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Research Article

PROTEOMIC EVALUATION OF ANTIOXIDANT ACTIVITIES OF NAP PEPTIDE IN RAT BRAIN CORTEX EXPOSED TO CHRONIC HYPOBARIC HYPOXIA

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Abstract: High altitude exposure results in decreased partial pressure of oxygen and increased formation of reactive oxygen and nitrogen species, which causes oxidative damage to lipids, proteins and DNA. Exposure to high altitude appears to decrease the activity and effectiveness of antioxidant enzyme system. The antioxidant system is limited in brain tissue and is very much susceptible to hypoxic stress. High metabolic rate along with a rich store of polyunsaturated fatty acids makes brain tissues a vulnerable target to oxidative damage. NAP peptide in particular has been reported to cross the blood–brain barrier and it improves cognitive functions and neuronal bioenergetics mechanisms. However, the therapeutic potential of NAP in hypoxic stress and the precise mechanism involved still remains unexplored. Our *in vivo* results show that the significant changes (antioxidant status of brain) were observed at 14 days hypoxic exposure. In order to monitor the proteins of rat brain cortex, adult Sprague Dwaly male rats were exposed to stimulated condition of high altitude (25000ft, 28±2°C) for 14 days alongside intranasal administration of NAP peptide (2µg/kg body weight/day). In this study, we identified 25 differentially expressed proteins which were antioxidant in nature and associated with energy metabolism.

***Keywords*:** NAP peptide; hypobaric hypoxia; brain; cortex; proteomics, Prdx5.

Introduction

The lower partial pressure of oxygen at high altitudes results in compromised oxygen supply to brain. This condition of hypobaric hypoxia leads to a number of structural modifications in brain and neurological conditions like headache, acute mountain sickness (AMS), cerebral edema (HACE), mental confusion, memory deficit, cerebral hemorrhages and sleep disturbances (Hackett and Roach, 2004; Sharp and Bernaudin, 2004; Weil, 2004; Wilson *et al.*, 2009). Moreover, paucity of molecular oxygen as terminal electron acceptor for Krebs cycle act as electron sink in mitochondria and leads to formation of reactive oxygen and nitrogen species (Bailey and Davies, 2001; Magalhaes *et al.*, 2005; Moller *et al.*, 2001). The high metabolic rate along with a rich store of polyunsaturated fatty acids makes brain vulnerable to hypoxia-induced oxidative stress (Calabrese *et al.*, 2000; Marttila *et al.*, 1988).

Biochemical studies have revealed that brain regions like hippocampus, cortex, cerebellum and striatum are vulnerable to chronic hypobaric hypoxia induced oxidative stress (Hota *et al.*, 2007; Maiti *et al.*, 2006). Maiti *et al.* have reported that exposure to 20,000 ft (349.3 mmHg) for 3 and 7 days results in increased free radical production and lipid peroxidation in brain regions. Similar results were also reported by Hota *et al.* (2007) for exposure to 25,000 ft (282 mmHg) for 3, 7 and 14 days. Several other studies also corroborating

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previous reports that hypoxic exposure induces oxidative stress (Dosek *et al.*, 2007), increased lipid peroxidation, elevated erythropoietin (EPO) levels (Behn *et al.*, 2007; Huang *et al.*, 2008) and tissue damage (Hellewell *et al.*, 2010). These studies suggest that significant reduction of antioxidant defense enzymes such as reduced glutathione (GSH), glutathione peroxidase (GPx), glutathione reductase (GR) and superoxide dismutase (SOD) enhances brain susceptibility to hypobaric hypoxia. The hippocampus and cortex are more sensitive to hypoxia-induced oxidative stress than other regions of CNS because glutamate receptors are more in these areas (Erecinska and Silver, 1996; Hota *et al.*, 2007; Maiti *et al.*, 2006; Ramanathan *et al.*, 2005). Hence, supplementation of antioxidants has been suggested to minimize the oxidative damage.

Several antioxidants like vitamin E and C, β-carotene, Acetyl-l-carnitine (ALCAR), N-acetyl-l-cysteine, dimethyl sulfoxide, 1, 2-dihydroxy benzene-3, 5-disulphonate and superoxide dismutase has been tested in animal models with limited success (Barhwal *et al.*, 2008; Barhwal *et al.*, 2007; Bautista-Ortega and Ruiz-Feria, 2010; Pialoux *et al.*, 2010; Pialoux *et al.*, 2009). We have previously demonstrated that intranasal administration of NAP peptide (active fragment of the glial-derived Activity-Dependent Neuroprotective Protein, ADNP) reaches brain and exerts neuroprotection during hypobaric hypoxia (Sharma *et al.*, 2011). The effects include decreased ROS and lipid peroxidation, maintenance of GSH/GSSG homeostasis and activation of Nrf2-Hmox1 pathway. Furthermore, NAP supplementation also improved memory impairment during chronic hypoxia exposure. However, the neuroprotective efficacies of NAP peptide at proteome level still need to be explored.

Conventional 2-DE with mass spectrometry makes proteomics a powerful tool to investigate dysregulated proteins. Burgula *et al.* have investigated proteome profiling of brain cortex for short time duration of hypobaric hypoxia and identified two proteins only (Burgula *et al.*, 2010). In contrast, Hernández *et al.* identified several proteins associated with glycolysis, TCA cycle, cytoskeleton and oxidative phosphorylation (Hernandez *et al.*, 2012) during 5 days of hypoxia exposure. Because none of the report described proteome alterations during prolonged hypobaric hypoxia exposure, we investigated the brain cortex proteome under chronic hypoxia exposure (25000 ft, 282 mmHg for 14 days) and further modulation by NAP supplementation. Our studies reveal that NAP peptide provides a comprehensive protection against hypobaric hypoxia induced oxidative stress.

