg protein), the HSF-1 levels were almost 2-fold higher in susceptible animals (962.70±51.32 ng/mg protein, $p<0.001$); in tolerant animals, the increase was more than 3-fold (1,439.00±76.65 ng/mg protein, $p<0.001$) (Fig. 2a). Animals having a gasping time between 10 and 25 min also showed

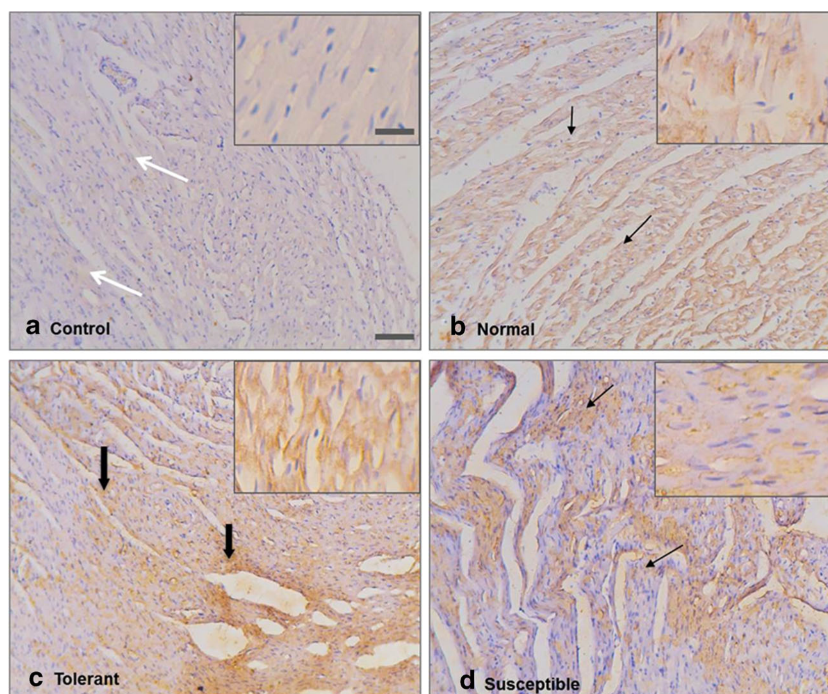

Fig. 1 Sublethal hypobaric hypoxia induces a differential upregulation of Hsp70 in the myocardial tissue sections. Heart right ventricular sections from hypoxia exposed animals were immunostained with a mouse monoclonal anti-Hsp70 antibody and were counterstained with hematoxylin stain for nucleus. The *scale bar* at the bottom represents 100 μm in (**a**–**d**). To highlight the difference in staining, *inset* shows a magnified view of the image (*bar* represents 25 μm in all insets). **a** Constitutive expression of Hsp70 observed in unexposed control tissue sections, indicated by *white arrows*. **b**–**d** *Black arrows* indicate regions of positive staining seen in the cardiac tissue sections (8 μm). *Bold black arrows* in (**c**) show presence of highly stained regions in the section

a significant increase in HSF-1 levels as compared with control (1,207.00±34.60 ng/mg protein, $p<0.001$). Although being a constitutive chaperone in the tissue, expression of Hsp70 was also observed in the susceptible animals having gasping time even less than 10 min, its significantly higher expression in tolerant animals was validated through dot blot analysis (Fig. 2b). Downstream upregulation of other members of chaperone family, such as Hsp60/HSPD1 and Hsp90/HSPC, was identified in the exposed myocardial tissues through dot blot analysis and immunoblotting. As shown in Fig. 2c, there is discernible higher expression of Hsp90 in the tolerant animals as compared to the animals with a higher susceptibility to hypoxia.

Hsp27/HSPB1 belongs to the family of small HSPs which have been recognized in playing a cytoprotective role in the cell (Willis and Patterson 2010). Evaluating the role of these chaperones in hypoxic tolerance, we found that the tolerant animals in our study showed an almost 3-fold increase in Hsp27/HSPB1 levels (41.10±1.93 ng/mg protein) as compared with susceptible animals (15.96±0.38 ng/mg protein, $p<0.001$) (Fig. 2d). On probing with an anti-rabbit Hsp27 antibody, a significant increase observed in the expression of the protein in tolerant animals ($p<0.001$) as compared with susceptible animals, validated our ELISA results (Fig. 2e).

