

Original Article

Experimental Studies on The Role of Nitric Oxide (NO) in High Altitude Stress Induced Physiological and Behavioral Changes in Rats

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Abstract

The present study evaluated the effects of acute high altitude stress induced physiological and neurobehavioral changes and the possible involvement of nitric oxide (NO) in the regulation of such effects in rats. High altitude was simulated in a high altitude/hypoxia chamber and the effects of such stress and its modulation by NO-ergic agents were assessed on plasma corticosterone, anxiety and oxidative stress markers in rats. Acute high altitude stress induced reductions in open arm entries and open arm time in the elevated plus maze (EPM) test-suggestive of anxiety like behavior and was associated with increased levels of plasma corticosterone. Assay of brain homogenates showed lowered levels of NO metabolites (NO_x) and GSH but elevated MDA. Pretreatment with the NO-mimetic, L-arginine (500 mg/kg) attenuated, whereas, the NOS inhibitor, L-NAME (50 mg/kg) aggravated both behavioral and biochemical changes induced by such high altitude exposure. Further, when combined with restraint stress (RS), these neurobehavioral and biochemical changes were further accentuated when compared to high altitude per se. Similarly, pretreatment with the L-Arginine neutralized the effects of high altitude + RS, whereas, L-NAME potentiated the behavioral and biochemical effects of the combined stressors. These results suggest that acute high altitude stress induced HPA axis activation, angiogenesis and oxidative stress which were under the regulatory influence of NO.

Introduction

The lowest portion of the atmosphere, troposphere envelopes the earth's entire surface. As altitude increases barometric pressure falls within

troposphere. With decrease in barometric pressure, partial pressure of oxygen decreases proportionately resulting in a condition called as hypobaric hypoxia (1). Environmental stressors like ascent to high altitude (and the resultant hypoxia) can disrupt the physiological homeostasis of the biological system and lead to a variety of physiological and behavioral responses as well as pathophysiological states. For example, such hypobaric hypoxia is known to disrupt the hemodynamic balance of the cardiorespiratory system and precipitate pulmonary edema (2). The CNS, in particular, is highly vulnerable to such hypoxic insults and could result in complex

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(Received on April 12, 2015)

neurobehavioral alterations. Neurobehavioral dysfunctions and high-altitude sickness that include phenomenon like negative mood states and inefficient cognitive performance are well documented subsequent to high altitude exposure (3, 4). Individuals known to spend long-term periods at a high altitude as well as the local residents at high altitude could also develop chronic mountain sickness accompanied with severe symptomatic polycythemia and hypoxemia (5). Acute Mountain Sickness and impaired neurobehavioral functions caused by high altitude exposure consequently are known to affect medical assistance and military support in emergency conditions.

Emotional and environmental stressors reportedly influence brain function and is known to be a key factor in the genesis of neuropsychiatric disorders. Further, emotional stressors could also compound the effects of environmental stress of high altitude stress on the organism (6).

Nitric oxide (NO) is a unique bioregulator molecule with complex physiological effects and having pathophysiological significance. Initially discovered as a vasodilator entity in the cardiovascular system, its role as a neuromodulator has now been widely documented.

The changes in the central and peripheral nervous systems consequent on exposure to high altitude simulation may be mediated by the endogenous generation of nitric oxide (NO), which is an intercellular messenger and potent vasodilator. NO is synthesized from L-arginine in the mammalian brain as well as in invertebrate neural structures by the enzyme nitric oxide synthase (NOS) (7-9).

Stressful conditions also generate reactive oxygen species (ROS). Reactive oxygen species are known to be generated continually as by-products during aerobic metabolism. Some of the various factors involved are exposure to UV light, hypoxia, pollution and many other stressors (10). Strenuous physical activity is also known to increase oxidative stress that might be because of 10 to 15 fold increased oxygen utilization in order to meet the energy demands. This is also coupled with small amount

(1–2%) of “electron leakage” through the electron transport chain with subsequent direct reduction of molecular oxygen to the superoxide anion (11). These ROS show a high chemical reactivity and the brain, in particular, is highly susceptible to such oxidative damage, due to its high polyunsaturated fatty acid (PUFA) content and a weak anti-oxidant defense system. The balance between pro-oxidant/antioxidant is thought to be crucial in processes like cell death, motor neuron disease, axonal injury and neurodegeneration. The anti-oxidants are also known to directly or indirectly protect the cells from the adverse effects of drugs, xenobiotics, carcinogens as well as toxic radical reactions (12).