Materials and Methods

Hypobaric hypoxia exposure and NAP supplementation schedule - Male adult Sprague-Dawley rats (220 ± 10 g) were maintained under 12 h light/dark cycles and were provided with food and water at *ad libitum*. All experimental protocols were approved by the Animal Use and Care Committee of Defence Institute of Physiology and Allied Sciences. To study the effect of NAP on chronic hypobaric hypoxia exposure, 18 male Sprague-Dawley rats (220 ± 10 g) rats were randomly divided into three groups. Group I served as normoxia group (n = 6 rats), Group II served as hypoxia group where the rats were exposed to simulated hypobaric hypoxia for 14 days (n = 6) at 25,000 ft (7600 m, 282 mm Hg) in a specially designed animal decompression chamber. The temperature and humidity were maintained at 28 ± 2 °C and 60 ± 5% respectively. Group III served as NAP supplemented hypoxia group (NAP + Hypoxia) wherein the rats were supplemented with intranasal NAP administration (2 µg/kg body weight, once in a day, described previously, Sharma NK *et al.*, 2011) along with hypoxic group (n = 6). For supplementation of NAP peptide, food and water the pressure was brought to normobaric conditions for 30 min every day. The rate of ascent to altitude was maintained at the rate of 300 m/min and it took a period of 20-25 min to reach the desired altitude. After the completion of exposure, rats were sacrificed by cervical dislocation and the brain were collected, snap-freezed in liquid nitrogen and stored for further use.

Sample Preparation for 2D Gel Electrophoresis - The brain cortex (100mg) homogenized on ice in 500 µl lysis buffer [(40 mMTris (pH 7.5), 8 M urea,

2.5 M thiourea, 3% 3-[(3- holamidopropyl) dimethylammonio]- 1-propanesulfonate (CHAPS), 10 mM dithiothreitol (DTT), 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF)], and protease inhibitor cocktail (Sigma, St. Louis, USA). The homogenate was then centrifuged at 15000xg and 4 °C for 45 minutes. The supernatant was transferred in a fresh tube and protein concentration was estimated by Bradford reagent.

Protein Separation - Protein sample (200 µg) was mixed with rehydration buffer containing 7 M urea, 2 M thiourea, 1.2% w/v CHAPS, 0.4% w/v ABS-14, 20 mM dithiothreitol (DTT), 0.25% v/v pH 3-10 ampholytes, 0.005% w/v bromophenol blue (BPB). The IPG strips (Immobiline DryStrip, pH 3-10, 18cm, GE Healthcare, Sweden) were passively rehydrated with protein containing rehydration buffer for 18 hours at 20°C. Following rehydration, strips were focused at 500 V for 7 h (slow), 1000 V for 1 h (linear), 8000 V 3 h (gradient), 8000 V 3 h (linear), 10000 V 2 h (gradient) and 10000 V 1h (linear), with the total of 65 -70 KV h using IPGphor system (Amersham Pharmacia Biotech). Prior to second dimensional separation, strips were equilibrated first with equilibration buffer (6 M urea, 30% v/v glycerol, 2% (w/v) SDS, 50 mM Tris pH 8.8) containing 1% (w/v) DTT for 15 min and then with equilibration buffer containing 2.5% (w/v) Iodoacetamide (IAA) for 15 min. The second dimension was carried out using EttanDaltSix Electrophoresis System (GE Healthcare, Uppsala, Sweden). The strip was then loaded onto 12% SDS-polyacrylamide gel and sealed with 0.5% agarose gel. The equilibrated IPG strips were subjected to second dimension separation on 12% SDS PAGE. The gels were manually silver stained as described earlier (Ahmad *et al.*, 2011) and image was scanned using Investigator™ ProPic II (Genomic Solutions). Differential spot pattern was analysed by Progenesis Same spot analysis software (Nonlinear Dynamics, version 4.0). Spot assignment, statistical calculations and background correction were performed using the default parameters.

In-gel Digestion - All differentially expressed spots were excised manually and washed several times with ultrapure water. Silver stained gel pieces were destained by Formers reagent, freshly prepared (equal amount of 30 mM Potassium ferricyanide and 100 mM Sodium thiosulfate). The destained gel spots were washed thrice with the ultrapure water and subsequently dehydrate and rehydrate by actonitrile and 10mM ammonium bicarbonate, respectively and subjected to vacuum drying by speed vac (Heto MAXI dry plus, UK). The dried gel pieces rehydrated in trypsin (20ng/µl, Sequencing grade, Promega, Madison, WI) for 30 minutes at 4 °C, equal amount of 25mM ammonium bicarbonate were added and incubated for overnight at 37 °C. The digested peptides were extracted by sonication with 50% Acetonitrile and 0.1% TFA and were dried in Speed Vac.