Hypoxic stress induces myocardial NO generation

Having observed a differential upregulation of HSR in the tolerant and susceptible animals, we measured the NO levels in the cardiac tissues of these animals. We found that while tolerant animals showed a 2-fold increase in NO generation (3.52±0.15 vs. control 1.71±0.08 nmol/mL nitrite, $p<0.001$), the elevation in the susceptible animals was not significant (2.09±0.07 nmol/mL nitrite) (Fig. 3a).

Analyzing the eNOS levels in the tissue homogenates, we found that with reference to control (3,798.00±565.30 μIU/mL), there was a significant 6-fold increase (24,198.00±2,725.00 μIU/mL, $p<0.001$) in eNOS in the tolerant animals (Fig. 3b). Although a considerable increase was also observed in the eNOS expression in the susceptible (14,787.00±898.50 μIU/mL) and normal (8,414.00±758.00 μIU/mL) animals, the distinctly higher expression of eNOS in the animals with a greater ability to withstand hypoxic stress correlates with the higher NO production in this group. We corroborated the higher expression of eNOS through immunoblotting with a monoclonal antibody against eNOS and found a substantially enhanced expression in the tolerant animals as compared with susceptible animals ($p<0.001$; Fig. 3c).

Another isoform of NOS is the inducible NOS, which showed a 2-fold higher expression on hypoxic exposure

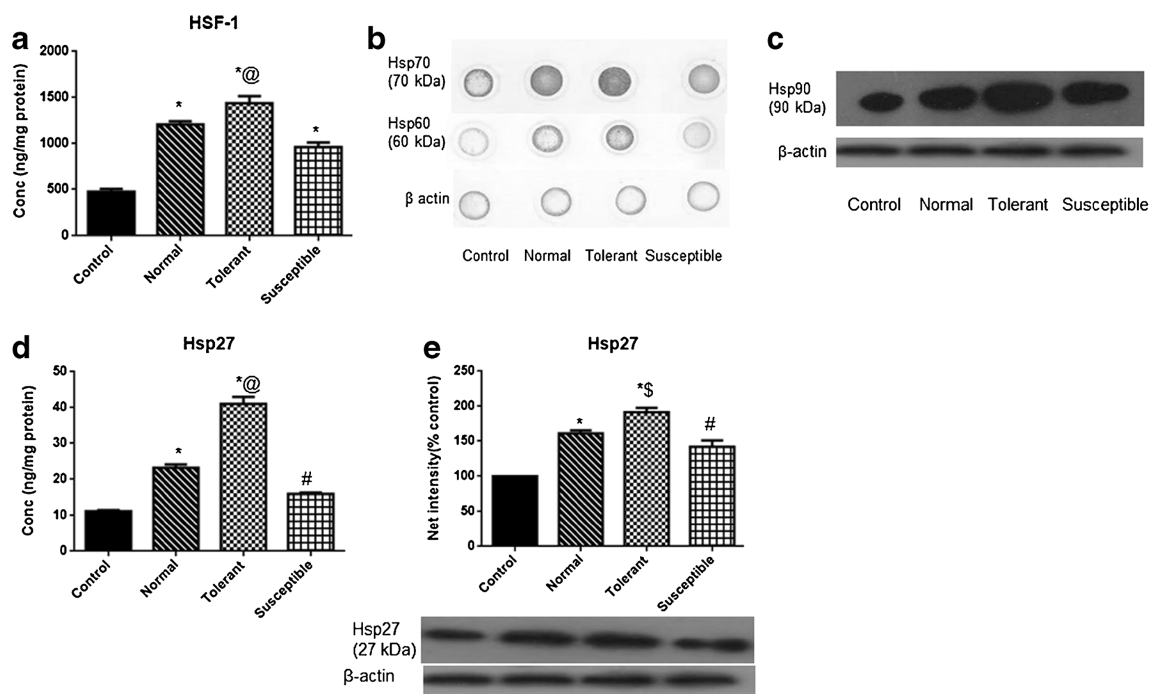

Fig. 2 Hypobaric hypoxia induces differential activation of the HSR. Values are given as mean±SEM. **a** ELISA-based quantification of heat shock factor-1 (*HSF-1*) in the nuclear extracts from the exposed and control cardiac tissues. **b** Representative image of dot blot analysis using monoclonal Hsp70 and Hsp60 antibody to semiquantitatively identify the change in expression. β-actin was taken as loading control. **c** Representative immunoblot showing expression of Hsp90 in cytosolic extracts of rat hearts exposed to acute hypobaric hypoxia. β-actin taken as loading control. **d** ELISA-based quantitative measurement of Hsp27 levels in the myocardial tissues from control and hypoxia-exposed animals. **e** Representative immunoblot showing expression of Hsp27 in myocardial cytosolic extracts. β-actin taken as loading control. *Graphs* show the integrated optical densitometric values performed using ImageJ software. Change in expression is expressed as net intensity (% control). The data presented are the mean with standard error (*bars*) of n=3 independent experiments. *p<0.001 vs. control; #p<0.05 vs. control; @p<0.001 vs. susceptible; $$p$<0.005 vs. susceptible

(p<0.001 vs. control) in the animals with better tolerance to hypoxic stress (Fig. 3d).