Earlier studies from our laboratory have shown that NO may act as an important regulatory molecule during emotional stressors like restraint stress, and its interactions with reactive oxygen species have been suggested during such stress induced angiogenesis (13,14,15).

In the present study, the possible role of NO was investigated on high altitude stress induced physiological and neurobehavioral changes in rats. The influence of such stress of high altitude alone and in combination with emotional stressors like restraint stress was evaluated on plasma corticosterone and anxiety like behavior. Further, alteration of oxidative stress markers in the brain during such hypoxic insults were also assessed. The effects of NO modulators on both behavioral and biochemical parameters during such environmental (simulated high altitude) and emotional (restraint) stress were also evaluated for studying possible interactions of NO and oxidative stress.

Materials and methods

Experimental animals

Male Wistar rats (150-200g) were used for the study. The animals (n=6 per group) were maintained under standard laboratory conditions (12 h light/dark cycle at 22±2°C) and had free access to food and water throughout the experiments. Animal care was as per Indian National Science Academy (INSA) Guidelines for Care and Use of Animals in Scientific Research.

The study protocol was approved by the Institutional Animal Ethical Committee (IAEC) of the VPCI.

Drugs and chemicals

The drugs used in the study were L-Arginine (500 mg/kg) and L-NAME (50 mg/kg) which were procured from Sigma-Aldrich (St. Louis, USA). Both drugs were dissolved in distilled water and injected intraperitoneally (i.p.), injecting volume was 2 ml/kg. The pretreatment times were 60 min in each case before exposure to stress. All other routine chemicals required for the biochemical assays were procured from SRL Labs, New Delhi.

Stress procedures

High Altitude simulation was done in especially designed hypoxia chamber (Seven Star, Model No-7001) in which rats were exposed to a single 6 hr of hypobaric hypoxia exposure (x 1 day) at $26\pm 2^\circ\text{C}$ temperature, $60\pm 10\%$ relative humidity and airflow rate of 400 ml/min. Animals were exposed to two levels of simulated high altitude, viz. 8000 ft (564 mmHg) (H-I) and 12000 ft (451 mmHg) (H-II).

In another stress protocol, high altitude simulation (H-I or H-II) was combined with restraint stress (RS) for 6 hr for 1 day. Restraint stress (RS) was given by immobilizing the animal in adjustable plexiglas restrainers (INCO, Ambala), in a manner so that movement were restricted except that of the tail with minimal pain.

Neurobehavioral studies

Elevated Plus Maze (EPM) test – The EPM consisted of two open arms of 40×40 cm, crossed with two similar closed arms with wall of 40 cm height. The arms were connected so that the maze had a plus sign look. The entire maze was elevated 50 cm above the ground level and placed in a quiet and dimly lit room (16). Vehicle (control) or drug treated rats were placed individually in the center of the maze facing the closed arms. The following parameters were measured: number of open arm entries and time spent in the open arms. The duration of the

test was 5 min. The percentage of open arm entries during this 5 min exposure to the EPM was calculated from open-arm entries divided by the total number of entries in both open and closed arms. The time spent on open-arm exploration divided by total time spent in both open and closed arms, were also calculated.

Biochemical studies

Immediately after the behavioral testing in the EPM, the rats were decapitated under ether anesthesia. Blood was collected after cardiac puncture in heparinized tubes. Cells were removed from plasma by centrifugation for 10 minutes at 1,000-2,000 x g using a refrigerated centrifuge. After centrifugation, the supernatant (plasma) was separated and stored at -80°C . After blood collection, the animals were decapitated and brains were dissected out, cleaned with ice cold saline and stored at -80°C . Brain samples were thawed and homogenized in a proportion of 1:10(w/v) ice cold phosphate buffer (0.1 M, pH 7.4). The brain was cleaned with ice cold saline and stored at -80°C . Brain samples were thawed and homogenized with 10 ml of ice cold 0.1M phosphate buffer (pH 7.4). Aliquots of homogenates were used to determine the oxidative stress markers, viz. MDA, GSH and stable NO metabolites (NOx).