Identification of differentially expressed proteins - The dried peptides were dissolved in 0.1% TFA and 1 µL was mixed with equal volume of CHCA matrix saturated with 50% ACN in 0.1% TFA. The resulting 2 µL solution was plated onto a MALDI target plate (Bruker Daltonics, Germany) and air dried. The spectra were acquired by MALDI TOF/TOF (Ultraflex III, Bruker Daltonics, Bremen, Germany) in the positive ion mode at the accelerating voltage of 25kV under the Flex Control software (Version 3.0, Bruker Daltonics) as described earlier (Ahmad *et al.*, 2011). The generated peptide mass list was searched with MASCOT (*http://www.matrixscience.com*) using Uniprot/Swiss-Prot protein database to find and match the protein identity with the following search parameters; *Rattus norvegicus* as taxonomy with ± 100 ppm peptide mass tolerance, 1 maximum missed cleavage sites permitted and carbamidomethyl (cysteine) as fixed modification. Protein identification was accepted only when the probability based Mowse score was greater than the score fixed by MASCOT as significant with $p < 0.05$. Gene ontology (GO) annonations (functional distribution) for differentially expressed proteins were assigned using Toppgene suite with manually searched from uniprot/swissprot database.

Network construction and pathway analysis - The gene list was generated, corresponding to differentially expressed proteins. The list was then analyzed by Ingenuity Pathways Analysis. These genes, called focus genes, were overlaid onto a global molecular network developed from

information contained in the Ingenuity Pathways Knowledge Base. Genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least 1 reference from the literature or from canonical information stored in the Ingenuity Pathways Knowledge Base. The intensity of the node colour indicates the degree of up- (red) or down- (green) regulation. Nodes are displayed using various shapes that represent the functional class of the gene product.

Protein expression analysis by Western blots - For western blot, cortex was homogenized on ice in RIPA buffer and centrifuged at 13,000 g for 20 minutes at 4 °C. The supernatant was collected and protein concentration was determined by the Bradford reagent. Protein (40 µg) from each sample were separated on a 10% SDS-polyacrylamide gel and electro transferred onto nitrocellulose membranes. Nonspecific binding of protein was blocked by saturating the Nitrocellulose membranes with 5% BSA overnight at 4°C. Next, membranes were incubated with primary antibody for 2 h at room temperature, then washed thrice (5 min each) in PBST to remove unbound antibody and incubated in secondary antibody at room temperature for 2 h. Membranes were washed thrice (5 min each) with PBST and visualized on X-ray films using chemiluminiscent peroxidase substrate. Autoradiogram signals were captured using a gel documentation system (Model Omega, Ultra Lum Inc., USA) and quantitative densitometric analysis was done with ImageJ software (Version 1.6, National Institutes of Health, Bethesda, MD).

Statistical analysis - The results of western blot are representations of three independent experiments (Mean ± SEM). Statistical analyses were performed with Turkey's t-test and a P value of <0.05 was considered significant.

Result

Proteome changes in rat brain cortex exposed to chronic hypobaric hypoxia

To identify proteome changes due to chronic exposure to hypobaric hypoxia for 14 days, we separated total protein extract of cortex by conventional two-dimensional electrophoresis (2-DE) after 0 day (normoxia), 14 days and NAP supplemented during hypoxia exposures. A total of 3 samples from each group were analyzed in duplicate for 2-DE. To comparison of hypoxic and NAP supplemented proteome with normoxia enabled us in the identification of 44 differentially expressed protein spots. Out of 44 proteins spot, we could identify only 25 proteins (Table 1, Figure 1). Among the differentially expressed protein spots 2, 1 and 3 spots were unique with corresponding to normoxia, hypoxia and NAP supplemented hypoxia exposed, along with 8 protein spots were common with correspond to NAP supplemented and normoxia (Figure 2). In the hypoxic exposure, 11 proteins (Pdia3, Rbm34, Uchl1, Serpina3n, Ldhb, Eno1, Atp5h, Gnao1, Ywhaz, Ckb, and Cyp11b1) were up-regulated and 7 proteins (Pgam2, Dynll2, Mif, Rps27a, Prdx5, Ddt and Rab3d) were down-regulated. In contrast, 14 proteins (Pgam1, Pgam2, Dynll2, Tpi1, Cep63, Cox5a, Rps27a, Pdia3, Rbm34, Uchl1, Serpina3n, Eno1, Atp5h, and Cyp11b1) proteins were up-regulated and 4 proteins (Ddt, Calm1, Ywhae and Rab3d) were down-regulated in NAP supplemented group. Comparing of hypoxic and NAP treated cortical proteome we have identified 7 common up-regulating proteins (Pdia3, Rbm34, Uchl1, Serpina3n, Eno1, Atp5h, and Cyp11b1)

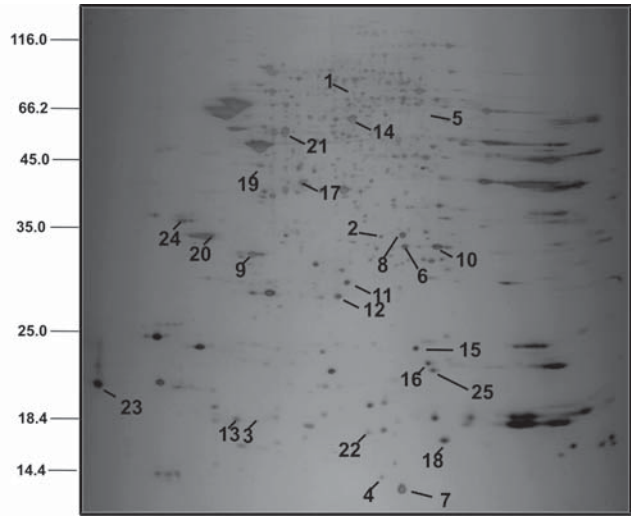


Figure 1: **The representative image of 2-DE proteome profile of brain cortex exposed to chronic hypoxia. Number marked on protein spots were reproducibly altered in response to hypoxia or NAP supplementation. The proteins were resolved according to their isoelectric point (pI) in the first and their Mw on 12% SDS-PAGE followed by silver staining**