Acute hypoxia induces a differential inflammatory response

NF-κB is a key transcription factor that plays a central role in inflammatory responses against various stress stimuli including hypoxia (Jones et al. 2003; Gordon et al. 2011). We find in our study, a considerable decrease in phosphorylated IκB expression from control in all the groups of hypoxia exposed animals (Fig. 4a, b; p<0.001). Evaluating the NF-κB expression in both the cytosol and nuclear extracts, we find that while there was a significant fall in cytosolic NF-κB levels in the susceptible animals; there was an appreciable increase in its expression in the nuclear fraction, as compared with the control (p<0.001). Conversely, nuclear NF-κB levels in the cardiac tissues of the tolerant animals did not show any significant change (Fig. 4c, d). Interestingly, the susceptible animals also showed a more than 4-fold elevation in the expression of the proinflammatory cytokine TNF-α (3,055.00±151.10, vs. control 688.50±47.78 pg/mg protein, p<0.001) (Fig. 4e). Notably, the increase in TNF-α expression was also significant (p<0.001) in the tolerant and normal animals (1,566.09±61.37 and 2,401.00±95.69 pg/mg protein, respectively).

Sublethal hypoxic stress differentially upregulates circulatory stress markers

Measuring the levels of ROS generation in the plasma of hypoxia-exposed animals using a nonfluorescent lipophilic dye, DCFH-DA, we found a 4-fold higher generation of free radical species in the susceptible animals (p<0.001 vs. control) (Fig. 5a). Although hypoxia also caused an increase in ROS levels in the tolerant and normal animals, the levels in the susceptible animals were appreciably higher (p<0.001 vs. tolerant). Taking a cue from the higher oxidative stress in these animals, we measured the levels of circulatory stress marker, corticosterone. This steroid hormone, which is considered to be one of the first markers of any kind of cellular stress and injury, was found to be significantly heightened (2,317.00±84.00 ng/mL; Fig. 5b) in the susceptible animals as compared with control (712.60±70.49 ng/mL, p<0.001) as well as tolerant animals (1,573.00±65.09 ng/mL, p<0.001). Erythropoietin, the pivotal regulator of erythropoiesis, was measured in the plasma of the exposed as well as the control animals. While an upregulation in its expression was observed

