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Molecular Mechanisms of Acclimatization to High Altitude Hypoxia: Role of Hypoxia Mimetic Cobalt Chloride

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1. INTRODUCTION

Oxygen is a fundamental requirement for cellular respiration and any deficiency in oxygen concentration in the body as a whole (generalised hypoxia) or a region (tissue hypoxia) is sufficient to cause an impairment of function. Hypoxia is caused by:

(i) the reduction in partial pressure of oxygen,
(ii) inadequate oxygen transport,
(iii) the inability of the tissue to use oxygen.

The lowered barometric pressure of the atmosphere (760 mm *Hg* at sea level to ~250 mm Hg at Everest) results in diminished alveolar oxygen tension (hypobaric hypoxia) and as a consequence, arterial partial pressure of oxygen (PaO_2) drops dramatically with increase in altitude. While normal PaO_2 at sea level is about 90-95 mm Hg, it plummets to 35 mm *Hg* at 20,140 feet above sea level. In addition to dramatic decreases in PaO_2, higher altitudes also trigger a greater decrease in oxygen saturation (PO_2) (150 mm Hg at sea level to ~40 mm *Hg* at Everest), thus putting the body in further jeopardy. Hypobaric hypoxia becomes progressively more severe with increasing altitude which stresses biological systems because of non-availability of a steady, uninterrupted supply of oxygen for mitochondrial metabolism.

High-altitude illness is the collective term for the syndromes that can affect unacclimatised individuals shortly after ascent to high altitude, viz., acute mountain sickness (AMS), high-altitude cerebral edema (HACE), and high-altitude pulmonary edema (HAPE). High-altitude illness is more likely to occur at altitudes higher than 2500 m but is being increasingly recognized at altitudes between 1500 m and 2500 m. However, in most cases it will result in mild AMS, the symptoms typically include headache, gastrointestinal symptoms (anorexia, nausea, or vomiting), insomnia, dizziness, and lassitude or fatigue but rarely it may progress to more severe forms, viz., HAPE and HACE, which can be life-hreatening.

The most important risk factors for the development of high-altitude illness are rate of ascent, altitude reached (especially the altitude where individual sleeps/rests), and individual susceptibility. HACE, clinically-defined as the onset of ataxia (loss of coordination), altered consciousness, severe headache, hallucinations and even seizures, has been considered as the end stage of AMS, eventually leading to death caused by brain herniation. Fluid accumulation in the brain may be caused by cytotoxic edema (cell swelling due to increased intracellular osmolarity), vasogenic oedema (leak of the blood-brain barrier with extravasation of proteins and fluid into the interstitial space), or both.

HAPE, characterised by tightness in chest, cough, gurgling sound, difficulty in breathing, typically develops 2-4 days after arrival at high altitude and as the disease progresses frothy pink sputum develop which is the hallmark of HAPE. Although the exact mechanism underlying the development of HAPE remains unclear, clinical investigations suggest that HAPE is a form of non-cardiogenic pulmonary edema with increased pulmonary vascular permeability and pulmonary hypertension due to excessive hypoxic pulmonary vasoconstriction. This leads to vascular leakage through overperfusion, capillary stress failure, or both, resulting in high concentration of vascular proteins and red blood cells in the alveolar fluid. Beside hypoxia induced damage to endothelial cells, activation of cytokines, chemokines and cell adhesion molecules may orchestrate the lung inflammatory response. It cannot be ruled out that reactive oxygen (ROS) and nitrogen species (RONS) are also involved and may even play a causative role in AMS, HAPE and HACE. ROS can contribute to 'opening up' of the blood-brain barrier, allowing neurotoxins, endotoxin and inflammatory cells to enter the brain.

As millions of visitors as tourists, trekkers, mountaineers or defence personnel travel to high altitude locations each year, these high altitude maladies pose a public health problem and have severe economic consequences. Recovery occurs with descent, oxygen inhalation or bed rest but where descent is not possible and oxygen is not available, deaths continue to occur. Hypoxia is thus a life threatening stress that has to be dealt with at both cellular and systemic levels.