Table 1
Cerebral cortex protein alterations in response to hypobaric hypoxia

Spot ID	*SwissProt ID*	*Score*	*Protein Name*	*Matched peptide /% coverage*	*Calculated Mass (kD)/ pI Value*	*Observed Mass(kD)/ pI Value*	*Fold Change 14DH*	*Fold Change 14DHT*	*Function*
1.	P11598	52	Protein disulfide-isomerase (EC 5.3.4.1) ER60 precursor	9/18	57.04/6.38	62/6.53	1.32	2.99	Intramolecular oxidoreductase
2.	JC1132	58	Phosphoglycerate mutase (EC 5.4.2.1) B chain - rat	7/44	28.92/6.67	31/6.85	…	2.5	Glycolysis, Hydrolase, Isomerase
3.	P15393	52	Cytochrome P450 11B1	11/26	23.23/5.98	16/5.16	1.19	2.43	Oxidation-reduction process
4.	Q78P75	106	Dynein light chain 2, cytoplasmic	7/35	10.45/6.81	7/6.82	0.71	1.46	Synaptic target recognition
5.	Q5M9F1	41	RNA-binding protein 34	6/20	47.74/10.2	52/7.66	1.19	1.44	Acetyl-CoA C-acyltransferase activity
6.	P48500	141	Triosephosphate isomerase	11/54	27.34/6.89	30/7.18	…	1.37	Fatty acid biosynthetic process
7.	Q4KLY0	72	Centrosomal protein of 63 kDa	8/21	63.04/5.54	4/7.11	…	1.34	Signal transduction in response to DNA damage
8.	P25113	111	Phosphoglycerate mutase 1	9/34	28.92/6.67	31/7.16	…	1.32	Phosphoglycerate mutase activity
9.	P11240	72	Ubiquitin carboxyl-terminal hydrolase isozyme L1	7/36	25.16/5.14	30/5.24	1.22	1.32	Ubiquitin-dependent protein catabolic process
10.	P48500	120	Triosephosphate isomerase	11/62	27.34/6.89	30/7.65	…	1.31	Fatty acid biosynthetic process
11.	P05544	60	Serine protease inhibitor A3L	8/47	46.41/5.48	27/6.37	…	1.23	Reduced during acute inflammation
12.	P31399	50	ATP synthase subunit d, mitochondrial	4/31	18.80/6.17	26/6.25	1.16	1.23	ATP synthesis coupled proton transport
13.	P11240	47	Cytochrome c oxidase subunit 5A, mitochondrial	5/48	16.34/6.08	17/4.95	…	1.23	Cytochrome-c oxidase activity
14.	P04764	273	Alpha-enolase	26/67	47.44/6.16	52/6.52	1.16	1.58	Role in glycolysis, growth control, hypoxia tolerance.
15.	P62982	169	Ubiquitin-40S ribosomal protein S27a	5/30	18.28/9.68	21/7.32	0.48	1.15	Metal ion binding
16.	Q9R063	72	Peroxiredoxin 5	6/40	22.53/8.94	19/7.58	0.44	1.15	Antioxidant activity;electron transporter activity
17.	P42123	154	L-lactate dehydrogenase B chain	14/49	36.87/5.7	37/5.82	1.22	…	Oxidation-reduction process
18.	P30904	59	Macrophage migration inhibitory factor	5/41	12.64/6.79	13/7.74	0.55	…	Positive regulation of acute inflammatory response,cellular response to hydrogen peroxide
19.	P59215	52	Guanine nucleotide-binding protein G(o) subunit alpha	9/26	40.61/5.34	40/5.32	1.19	…	Response to hydrogen peroxide
20.	P63102	137	14-3-3 protein zeta/delta	20/55	27.92/4.73	32/4.5	1.19	…	Postsynaptic density
21.	P07335	132	Creatine kinase B-type	14/51	42.98/5.39	45/5.6	1.66	…	Energy transduction in tissues with large fluctuating energy demands
22.	P80254	51	D-dopachrome decarboxylase	4/50	13.23/6.09	14/6.63	0.43	…	Inflammatory response
23.	P62161	170	Calmodulin	7/19	16.82/4.09	19/3.24	…	…	Calcium-dependent protein binding
24.	P62260	157	14-3-3 protein epsilon	12/40	29.32/4.63	33/4.29	…	0.83	Signal transduction
25.	Q63942	48	GTP-binding protein Rab-3D	4/27	24.50/4.75	19/7.73	0.39	0.3	Protein transport

For each identified protein, an abbreviation, the 2-DE spot number, the Swiss-Prot accession number, the expression group (14 days hypoxia and NAP supplemented hypoxia), the number of peptides identified through MALDI-MS, the sequence coverage (Seq. cov.), no. of matched peptides, MALDI-MS identification score, and the fold change of an individual protein between hypobaric hypoxia exposed and controls are presented (Significant change considered only if fold change ≥ 1.15 and $p \leq 0.05$).

along with 5 (Mif, Gnao1, Ldhb, Ckb and Ywhaz) proteins were specific for hypoxic exposed group and 7 (Cep63, Ywhae, Pgam1, Tpi1, Cox5a, Tpi1 and Calm1) proteins specific for NAP supplemented group.