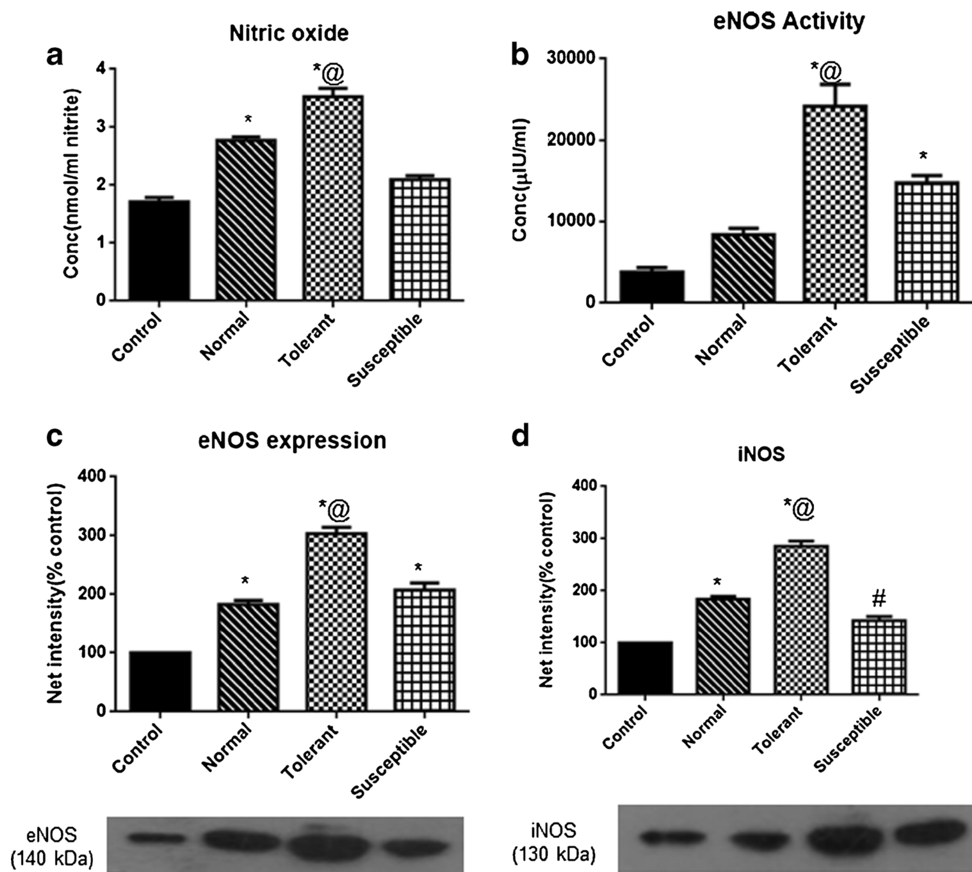

Fig. 3 Acute hypoxia elevates nitric oxide generation in rodent heart through activation of nitric oxide synthases. **a** Nitric oxide levels in the rat myocardium measured using Griess reagent. **b** eNOS levels in the rat heart homogenates quantified by ELISA. **c** Expression of eNOS validated by immunoblotting. *Representative blot image* shows change in expression. β-actin taken as loading control. *Graphs* show the integrated optical densitometric values measured using ImageJ software. Change in expression is expressed as net intensity (% control). The data is mean with standard error (*bars*) of $n=3$ independent experiments. **d** Representative western blot for expression of inducible NOS in myocardial cytosolic extract. *Graph* represents the densitometric analysis, expressed as net intensity (% control). Values are given as mean±SEM. *$p<0.001$ vs. control; #$p<0.05$ vs. control; @$p<0.001$ vs. susceptible

in all animals exposed to an altitude of 9,754 m (Fig. 5c), the most distinct increase was evident in the tolerant animals with a gasping time of more than 25 min ($p<0.001$). Evaluating the expression of endothelin-1 in the plasma of the exposed animals, all animals showed a markedly higher level following hypoxic exposure (Fig. 5d). Significantly, the increase was maximal, almost 12-fold, in the susceptible animals with a lower ability to survive under acute hypoxic stress ($p<0.001$).

Discussion

Characterized by hypobaric hypoxia, the severe environment in the high altitude milieu poses a challenge to the survival of natives as well as sojourners. We have earlier identified specific molecules that impart tolerance or susceptibility to such stressors, which may be important for predicting the tolerance/susceptibility to hypoxic stress (Jain et al. 2013a). We have also postulated the role of altered protein folding homeostasis in making a significant contribution to increased hypoxic susceptibility (Jain et al. 2013b). Our present study was designed to delineate the role played by the HSR and its downstream signaling pathways in imparting tolerance to stress under high altitude conditions. We found that the animals with a better sustenance under acute hypoxic conditions had an elevated HSR as observed through a significant upregulation of the transcription factor HSF-1 and the chaperones Hsp70, Hsp90, and Hsp27. The increased activation of Hsp90 in tolerant animals led to the enhanced NO production mediated through the activity of the eNOS. Increased iNOS activation under hypoxic conditions also contributed to higher NO production and its cardioprotective effect on the vasculature. Activation of Hsp70 in these tolerant animals inhibited the translocation of NF-κB, thereby attenuating the inflammatory response. Conversely, animals with a gasping time of less than 10 min, showed evidence of an inflammatory response mediated by the activation of NF-κB and upregulation of proinflammatory TNF-α (Fig. 6). These animals also had elevated free radical generation, corticosterone, and endothelin-1 plasma levels, characteristic of a high-altitude stress response.

Hypoxia induces a number of cellular responses to compensate for the decrease in oxygen availability. Molecular chaperones play a prominent role in physiological responses to environmental stress (Welch 1992). A highly conserved, ubiquitiously expressed family of stress–response proteins, HSPs are expressed at low levels under normal conditions. Elevated expression of Hsp70 has been shown to be protective against myocardial damage and forms the first line of defense against cellular oxidative injury (Dillmann and Mestril 1995; Christians et al. 2002). The stress induced regulation of the