Plasma corticosterone assay

Corticosterone is a sensitive and consistent biomarker for stress in rats and this assay was carried out in plasma samples using commercially available ELISA kit (Qayee-Bio). The assay was based on a double-antibody sandwich ELISA. Briefly, to the precoated enzyme wells (corticosterone antibodies), HRP labeled corticosterone antibodies, test sample and standard were added. On addition of chromogen solutions, the colour of the liquid changed. The product of this enzyme reaction was a yellow coloured product and the absorbance was taken at 450 nm by a multimode ELISA reader (Spectramax 3, Molecular Devices, USA). The concentration of corticosterone binding to the antibody coated wells is directly proportional to the colour developed after enzyme substrate reaction. The results were expressed as $\mu\text{g/dl}$.

Malondialdehyde (MDA)

Malondialdehyde, a measure of lipid peroxidation was determined as described by Ohkawa et al (18), briefly the reaction mixture consisted of 0.2 ml of 8.1% sodium lauryl sulphate, 1.5 ml of 20 % acetic acid (pH 3.5) and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid was added to 0.2 ml of processed brain homogenate. The mixture was made up to 4.0 ml with distilled water and heated at 95°C for 60 min. After cooling with tap water, 5 ml of n -butanol and pyridine (15:1 v/v) and 1 ml of distilled water was added and centrifuged. The organic layer was separated out and its absorbance was measured at 532 nm using a UV- Visible spectrophotometer (UV 5740 SS, ECIL) and MDA content was expressed as nmole/mg protein. Tissue protein was estimated using Lowry method of protein assays (17).

Reduced glutathione (GSH)

Reduced glutathione was measured according to the method of Ellman (19). An equal quantity of homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate the proteins.

To 0.1 ml of this supernatant, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5,5-dithiobis (2-nitrobenzoic acid) and 0.4 ml of double distilled water was added. The mixture was vortexed and absorbance read at 412 nm within 15 min. The concentration of reduced glutathione was expressed as $\mu\text{M/g}$ tissue.

Nitric Oxide metabolites (NOx)

Brain NOx (stable metabolites of NO) content was determined by the method of Tracey et al. (20) which coupled Aspergillus nitrate reductase with NADPH and FAD to convert all nitrates present in sample to nitrites. Assay mixture consisted of 50 μl brain supernatant, 10 μl nitrate reductase (2 U/ml) and 20 μl of 310 mM/l potassium phosphate buffer in total assay volume of 100 μl . The samples were incubated at 37°C for 1 hr in the dark, 5 μl of 1 M/l zinc sulphate was added to precipitate the protein in the sample. The microtubes were centrifuged at 6000 x g for 5 minutes at 4°C. Supernatant from each microtube was then transferred to a microplate (96 well plate).

To this supernatant 100 μl of Griess reagent was added for colour development. Readings were taken after 10 minutes at 540 nm. Standard curve was generated using known concentration of sodium nitrate that converted to NOx content by using nitrate standard curve. The results were expressed in nmole/mg protein. Brain protein was estimated by the method of Lowry et al. (1951) at 540 nm.

Statistical analysis

The data were expressed as Mean \pm S.E.M. The behavioral and biochemical data were analysed using One-way ANOVA, followed by Tukey's test for inter-group comparisons. A p value of at least 0.05 was considered as the level of significance in all statistical tests.

Results

Effects of high altitude (H-I or H-II), restraint stress (RS) and nitric oxide (NO) modulators in the elevated plus maze test