Functional annotation and canonical pathways

The functional annotations revealed alterations in hypoxia and NAP supplemented group. Chronic hypoxia exposed group showed unique molecular functions like dopachrome isomerase activity while NAP treated grouped showed bisphosphoglycerate mutase activity and Oxidoreductase activity. Some molecular functions like isomerase activity and intramolecular oxidoreductase activity were detected in both groups but varying in the expression. Several biological processes were identified. Hypoxia exposed group showed significant alteration only in glycolysis. In contrast, number of biological process like glycolysis, generation of precursor metabolites and energy, gluconeogenesis, oxidoreduction coenzyme metabolic process, carbohydrate catabolic process and oxidation-reduction process detected in NAP supplemented group. To explore of cellular pathways that may be regulated during long-term hypoxia, we uploaded all identified proteins to the IPA (Ingenuity Pathway Analysis). The top significant function associated with hypoxia exposure is Free Radical Scavenging, Cellular Assembly and Organization, Carbohydrate Metabolism. Similarly, top significant function associated with NAP supplemented group is Cell Death, Cell Cycle and Cancer. We also visualized the molecular networks for hypoxia as well as NAP supplemented group. For hypoxia exposed cortex, proteins such as Ddt, Mif, Pdia3, ldhb and Rab3d act as central node. Similarly, for NAP supplemented group, proteins Eno1, Pgam1, Pgam2 and Tpt1act as central node (Figure 3).

Next we identified canonical pathways associated with hypoxia and treated groups. In hypoxia exposed group, canonical pathways includes Hif1a signaling, 14-3-3 mediated signaling, mitochondrial dysfunction, gap junction signaling and CREB signaling in neurons were detected. Similarly in the NAP supplemented group, neuroprotective role of THOP 1 in AD, 14-3-3 mediated signaling, oxidative phosphorylation, protein kinase A signaling, cell cycle G2/M DNA damage check point regulation, VEGF signaling, glycerophospholipid metabolism, mitochondrial dysfunction and acute phase response signaling were detected (Figure 4).

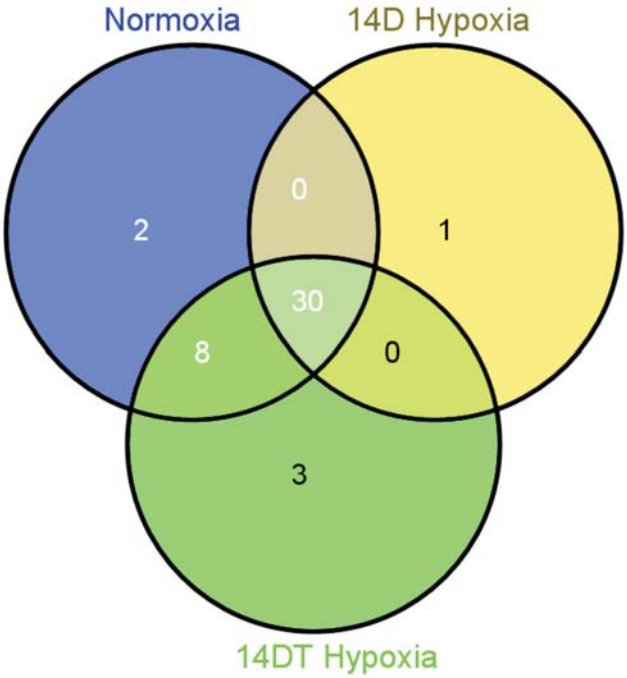
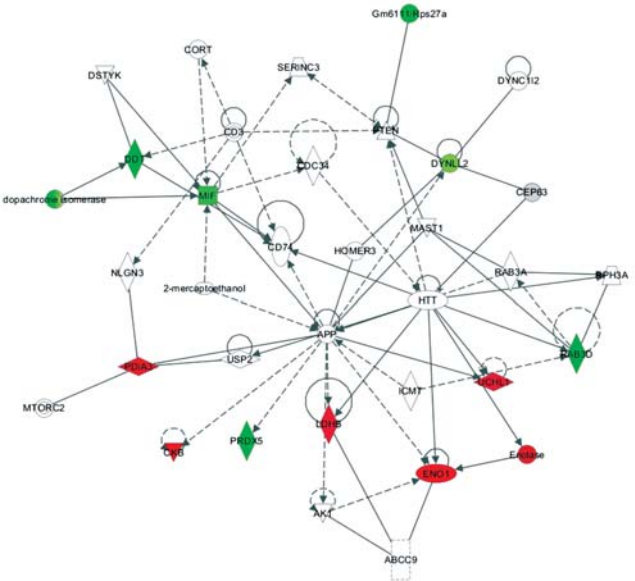
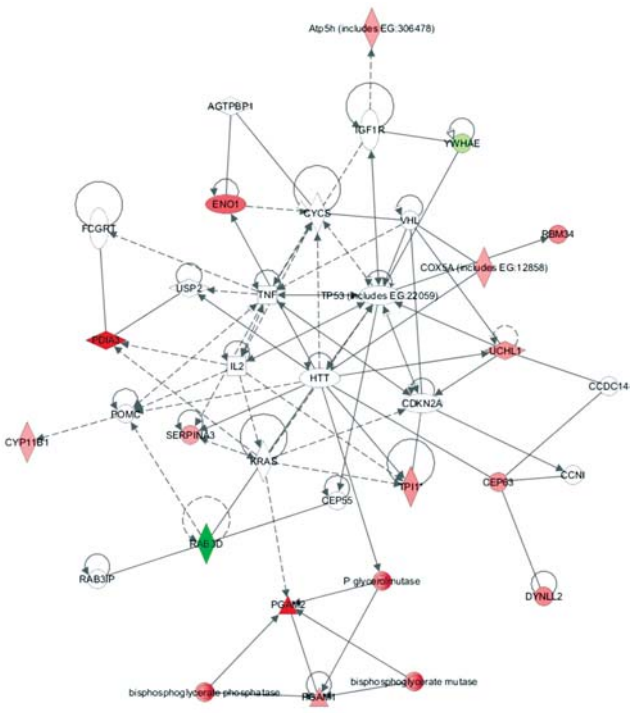