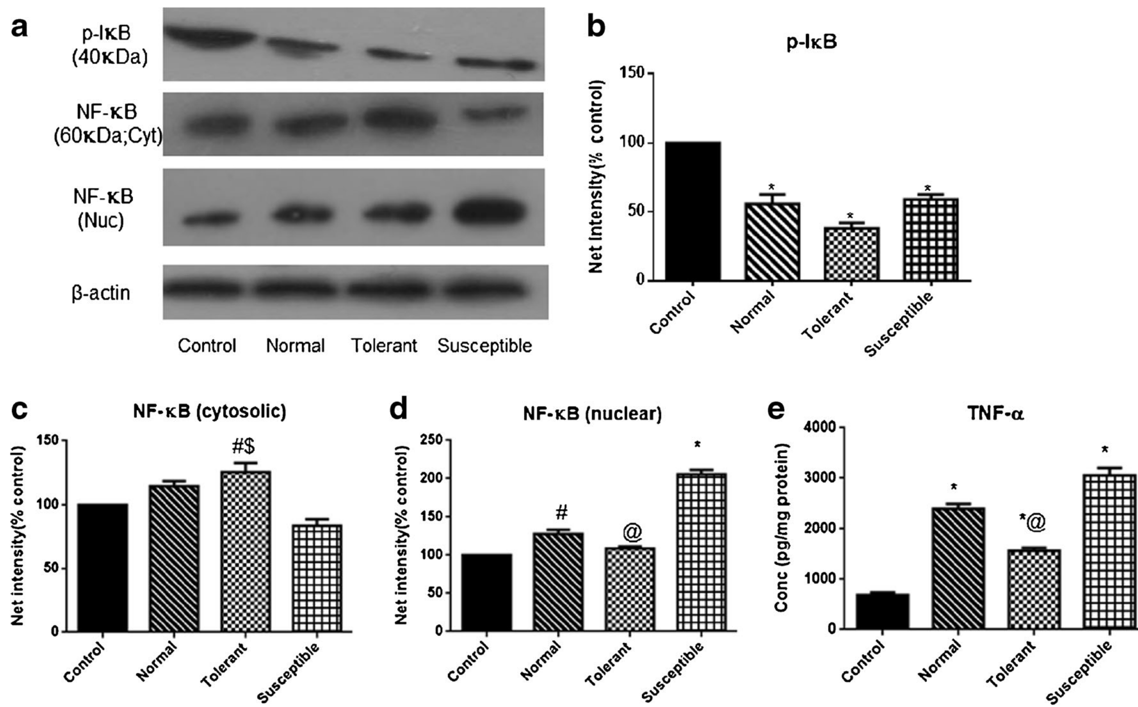

Fig. 4 Hypobaric hypoxia mediates a differential inflammatory response in the rodent heart. **a** Representative western blots for expression of inflammatory markers IκB and NFκB in myocardial cytosolic and nuclear extracts, respectively. Loading was normalized with reference to β-actin. **b–d** *Graphs* represent semiquantitative densitometric analysis of IκB in cytosolic fraction and NF-κB protein expression in both the cytosolic and nuclear fractions. The net intensity is calculated based on results in $n=3$ independent experiments and shown as mean with standard error. **e** Quantitative measurement of proinflammatory cytokine TNF-α levels. All values presented as mean±SEM. *$p<0.001$ vs. control; #$p<0.05$ vs. control; @$p<0.001$ vs. susceptible; $\$p<0.005$ vs. susceptible