2. HYPOXIC ADAPTATION

2.1. HIF-1: Mediator of Hypoxic Response

Hypoxia elicits variety of adaptive responses at different levels in the body that enhance cell survival. At the organism level there is increase in ventilation, increased erythropoeisis and neovascularisaton, which in combination lead to increased oxygen delivery from the atmosphere to the tissue. At the cellular level, adaptation involves activated gycolysis, increased glucose uptake thus maintaining ATP despite low oxygen availability and the expression of cell survival and cell death related proteins. The regulation of the proteins required for hypoxic adaptation occur at gene level. Remarkably, the hypoxic induction of all these diverse genes appear to depend on a common mode of oxygen sensing and signal transduction mechanism mediated by activation of a critical transcription factor, hypoxia inducible factor (Hif1) . It is a redox sensitive protein that binds to hypoxia responsive element in different hypoxia responsive genes including erythropoietin (Epo), vascular endothelial growth factor (VEGF), Nitric oxide synthase (NOS), heme oxygenase (HO-1), glucose transporter-1 (Glut-1), and several glycolytic enzymes, thus activating their transcription. HIF-1 is a heterodimer composed of two proteins called HIF-1á and HIF-1ß. HIF-1á is rapidly degraded under normoxia by hydroxylation of proline residue by prolyl hydroxylases within a highly conserved region in its oxygen dependent degradation domain (ODDD). The hydroxylated protein interacts with pVHL protein and is then degraded by proteasome pathway. Under hypoxic conditions, HIF-1á is not hydroxylated because of limitation of the major substrate oxygen. The unmodified protein escapes the pVHL-binding, ubiquitination and degradation and then dimerizes with HIF-1ß and stimulates the transcription of its target genes. Transition metals (*Co, Ni, Mn*) and iron chelators mimic hypoxia by causing the stabilization of HIF-1á, thus allowing its accumulation, nuclear translocation and binding to HIF-1ß to form the transcriptionally active HIF-1 complex.

2.2. Pre-conditioning

Pre-conditioning is a process by which a tissue is rendered more tolerant to a subsequent lethal insult such as hypoxia/ischemia. Tolerance can be attained by subjecting tissues to a sub-lethal stress that results in intracellular adaptation and enhanced endogenous defense mechanism. The best way to acclimatize the humans to high altitude hypoxia is to induce necessary physiological and genetic changes in the body of the humans before they are inducted to high altitude. This can be achieved by hypoxia pre-conditioning.

Hypoxia pre-conditioning has been shown to produce tolerance against hypoxic-ischemic brain injury in new born rats. Gradual ascent, allowing time for acclimatization, is the best strategy for preventing high altitude illness. Several studies revealed that hypoxia preconditioning protects brain and heart from several types of injury including ischemia, seizures, edema.

2.2.1. Hypoxia Mimetics

The hypoxia preconditioning has potential clinical usefulness and can be mimicked by many divalent metals as cobalt, nickel, cadmium and zinc that act as hypoxic mimetics by stabilizing HIF-1á even under hypoxia. Among all the metals reported, cobalt is one of the classic examples of a hypoxic mimetic.

Cobalt (*Co*) is a silvery-white, hard metal with an atomic number of 27 and an atomic weight of 58.93. Common compounds of cobalt have an oxidation state of +2 or +3. The +2 valence is stable in aqueous solution and is the major form of cobalt found in simple salts. Cobalt is as an essential component of vitamin B12 (cobalamin). Mammals lack the ability to synthesize vitamin B12, and nonruminant animals require a dietary source of vitamin B12. Absorbed cobalt is primarily excreted in urine with small amounts excreted via fecal endogenous routes. Cobalt concentrations in tissues is generally low (1mg/kg DM or less). The amount of cobalt normally stored in human body is around 1.5 mg.

Cobalt toxicosis in animals is very rare because concentrations of cobalt normally present in animal diets are much lower than those needed to cause toxicosis. Cobalt toxicosis is not likely to occur in nonruminants unless environmental contamination of feed or water occurs.

Various hypotheses had been proposed to describe the mechanism of action of *Co* in stabilizing HIF-1á.

(i) $CoCl_2$ stabilises Hypoxia-inducible factor-1 (HIF-1á) by antagonizing Fe^{+2}, which is an essential cofactor along with oxygen for prolyl hydroxylases (PHDs) that degrade HIF-1á.

(ii) Partial inhibition of PHDs, depletion of ascorbate, which is required to maintain the HIF-PHDs and FIH (factor inhibiting HIF) in an active state.

(iii) Direct binding of cobalt to HIF-1á, which may prevent its degradation by VHL-dependent and VHL-independent pathways.