Figure 2: **Venn diagram representation for differentially expressed protein spots**



(A)



(B)

Figure 3: **Ingenuity pathway analyses (IPA) biological network of brain cortex** (A) **"Free Radical Scavenging, Cellular Assembly and Organization, Carbohydrate Metabolism" for hypoxia exposed, and** (B) **Cell Death, Cell Cycle, Cancer in NAP supplemented cortical region. Proteins are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). The intensity of the node color (green for down and red for up-regulation) indicates the labeling intensity**

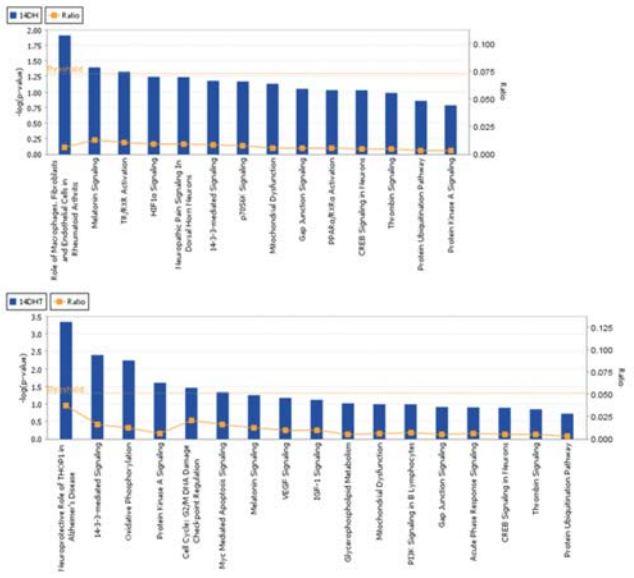


Figure 4: **Canonical pathways of differentially expressed proteins by IPA. X axis represents pathways and Y axis represents –log (p-value)**

Validation of differentially expressed protein by western blotting

For further confirmation, we selected two proteins on the basis of expression level in Table 1 and have important role in antioxidant properties (Figure 5). The western blot revealed 263% and 386% increase of Eno1 level in corresponding to Hypoxia and NAP supplemented group. In contrast, 43% and 11% decrease level of Prdx5 level in corresponding to Hypoxia and NAP supplemented group (Figure 6). These results showed antioxidant potential of NAP peptide.

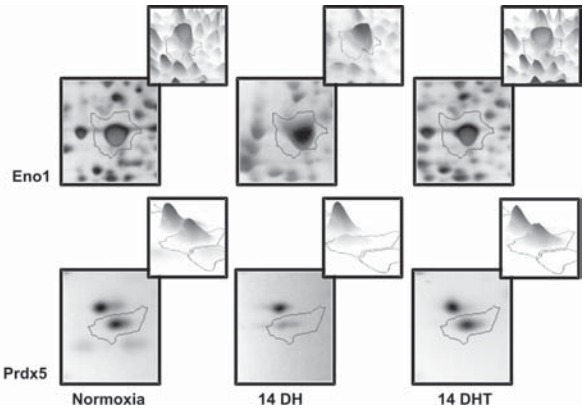


Figure 5: **Expression profiling of identified hypoxia-regulated proteins (Eno1 and Prdx5) in 2-DE exposed to chronic hypoxia and NAP supplemented group**

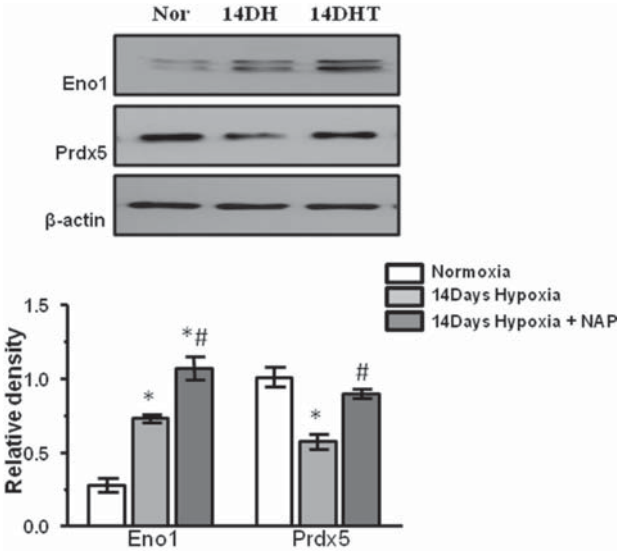


Figure 6: **Western blot analysis of altered protein (Eno1 and Prdx5), and densitometric analysis of immunoblots. Densitometric analysis of immunoblots in relative change with respect to control and signals were normalized against *β*-actin. The data is expressed as mean ± SEM.**
'*' refer as P≤0.05 with compare to normoxia and '#' refer as P≤0.05 with compare to 14 days hypoxia