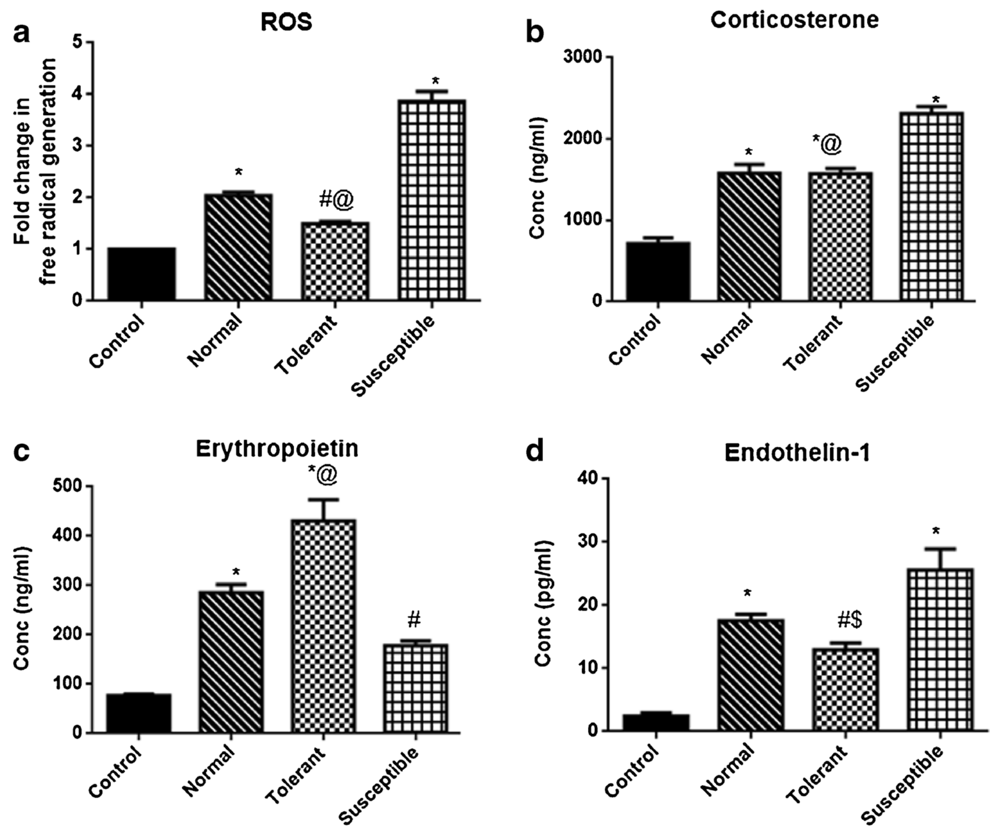

Fig. 5 Acute sub lethal hypobaric hypoxia differentially upregulates circulatory stress markers. **a** Reactive oxygen species generation in plasma samples collected from exposed animals measured using the dye DCFH-DA. **b** Quantitative measurement of stress hormone corticosterone. **c** ELISA based detection of erythropoietin in plasma samples. **d** Measurement of endothelin-1 levels in rat plasma. All values are given as mean±SEM. *$p<0.001$ vs. control; #$p<0.05$ vs. control; @$p<0.001$ vs. susceptible; $\$p<0.005$ vs. susceptible

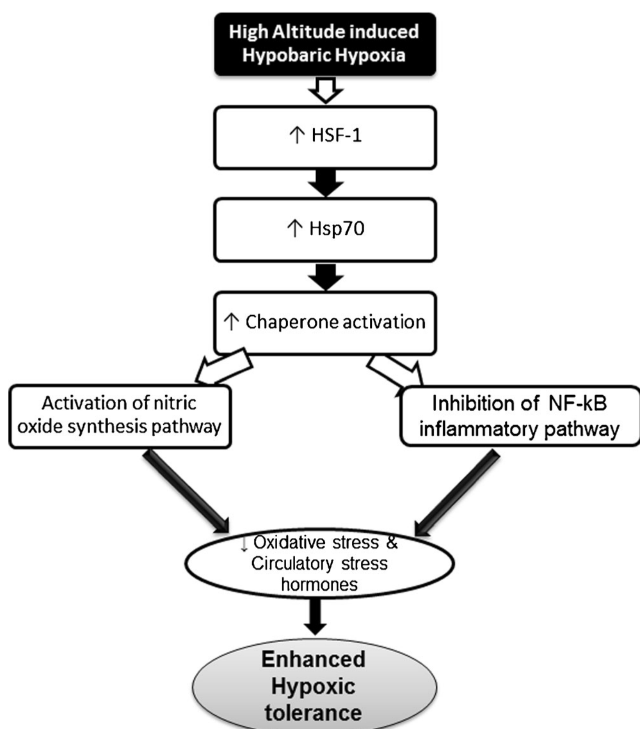

Fig. 6 Acute hypoxia induced differential activation of HSR and downstream signaling response mediates hypoxic tolerance