Acute (single) high altitude exposure at 8000 ft (H-I) induced a reduction in the open arm entries and time spent in open arm in the EPM to 45% and 92% of control levels, respectively. However, the percentage open arm entries and open arm time were further reduced after an exposure to 12000 ft (H-II) simulated high altitude to 26% and 39% of the respective control values (normoxia) ($p < 0.05$, in each case). Exposure to a combination of high altitude (H-I or H-II) and restraint stress (RS) showed that RS potentiated the effects of both H-I (8000 ft) and H-II (12000 ft) on neurobehavioral suppression in the EPM, and both H-I + RS and H-II + RS groups had markedly lower OAE and OAT values as compared to the control (normoxic) groups. There was a 68% reduction with H-I + RS as compared to a 55% reduction in OAE with H-I alone, whereas, the reduction in OAE in H-II and H-II + RS groups were 74% and 77% respectively ($p < 0.05$). Pre-treatment with the NO precursor, L-arginine (500 mg/kg) reverted back the high altitude (H-II) induced effects towards those seen in normoxic group (controls) and the data of these drug treated groups were significantly different from the values of the H-II groups alone

($p < 0.05$). On the other hand, pretreatment with the NOS inhibitor – L-NAME (50 mg/kg) further aggravated the response seen after of H-II alone with the OAE data being statistically significant ($p < 0.05$). Similar attenuations in the neurobehavioral suppression in the EPM were also seen after L-arginine in the H-II+RS group, whereas, L-NAME further aggravated this response, and in both cases the differences were statistically significant, when compared to the vehicle treated H-II+RS group ($p < 0.05$, in each case). These results are summarized in Table I.

Effects of high altitude (H-I or H-II), restraint stress (RS) and nitric oxide (NO) modulators on plasma corticosterone levels

Acute (single) high altitude exposure induced stressor intensity dependent elevations in plasma corticosterone levels as compared to controls. At simulated high altitude of 8000 ft (H-I) this stress hormone was increased by 23%, whereas, a 40% increase was seen after an exposure of 12000 ft (H-II) of simulated high altitude ($p < 0.05$, in the latter case). When H-I or H-II was combined with RS, the combined stressors showed a potentiating effect on plasma corticosterone levels when compared to those seen after either H-I or H-II alone. Specifically,

TABLE I: Effects of high altitude (H-I or H-II), restraint stress (RS) and NO modulators on neurobehavioral and endocrinal responses in rats.

Treatment (mg/kg)	Elevated Plus maze (EPM) data		Plasma Corticosterone ($\mu\text{g/dl}$)
	OAE (%)	OAT(%)	
Control	32.90 \pm 3.77	10.31 \pm 1.68	17.40 \pm 4.95
H-I	14.98 \pm 8.16	9.55 \pm 5.40	21.49 \pm 2.66
H-II	8.79 \pm 2.23*	4.06 \pm 2.60*	24.55 \pm 1.16*
H-II + L-Arginine (500)	34.86 \pm 8.60 ^a	4.75 \pm 1.41	20.89 \pm 1.56
H-II + L-NAME (50)	2.67 \pm 1.76 ^a	0.57 \pm 0.37 ^a	30.50 \pm 2.33 ^a
H-I + RS	10.71 \pm 3.88*	2.33 \pm 1.07*	26.61 \pm 6.30*
H-II + RS	7.81 \pm 4.13*	0.88 \pm 0.48*	31.22 \pm 3.66*
H-II + RS + L-Arginine (500)	30.20 \pm 10.75 ^b	6.92 \pm 2.60 ^b	23.83 \pm 1.50 ^b
H-II + RS + L-NAME (50)	1.44 \pm 0.83 ^b	0.48 \pm 0.31	36.50 \pm 3.50

All data are Mean \pm SE (n=6/group) OAE – Open arm entries; OAT – open arm time in EPM; * $p < 0.05$ vs controls; ^a $p < 0.05$ (compared to H-II); ^b $p < 0.05$ (compared to H-II+RS)

increases of 52% and 79% were observed with H-I + RS and H-II + RS groups, respectively, when compared to controls, which were greater than those seen after H-I or H-II per se ($p < 0.05$, in each case).

Pre-treatment of rats with L-arginine (500 mg/kg) resulted in differential attenuations in elevated plasma corticosterone levels that were seen after H-II, or H-II+RS groups, and the data of these drug treated groups were significantly different from the values of the H-II or H-II+RS per se groups ($p < 0.05$). On the other hand, pretreatment with the NOS inhibitor, L-NAME (50 mg/kg) resulted further increases in plasma corticosterone levels with 25% and 20% increases being observed in the drug treated groups when compared to respective per se H-II or H-II+RS groups, and these differences were statistically significant ($p < 0.05$). These results are summarized in Table I.