2.2.2. Cobalt Pre-conditioning

It has been known for a long time that cobalt increases erythropoietin production both in vitro and in vivo in normoxia. Cobalt had also been in use for the treatment of anaemia in infants and women. In the human hepatoma cell lines, production of erythropoietin mRNA was stimulated 6- to 12-fold in response to Co^{2+} in the absence of hypoxia. Chronic oral administration of $CoCl_2$ has been reported to induce polycythemia without an effect on body or heart weight in animals as well as humans. Administration of $CoCl_2$ (Co^{2+}) in 7 days old rats was shown to provide protection against ischemia reperfusion injury in brain. It has recently been reported that pretreatment with a low dose of cobalt in mice induced cardiac preconditioning and this protective effect of $CoCl_2$ is achieved through selective activation of HIF-1á signaling. Also administration of cobalt resulted in a marked protection against ischemic renal injury. Cobalt was also shown to be cytoprotective against tert-butylhydroperoxide induced oxidative stress

in HepG2 cells and also resulted in induction of renoprotective genes in rats when $CoCl_2$ was given with drinking water for 13 days. Similarly it has been reported to improve cardiac contractile function in rats when administered with water containing 0.01% $CoCl_2$ for 6-7 weeks.

Chemical preconditioning has the several advantages over physical preconditioning:

(i) reduced acclimatization schedule at altitude, leading to decreased loss of man days
(ii) number of people that can be preconditioned is not limited as compared to that of physical preconditioning in simulation chambers
(iii) easy to implement and
(iv) economical.

However, most of the studies on hypoxia mimetics especially on cobalt are focused on ischemia/reperfusion injury and there is paucity of data on the efficacy of cobalt in facilitating acclimatization to high altitude (hypobaric hypoxia) and its efficacy in prevention of HA induced ailments.In view of above the study was undertaken with the following objectives using adult male Sprague-Dawley rats as model system.

1. To determine the effect of $CoCl_2$ preconditioning in facilitating acclimatization to hypoxia.
2. To study the efficacy of $CoCl_2$ preconditioning in prevention of hypoxia induced oxidative stress.
3. To study the efficacy of $CoCl_2$ preconditioning in preventing HACE and HAPE and the role of NFêB in the associated pathophysiology.

2.3. Possible Outcome

With an explosive boast in tourism industry at high altitude (HA) there is a likelihood of tourists being suddenly exposed to hypoxia after rapid transition from sea level. HA sports activity as skiing and certain sports meet held at HA venues may have deleterious effect on health and performance of the sea level altitude athletes, and increased occurrence of HA maladies. Investigations of the processes of acclimatization of man to HA, and the associated pathophysiology and the molecular mechanism involved may pave way for understanding the physiology of other diseases involving oxygen deprivation as stroke, cardiopulmonary disorders, cancer to name a few. This would in-turn result in identification of new targets and development of an effective therapeutic regimen for effective control of such ailments.

3. EFFECT OF COBALT SUPPLEMENTATION

Effect of Cobalt supplementation promotes tolerance and facilitates acclimatization to hypobaric hypoxia in rat brain Male Sprague-Dawley rats (175-200g) maintained in the institute's animal house were used for all experiments. Rats were fed with varying doses of $CoCl_2.6H_2O$ dissolved in sterile distilled water using a gastric catheter for different time period. Hypoxic tolerance was determined by measuring the gasping time (GT) and hypoxic survival time (HST) by exposing animals one at a time to simulated hypobaric hypoxia of 10,668 m in an animal decompression chamber at 32^0C. The animals were exposed to a very high altitude of 10668 m to determine the hypoxic tolerance as smaller animals have higher capillary density in tissues, which make them more resistant to hypoxia than man.

Time taken for appearance of first sign of gasping followed by survival time was recorded using electronic stopwatch. Upon exposure to hypoxia, the control rats started gasping after 45-50 min and all of them died within 2-3 min after the onset of gasping. However, administration of $CoCl_2$ at 12.5 mg cobalt kg-1 BW significantly improved GT and HST ($p < 0.001$) by about 3-4 times as compared to the control rats. The optimum time period with respect to HGT and HST was found to be 7 days. Therefore, all the subsequent experiments were conducted by feeding rats with 12.5 mg/Kg BW for 7 days.

The rats were randomly divided into 4 experimental groups (n=6):

(i) Control (normoxia)
(ii) Hypoxia
(iii) $CoCl_2$ supplemented group under normoxia (12.5 mg cobalt kg-1 BW, 7days)
(iv) $CoCl_2$ supplemented group exposed to hypoxia (12.5 mg cobalt kg-1 BW was administered for 7 days along with exposure to hypoxia for the last 2 days of cobalt supplementation).

The administration of cobalt was started 5 days before exposing the animals to hypobaric hypoxia and 2 days during hypoxia so that protective mechanisms were ready when the animals were exposed to high altitude hypoxia.

The animals were exposed to a simulated altitude of 7619 m where the temperature and humidity were maintained at 28°C and 55-60% respectively. The rate of ascent was 300 m/min. The rats were taken out of hypoxic chamber once after 24 h exposure for 15 min for replenishing food and water. After hypoxic exposure, the rats were sacrificed and brain and lung were dissected out. All the experiments were performed on two different occasions and data were presented as mean ± SD. One-way analysis of variance with post-hoc Bonferroni analysis was used to determine statistical significance between groups.