Discussion

The decrease in partial pressure of oxygen at high altitude leads to increase reactive oxygen species (ROS) which induces oxidative stress. The effect of oxidative stress may vary in altitude, duration of time whether acute or chronic and individual health. The disturbance in oxygen supply during hypoxia causes a number of disorders like headache, acute mountain sickness (AMS), cerebral edema (HACE), mental confusion, memory deficit, cerebral hemorrhages and sleep disturbances (Hackett and Roach, 2004; Sharp and Bernaudin, 2004; Weil, 2004; Wilson *et al.*, 2009). Oxidative stress can affect any part of body but brain is most susceptible organ because high energy requirement, lack of oxygen storage and incapable of increasing capillaries makes brain most vulnerable organ. Hence, supplementation of an antioxidant has been suggested to minimize the hypoxia induced oxidative stress. Several antioxidants like vitamin E and C, β-carotene, Acetyl-l-carnitine (ALCAR), N-acetyl-l-cysteine, dimethyl sulfoxide, 1, 2-dihydroxy benzene-3, 5-disulphonate and superoxide dismutase has been studied (Barhwal *et al.*, 2008; Barhwal *et al.*, 2007; Bautista-Ortega and Ruiz-Feria, 2010; Pialoux *et al.*, 2010; Pialoux *et al.*, 2009). However, these compounds have limited success in minimizing oxidative stress during hypoxia exposure and unspecified mode of action and quantity put a question in mind. Our previous study revealed antioxidant potential of NAP peptide in femto-molar concentration during prolonged chronic exposure to hypoxia (Sharma *et al.*, 2011). In the present study, we investigated proteome profile of brain cortex and effect of NAP peptide under chronic hypobaric hypoxia exposure. Our present study also confirmed that chronic hypoxia exposure altered biological process, molecular function and signaling pathways. The GO molecular functions like bisphosphoglycerate mutase activity and oxidoreductase activity could be detected in NAP supplemented group indicating an active defense mechanism under chronic hypobaric hypoxia. This was further supported by biological process where we could detect only glycolysis in hypoxia. In contrast, NAP supplementation modulates many biological processes like glycolysis, oxidation-reduction and generation of precursor metabolites and energy (Table 2). The canonical pathways analysis demonstrate Hif1a signaling, 14-3-3 mediated signaling, mitochondrial dysfunction, gap junction signaling and CREB signaling in neurons in hypoxia exposed rats. Hif1a activates transcription of genes encoding proteins that mediate adaptive responses to reduced oxygen availability. Down-regulation of Hif1a signaling associated proteins results in increased ROS level. In contrast, up-regulation of antioxidant proteins with VEGF signalling and acute phase response signaling in NAP supplemented group enhance antioxidant status of brain during prolonged hypoxia exposure (Figure 4). Functional network studies revealed that most of the proteins related to free radical scavenging, cellular assembly and metabolism were down-regulated in hypoxia exposed rats. In contrast, these proteins were either up-or equal to normoxia in NAP supplemented rats suggests antioxidant potential of NAP peptide. Furthermore, many proteins related to cell cycle were up-regulated and cell death related proteins were down-regulated.

On the proteome profile comparison, major proteins associated with antioxidant in nature and related to energy metabolism in hypoxia and NAP supplemented hypoxia exposed rat brain cortex. Low level of oxygen modulates the reactive oxygen species (ROS). To neutralize the elevated level of RONS, there are several antioxidant proteins present in the cell. Sod and peroxiredoxins are the major proteins responsible for detoxification of ROS and converts to H_2O_2 and subsequently into water. Our previous study showed the up-regulation of Sod1, Cat and Nrf2 expressions in NAP supplemented group. Sod1 and Cat acts as primary defense against ROS while Nrf2 is a master regulator of antioxidant genes. In this study, we detected thiol-specific antioxidant protein of peroxiredoxin family. Prdxs have been shown to be involved in tumor cell proliferation under conditions of micro environmental stress such as hypoxia. Peroxiredoxins (Prdxs) are a new type of antioxidant protein that reduces ROS. There are six members in peroxiredoxin family such as Prdx1to Prdx6. Prdx1 and Prdx2 are mainly found in the cytoplasm, Prdx3 is present in mitochondria, and Prdx4 is secreted into the extracellular space. Prdx5 is an atypical, cytosolic