Effects of high altitude (H-I or H-II), restraint stress (RS) and nitric oxide (NO) modulators on total nitrates and nitrites (NOx) in brain

Exposure to high altitude (H-I or H-II) resulted in reductions in total nitrate and nitrite (NOx) levels in brain homogenates of rats. There were 4% and 30% reductions in NOx levels in the H-I and H-II groups when compared to control (normoxic) values, with the data of the latter group being statistically significant ($p < 0.05$). Pretreatment with the NO mimetic, L-Arginine, markedly attenuated these high altitude hypoxia-induced reductions in brain NOx levels and the data of the L-arginine + H-II group were found to be significantly higher than that of H-II alone ($p < 0.05$). On the other hand, NOS inhibitor, L-NAME pretreatment induced further reductions in brain NOx levels beyond that seen in the H-II per se group ($p < 0.05$). When H-I or H-II was combined with restraint stress (RS), a clear potentiation in the reductions in brain NOx levels was seen and the data of the H-I+RS and H-II+RS groups were lower than those of the normoxic controls ($p < 0.05$). As compared to 4% and 30% reductions seen after H-I or H-II, the combined stress of H-I+ RS and H-II+RS resulted in 29% and 38% reductions in brain NOx levels, respectively, as compared to controls ($p < 0.05$). Pretreatment with L-arginine attenuated the

H-II+RS induced suppressions in brain NOx when compared to the H-II+RS. On the other hand, the NOS inhibitor, L-NAME tended to aggravate these effects, but these differences were not statistically significant ($p>0.05$). These results are summarized in Table II.

Effects of high altitude, restraint stress and nitric oxide modulators on oxidative stress markers

GSH

Exposure to acute high altitude (H-I or H-II) resulted in marked changes in oxidative stress markers in brain homogenates of these rats when compared to normoxic controls. Specifically, there was a 44% reduction in GSH values after H-I and a 47% reduction after H-II exposure, the data of both H-I and H-II groups being statistically significant when compared to controls ($p<0.05$). When RS was combined with either H-I or H-II greater suppressions in brain GSH levels were seen as compared to that seen after H-I or H-II alone – indicating a potentiating effect of RS. Pretreatment with L-arginine (500 mg/kg) reversed both the H-II and H-II+RS induced suppressions in GSH levels and these differences were statistically significant ($p<0.05$). On the other hand, L-NAME (50 mg/kg) showed differential nature of GSH lowering effects after H-II or H-II+RS. When H-II was combined with RS, the GSH levels were lower than that seen with H-II alone or controls, with

TABLE II : Effects of high altitude (H-I or H-II), restraint stress (RS) and NO modulators on GSH, MDA and NOx levels in brain homogenates of rats.

Treatment (mg/kg)	GSH ($\mu\text{mol/g}$ tissue)	MDA (nmol/mg protein)	NOx (nmol/mg protein)
Control	6.45 \pm 0.49	4.61 \pm 0.23	4.75 \pm 0.39
H-I	3.65 \pm 0.31*	4.93 \pm 0.35	4.60 \pm 0.26
H-II	3.43 \pm 0.39*	6.51 \pm 0.42*	3.33 \pm 0.23*
H-II + L-Arginine (500)	6.36 \pm 0.23 ^a	4.75 \pm 0.27	5.23 \pm 0.28 ^a
H-II + L-NAME (50)	2.46 \pm 0.31	7.34 \pm 0.59	2.33 \pm 0.25
H-I + RS	1.94 \pm 1.17*	6.86 \pm 0.47*	2.98 \pm 0.11*
H-II + RS	2.55 \pm 0.23*	7.16 \pm 0.50*	3.36 \pm 0.13*
H-II + RS + L-Arginine (500)	5.50 \pm 0.33 ^b	6.03 \pm 0.30	4.01 \pm 0.12
H-II + RS + L-NAME (50)	1.73 \pm 0.33	7.70 \pm 0.26	2.15 \pm 0.24

All data are Mean \pm SE (n=6/group).

* $p<0.05$ vs controls; ^a $p<0.05$ (compared to H-II); ^b $p<0.05$ (compared to H-II+RS)

reductions of 64% in comparison with control group ($p<0.05$). These results are summarized in Table II.