HSR is mediated primarily through the activation of the transcription factor HSF-1 (Ahn and Thiele 2003; Yan et al. 2002; Baird et al. 2006), which subsequently augments HSP synthesis though Hsp70 mediated action, promotes removal of misfolded proteins, enhances NO synthesis as well as regulates proinflammatory and apoptotic responses (Buzzard et al. 1998; Morimoto 1998; Barral et al. 2004; Chen and Meyrick 2004). Normally present in its inactive form in the cytosol, under cellular stress, this transcription factor translocates to the nucleus and induces chaperone expression (Baird et al. 2006). Though it is difficult to precisely delineate whether the higher nuclear concentration of HSF-1 in the cardiac tissues of tolerant animals is due to its initially elevated levels in the cytosol or better hypoxic stress sensing by these animals, we find evidence of increased HSPs in such animals. Higher expression of the HSPs in our study suggests that although hypoxia-induced oxidative stress is capable of activating HSR, better chaperone activity contributes significantly to enhanced hypoxic tolerance. While the multifaceted pro-survival effects of Hsp70 and Hsp90 have been widely examined, research is now focussed on the role of small HSPs in contributing to critical life and death decisions of the cell. Studies have shown that increased expression of Hsp27 or Hsp22 causes a significant decrease in myocardial susceptibility to pathophysiological injury after experimental ischemia in different animal models (Martin et al. 1997; Vander Heide 2002; Ghayour-Mobarhan et al. 2012). Tolerant animals in our study showed an almost 3-fold increase in Hsp27 levels as compared with susceptible animals. Collectively, the animals with an enhanced hypoxic tolerance display a discernible upregulation of the HSR as compared to animals with greater susceptibility to low oxygen tensions. While this upregulation may be attributed to a greater ability to sense and respond to hypoxic stress in tolerant animals, it is possible that these animals, with a longer duration of exposure to acute hypoxia are better equipped to increase the synthesis of the HSPs.

One mechanism responsible for the myocardial protection afforded by HSPs may be linked to the activation of NO and NOS pathways. Although the exact mechanism involved in the interaction between NO and the HSPs is unknown, evidence suggests that Hsp70 expression is paralleled by an increase in NO production (Malyshev et al. 1995; Bellmann et al. 2000). It is possible that NO itself triggers Hsp70 expression and synthesis and may ultimately protect the cardiomyocytes against the cytotoxic effects of TNF-α, a cytokine with a central role in inflammation and apoptosis (Kim et al. 1997; Bellmann et al. 2000; Valen et al. 2000). In congruence with numerous earlier studies highlighting the versatile cardioprotective role of NO against ischemic injury and apoptosis (Felaco et al. 2000; Jones and Bolli 2006), we found a considerable increase in its levels in animals with a better ability to cope with hypoxia.

eNOS, the most abundant and widely distributed NOS isoform in the heart, is associated with the maintenance of basal, physiological cardiac function. Elevations in ischemia-induced levels of activated eNOS have been identified in cardiomyocytes, indicating eNOS as a significant source of hypoxia/ischemia-induced NO production in the heart (García-Cardena et al. 1998; Jones et al. 1999; Brunner et al. 2003). The distinctly higher expression of eNOS in the animals equipped with an enhanced ability to withstand hypoxic stress correlates with the higher NO production in this group. It is well documented that activated PKB/Akt is the most prominent kinase responsible for phosphorylation of NOS and it has been demonstrated that hypoxia activates eNOS via Akt activation (Fulton et al. 1999). Studies have shown that inhibition of the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway resulted in a decreased NO production under hypoxia in cardiomyocytes and endothelial cells (Chen and Meyrick 2004). Hsp90, which forms a chaperone substrate protein complex with Akt, is necessary for the proper functioning of Akt; loss of Hsp90-Akt binding results in Akt inactivation (Fulton et al. 1999; Sato et al. 2000). Previously, we have demonstrated that this pathway is elevated in the tolerant animals and promotes survival (Jain et al. 2013b). Observations from our present study suggest that hypobaric hypoxia differentially induces the HSR and Hsp90, which may activate the PI3-K/Akt pathway, thus leading to enhanced eNOS activation and NO generation in tolerant animals.

Having earlier shown the upregulation of HIF-1α in tolerant animals (Jain et al. 2013a), we postulated a role for the HIF-1α induced iNOS activation in the escalation of NO production. Concommitent with our hypothesis, we found a 2-fold higher iNOS expression on hypoxic exposure in the cardiac tissues from animals which could withstand the reduced oxygen availability for longer periods. Studies by Rus et al. (2011a, b) have shown a crucial cardioprotective role for iNOS under hypoxia reoxygenation injury using iNOS inhibitors. We propose that the enhanced eNOS and iNOS levels and NO generation, mediated in part by the activation of HSR, may be responsible for the better tolerance to acute sub lethal hypoxic stress. Indeed, as the susceptible animals are exposed to acute sub lethal hypoxic stress imposed at an altitude of 9,754 m for duration of less than even 10 min, it is interesting to hypothesize that these animals are unable to induce the expression of the NO synthases and generate NO. Identifying the cause–effect relationship between the tolerance to hypoxic stress and the time required to mount the defensive response against hypoxia may provide an integral insight into the individual variability in stress tolerance.