Table 2
Functional annotations of differentially expressed proteins

Category	*ID*	*Name*	*P-value*	*Hit Count Query*	*Hit Count Genome*	*Hit in Query List*
14 Days Hypoxia Exposure						
GO: Molecular Function	GO:0004167	Dopachrome isomerase activity	3.25E-04	2	3	MIF,DDT
GO: Molecular Function	GO:0016860	Intramolecular oxidoreductase activity	1.28E-03	3	46	PDIA3,MIF,DDT
GO: Molecular Function	GO:0016853	Isomerase activity	4.31E-02	3	149	PDIA3,MIF,DDT
GO: Biological Process	GO:0006096	Glycolysis	2.85E-02	3	68	LDHB,MIF,ENO1
14 Days Hypoxia Exposure with NAP Supplimentation						
GO: Molecular Function	GO:0016853	Isomerase activity	6.26E-05	5	149	PGAM2,PGAM1,PDIA3,TPI1,DDT
GO: Molecular Function	GO:0004082	Bisphosphoglycerate mutase activity	9.80E-04	2	4	PGAM2,PGAM1
GO: Molecular Function	GO:0016860	Intramolecular oxidoreductase activity	2.31E-03	3	46	PDIA3,TPI1,DDT
GO: Molecular Function	GO:0016491	Oxidoreductase activity	1.46E-02	6	752	COX5A,LDHB,PRDX5, YWHAE,PDIA3,CYP11B1
GO: Biological Process	GO:0006096	Glycolysis	5.38E-06	5	68	LDHB,PGAM2,PGAM1,TPI1,ENO1
GO: Biological Process	GO:0006091	Generation of precursor metabolites and energy	2.36E-04	7	471	COX5A,LDHB,PGAM2,PGAM1, ATP5H,TPI1,ENO1
GO: Biological Process	GO:0006733	Oxidoreduction coenzyme metabolic process	5.22E-04	4	69	LDHB,PRDX5,PGAM1,TPI1
GO: Biological Process	GO:0006739	NADP metabolic process	2.77E-03	3	30	PRDX5,PGAM1,TPI1
GO: Biological Process	GO:0044262	Cellular carbohydrate metabolic process	1.18E-02	6	554	LDHB,PGAM2,PGAM1,PDIA3,TPI1,ENO1
GO: Biological Process	GO:0055114	Oxidation-reduction process	3.31E-02	7	997	COX5A,LDHB,PRDX5,PGAM1,ATP5H, TPI1,CYP11B1

type, which possesses more effective antioxidant activity against ROS than other Prdxs. Prdx5 is a mitochondrial protein encoded by PRDX5 gene (Knoops *et al.*, 1999; Yamashita *et al.*, 1999). This gene encodes of antioxidant enzymes, which reduce hydrogen peroxide and alkyl hydroperoxides with reducing equivalents provided through the thioredoxin system. Peroxiredoxin-5/PRDX5 is involved in intracellular redox signaling (Declercq *et al.*, 2001; Noh *et al.*, 2001; Seo *et al.*, 2000). Exogenous supplementation of Prdx5 demonstrated neuroprotection against brain lesions in newborn mice (Plaisant *et al.*, 2003). The decreased expression of prdx5 revealed susceptibility of cortex to oxidative stress in chronic hypoxia (Figures 5 and 6).

A rapid decline in ATP levels under hypoxic conditions may cause increase anaerobic glycolysis pathways. Glycolysis related proteins such as Pgam1 and Pgam2 catalyzes the inter conversion of 3-phosphoglycerate to 2-phosphoglycerate, leading to a second equivalent of ATP produced in glycolysis. Alpha-enolase hydrolyzes 2-phosphoglycerate to phosphoenol pyruvate and maintains intracellular ATP levels during ischemic hypoxia and responsible for stress tolerance (Mizukami *et al.*, 2004). The increased expressions of these proteins with NAP supplementation confirmed the ability to maintain energy metabolism during prolonged hypoxia exposure. In contrast, down-regulation of Pgam1 and Pgam2 suggested less ATP production in hypoxia exposed groups. Increased expression of lactate dehydrogenase in hypoxic group suggested inter-conversion of pyruvate to lactate which is the marker of oxidative stress. Eno1 was up-regulated in both groups with varying level suggesting hypoxia tolerance ability of brain cortex through one or more of its nonglycolytic functions during prolonged hypoxia (Aaronson *et al.*, 1995). Several Studies demonstrated up-regulation of alpha-enolase expression at gene level as well as protein level. The increased expression of alpha-enolase helped in adaptation to hypoxia under Hif1a regulation (Sedoris *et al.*, 2010; Semenza *et al.*, 1996). Western blot analysis of Eno1revealed that NAP supplemented group has more hypoxia tolerance ability than hypoxia alone (Figure 6). Furthermore, protein-disulfide isomerase (Pdi) was up-regulated in NAP supplemented group. The increased Pdi expression showed its role in resistance to ischemic damage and beneficial effects against brain stroke (Tanaka *et al.*, 2000). These results revealed that supplementation of NAP peptide up-regulate several metabolism and antioxidant responsive proteins in comparison to hypoxia alone.

Conclusion

The present study revealed significant changes in proteome profile during long term hypoxia and modulation of antioxidant proteins with supplementation of NAP peptide. Our study highlights the antioxidant potential of NAP peptide. NAP maintained Prdx5 protein level with up-regulation of Eno1 expression. Moreover, NAP also maintained glycolytic enzymes during chronic hypobaric hypoxia exposure.

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Abbreviations

GSH, Glutathione reduced; GSSG, Glutathione oxidized; SOD, Superoxide dismutase; CNS, Central Nervous System; 2-DE, 2-Dimensional gel electrophoresis; ACN, Acetonitrile; MALDI-TOF/TOF, Matrix Assisted Laser Desorption Ionization-Time of Flight/Time of Flight.

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