MDA

Exposure to acute high altitude (H-I or H-II) resulted in marked elevations in brain MDA levels as compared to normoxic controls. The MDA levels in H-I and H-II groups were found to be elevated by 18% and 56% respectively, the data of latter being statistically significant ($p<0.05$). Pretreatment with L-arginine (500 mg/kg) induced marked reversals in these elevated MDA levels when compared to the H-I or H-II group data. On the other hand, the NOS inhibitor, L-NAME further aggravated the MDA levels as compared to H-II alone. When RS was combined with H-II, the MDA levels were further increased as compared to that seen with H-II alone with elevations being to the extent of 72% therefore indicating a potentiating effect ($p<0.05$). Pretreatments with the NO mimetic, L-arginine (500 mg/kg) attenuated the effects of H-II+ RS on MDA and reversed the effects towards normoxic levels. These results are summarized in Table II.

NOx

In the experimental groups H-II, H-I + RS and H-II + RS, there were marked suppressions in the nitric oxide metabolite (NOx) levels in brain homogenates when compared to controls. These reductions were to the extent of 30%, 39% and 30% respectively ($p<0.05$, in each case). Pretreatment with L-arginine (500 mg/kg) attenuated the H-II and H-II + RS induced lowering of brain NOx levels, with the effects in the former case being statistically significant ($p<0.05$). On the other hand, L-NAME pretreatment induced opposite effects on H-II and H-II + RS induced changes in brain NOx levels, and though there were 31% and 37% reductions in the NO metabolite levels after the NOS inhibitor, these differences were not statistically significant when compared to their respective control values ($p>0.05$). These results are summarized in Table II.

Discussion

High altitude exposure is an environmental stressor

that shows debilitating effects on the physiological as well as physical performances and may lead to health issues if the mechanism of acclimatization fails (21). Hypobaric hypoxia present at high altitude as well as simulated conditions is known to cause neurobehavioral changes like cognitive and mental dysfunctions accompanied with memory deficits and motor impairment (22, 23). Complex pathways are thought to regulate such responses and interactions between varieties of host factors are instrumental in deciding the nature and extent of the impact of such aversive inputs could have on the biological system. Nitric oxide (NO) is a ubiquitous neurotransmitter molecule that has multi-dimensional effects and NO modulators have been found to be experimentally very helpful in studying the NO-ergic mechanisms in experimental situations (13, 14). Though effects of NO are well documented in cardiorespiratory system, its regulatory role in hypoxia (of high altitude) induced neurobehavioral effects are not clearly defined. Thus the present study was designed to evaluate the role for NO in high altitude stress induced behavioral changes in rats. Further, possible interactions between environmental stressors (eg. high altitude) and emotional stressors (restraint), which actually co-exist in real life situations, were assessed during physiological and neurobehavioral effects and their regulation by NO and NO-mediated signaling mechanisms were also evaluated.

In our experiments, hypoxia of high altitude was simulated at two different levels viz. 8,000 ft and 12,000 ft, and the effects induced by these simulations were studied on the neurobehavioral profile. The Elevated Plus Maze test is a well validated model to study anxiety modulation and effects of anxiolytic/anxiogenic agents, and high altitude stress reduced both open arm entries and time spent in open arms – suggesting an anxiety like response. These anxiogenic effects seen were altitude dependent with H-II (12000 ft) showing greater effects than H-I (8000 ft). These experimental findings are generally in agreement with earlier studies that reported that exposure to high altitude caused changes in neurobehavioral paradigms (24, 25). Interestingly, this anxiogenic effect was further compounded in rats exposed to both restraint stress and high altitude simulation. The increase in

percentage of both open arm entries as well as the time spent in the open arms, i.e. aggravations in anxiety like behavior, shows a compounding effect of one stressor on the other.

The role of NO as a neuromodulator substance has been documented and its involvement has been suggested in neurological and psychiatric disorders (26). Further, the regulatory influence in emotional stress induced anxiety like behavior has also been reported. The NO/cGMP pathway has also been found to have an important role in modulation of anxiety like behaviour. Further, localization of NO synthase is known in brain areas like hypothalamus, amygdala and hippocampus (27). Thus, NO related anti-anxiety effects were assessed in these models of anxiety, with the aim being to explore the role of NO in neurobehavioral changes of high altitude stress.