Involved in the critical regulation of the cellular immune response, inflammatory signaling cascades as well as stress responses to external stimuli, NF-κB itself is regulated by the IκB (Haudek et al. 2001; Higuchi et al. 2002). It has been shown that the HSR inhibits the activation of the I kappa B kinase (IKK) complex thus resulting in the suppression of NF-κB pathway, release of proinflammatory cytokines and paradoxically the inhibition of iNOS (Wong et al. 1997). Although the tolerant animals in our study did not show a significant variation in the NF-κB expression in the nuclear fraction, the decreased cytosolic and increased nuclear content of the transcription factor in the myocardium from susceptible animals indicates its activation in the animals with low hypoxic tolerance. Greater TNF-α expression in the susceptible animals supports the activation of an inflammatory response. A proinflammatory cytokine, TNF-α, has been implicated in the pathogenesis of cardiovascular diseases, including acute myocardial infarction, chronic heart failure and atherosclerosis (reviewed by Kleinbongard et al. 2011). Shames et al. (1998) showed that cardiac tolerance to endotoxins is associated with an increased level of myocardial IκB, attenuated NF-κB activation, and decreased TNF-α production. Interestingly, the presence of such cytokines is capable of increasing HSP production as a signal of danger/stress to the cell. Studies have earlier reported that the cardioprotective action of Hsp70 is associated with diminished production of TNF-α (Nakano et al. 1996; Meng et al. 1999). Paradoxically, several investigators have implicated NO in TNF-induced myocardial dysfunction and apoptosis (Schulz et al. 1995; Ing et al. 1999). In our present study, we find that while eNOS and iNOS are both upregulated in the tolerant animals, there is a marked increase in the NF-κB and TNF-α level in the animals with low hypoxic tolerance, indicating that within the complex mammalian signaling cascades; more than a single pathway contributes to the stress response.

Using plasma as an efficient indicator of the pathophysiological state of all tissues, we further identified the variation in stress response under acute hypoxic stress through the evaluation of circulatory hormone levels in the exposed animals. Stressful stimuli including lack of oxygen availability at high altitude is bound to increase the stress hormones like catecholamines in the circulating blood. While oxidative stress and damaged proteins are known inducers of the HSR, stress hormones, including catecholamines and cortisol, also influence the transcription and translation of HSP genes and proteins. Corticosterone modulates the HSP expression through its glucocorticoid receptors, chaperoned mainly by Hsp70 and Hsp90. Interestingly, although most studies identifying its impact on HSPs have been conducted on fishes, cortisol mediates a suppressive role on HSP induction at the gene and protein level (LeBlanc et al. 2012). In our results, we find that the plasma corticosterone levels are appreciably higher in the susceptible animals, correlating inversely with the relatively lower chaperone activation in these animals.

Previous studies, have proposed a central role for altered oxygen delivery under hypoxic conditions in the successful adaptation to life at high altitude milieu (recently reviewed by Beall 2013). High-altitude natives have shown evidence of a higher expression of HIF-1α and its regulated proteins including erythropoietin, which forms one of the primary responders to hypoxic stress (Rodríguez et al. 2000; Scheinfeldt et al. 2012). Known to be cardioprotective against ischemic injury as well as hypoxic stress (Burger et al. 2009), in our study tolerant animals displayed much higher plasma levels of erythropoietin as compared with susceptible animals. Although the precise mechanism remains unexplored, research has shown that pretreatment with erythropoietin mediates a form of cardioprotection associated with enhanced Hsp70 and diminished NF-κB expression (Xu et al. 2005). Indeed, Burger et al. (2006) proposed an upregulation in the eNOS expression as the mechanism behind erythopoietin-mediated cardioprotection against ischemic injury. Contrasting with the higher levels of NO in the tolerant animals, an inverse pattern was seen in the plasma levels of endothelin-1, with the highest expression in the susceptible animals. Known to be a potent vasoconstrictor and a major player in the hypertensive pathophysiology, endothelin-1 overexpression has been identified to play cardioprotective role in hypoxic cardiomyocytes through the enhanced expression of Hsp70 and GRP78 (Li et al. 1994; Nakanishi et al. 1999; Pan et al. 2004). While the mechanism of how it exerts its protective action remains yet unexplored, it emphasizes on the multitude of interlinked pathways involved in the cellular response to stress.

Collectively, our findings for the first time highlight the differential regulation of signaling pathways by the stress-inducible HSR in providing protection against damaging environmental stimuli like acute sublethal hypobaric hypoxia. The enhanced activation of this response triggers an increase in NO generation while inhibiting proinflammatory effects; thus, this may form a basis for the better tolerance of organisms to the lack of oxygen availability.

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Declaration of interest The authors declare that no conflict of interest exists.

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