In this study, the precursor of NO, L-arginine reversed the effects of high altitude (and resultant hypoxia) induced neurobehavioral suppression on the EPM per se, as well as in combination with restraint stress. L-NAME, a non-specific NOS inhibitor, on the other hand, aggravated the hypoxia induced stress response in EPM. L-NAME is known to have an anxiogenic effect (14). These results indicate that there is a role of NO in high altitude stress induced anxiogenesis. Since, L-NAME also blocks the nNOS pathway, it is possible that nNOS might be involved in these high altitude induced neurobehavioral responses.

The hypothalamo-pituitary-adrenal axis (HPA) is one of the primary targets and effectors of the stress response. Activation of the HPA axis by stress releases causes the release of corticotrophin releasing factor (CRF) in the hypophysial portal system which in turn activates the anterior pituitary to release adrenocorticotrophic hormone (ACTH) in the systemic circulation. ACTH targets the adrenal cortex and stimulates steroid hormone release - cortisol in humans and corticosterone in rodents. This acts by regulating metabolic, cardiovascular, immune as well as behavioral responses during stress, and is widely recognized as a sensitive, reliable and consistent marker of

stress (38). Plasma corticosterone levels in the high altitude stress groups were found to be elevated in an intensity dependent manner (the increase in plasma corticosterone was higher at 12,000 ft). This confirmed that high altitude was an important environmental stressor capable of activating the HPA axis. When high altitude was combined with restraint stress, the levels of corticosterone were further augmented and greater elevations were seen at high altitude of 12000ft (H-II). This potentiating effect on plasma corticosterone suggested complimentary roles for both stressors (high altitude and restraint) on HPA axis activation. The NO mimetic, L-arginine, reversed the plasma corticosterone elevations at high altitude (12,000 ft, H-II) alone and when combined with restraint stress, whereas, L-NAME (NOS inhibitor) showed opposite effects. This is in agreement with earlier studies that showed a role for NO in attenuating the stress-induced activation of the HPA axis (37).

Many transcription factors are involved in physiological responses to hypoxia and an important role is played by HIF-1 in oxygen homeostasis and is induced by hypoxia in most cell types (28). HIF-1 is a heterodimeric transcription factor composed of HIF-1 α and HIF-1 β subunits. Hypoxia is a well-known factor to increase the levels of ROS in various cells, tissues and systems. Further, ROS are known to promote endothelial dysfunction through several mechanisms, including direct action on the vascular endothelium, promoting formation of lipid inflammatory mediators, and/or depleting the cellular levels of nitric oxide (NO), an endogenous antioxidant (29). In addition to this, HIF-1 α is thought to be

degraded by ROS (20). Earlier studies have implicated NO for a role in high altitude adaptation (30, 31). This effect of NO appears to be a result of time- and concentration-dependent delivery as well as the interaction with other biomolecules. Under normoxic conditions, NO is described to have stimulating properties on HIF-1 activity (32), which is contrasted by reports showing inhibitory actions of NO in combination with hypoxia and CoCl₂ (33, 34, 35). Our study extends these finding by establishing a correlation between the NO and ROS in high altitude simulated hypoxia. Our earlier studies with restraint stress also suggest generation of free radicals and increased anxiety in the EPM which was under the moderating influence of NO mimetics suggesting an anxiolytic effect for NO (11, 36, 37). In this study, RS induced potentiation of high altitude effects was also accompanied by oxidative stress and attenuated by L-arginine – again suggesting interactions between NO and ROS during combined stressors. Taken together, our study indicates that high altitude induced angiogenesis either alone or in combination with restraint stress is accompanied by brain oxidative stress and both behavioral and biochemical changes are attenuated by NO mimetics – suggesting a protective role for NO in such high altitude stress.

Acknowledgments

The authors thank the Defense Institute of Physiology and Allied Sciences (DIPAS, DRDO), Delhi, and Indian Council for Medical Sciences (ICMR), New Delhi, for providing the financial support for the study.

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