Supplementation of cobalt is known to increase erythropoeisis; therefore Hb and Hct values in blood of control and cobalt fed rats were determined. There was significant increase ($p < 0.01$) in Hb and Hct levels in blood of animals fed with cobalt as compared with control animals.

3.1. Effect of $CoCl_2$ supplementation on Hif - 1α and HIF-1α regulated genes expression:

The relative levels of HIF-1 and its regulated genes as EPO, Glut-1, VEGF and NOS which promote erythropoeisis, glucose transport and angiogenesis respectively were determined by RT-PCR and immunoblotting in brain and lung of control and cobalt fed animals. It was noticed that cobalt administration for 7 days resulted in a significant increase in Hif-1α levels. To know whether increased Hif-1α levels in hypoxic and cobalt supplemented groups

results in increased binding of Hif-1α to hypoxia response elements (HRE), gel shift assays were performed using highly specific oligonucleotide probe consisting of enhancer region of EPO gene. Exposure of animals to hypoxia resulted in an appreciable increase in DNA binding activity of Hif-1α. However, a much stronger increase in DNA binding activity of Hif-1α was seen in animals fed with cobalt during both normoxia and hypoxic exposure. This resulted in higher EPO levels in brain of cobalt administered animals. An increased EPO level in cobalt supplemented animals (even during normoxia) was in line with the observed higher blood haematocrit and haemoglobin levels which in turn increase the O_2 carrying capacity of the blood. This might be one of the reasons for induction of hypoxic tolerance (as shown by increased HGT and HST) in rats following cobalt preconditioning. Further, EPO has also been shown to act as neuroprotective and neurotrophic factor directly in brain. EPO improves synaptic transmission during oxygen and glucose deprivation. It can inhibit hypoxia-induced apoptosis and provide stroke tolerance.

Because translational regulation of m-RNA is an important step in the control of gene function, the level of protein expression was evaluated by immunoblotting. A significant increase in VEGF protein levels was found in brain of hypoxic and cobalt-supplemented animals relative to control animals. Higher VEGF levels enhance capillary density and hence better oxygen transport to the brain. Hypoxia also causes up-regulation in brain glycolysis which cannot be maintained without parallel increase in the rate of glucose transport across the blood-brain barrier (BBB). The m-RNA and protein levels of glucose transporter-1 (GLUT-1) mediating the transport of glucose from blood to brain were increased in cobalt + hypoxic groups as compared to the control group, indicating enhanced glucose uptake for continued energy generation in hypoxic environments. Thus the increased EPO, VEGF and Glut-1 protein levels in cobalt supplemented animals ensure increased oxygen transport, capillary density and glucose transport respectively to cope up the limited oxygen availability during hypoxia.

To further confirm whether cobalt administration results in better oxygen delivery, LDH and lactate levels were measured in brain. Interestingly, cobalt administration significantly attenuated the hypoxia induced increase in LDH and lactate levels as compared to animals. This indicated better oxygen availability to brain even during hypoxia in cobalt supplemented rats. We also observed a significant increase in brain nitrite levels in hypoxia and cobalt + hypoxia groups. To address the source of the increased nitrite levels, the relative levels of NOS-1, NOS-2 and NOS-3 were determined by RT-PCR and immunoblotting. The results revealed an appreciable increase in NOS-2 levels in brain of hypoxia and cobalt + hypoxia groups, while no appreciable change in NOS-1 and NOS-3 protein levels were noticed in brain of animals exposed to hypoxia. This suggested that the observed increase in nitrite during hypoxic exposure is due to the activation of NOS-2 (inducible nitric oxide synthase). Nitric oxide (*NO*), a putative neurotransmitter in the brain and peripheral nervous system is an important mediator of vascular homeostasis and blood flow. The potentially protective *NO* can also act as a superoxide radical scavenger and may inhibit platelet aggregation and neutrophil adhesion.

From this part of the study, it can be concluded that cobalt administration markedly increased hypoxic tolerance as compared to the control animals. It was evident that Hif-1α induction is primarily responsible for the observed phenomenon that led to up-regulation of EPO, VEGF, Glut-1 and NOS-2 which in turn were responsible for increased oxygen transport, oxygen delivery, glucose utilization and vascular tone respectively. The findings of the study will help in designing and development of novel therapeutic strategies to use cobalt either as drug or neutraceutical for promoting acclimatization to high altitude.

4. COBALT-CHLORIDE ATTENUATES HYPOBARIC HYPOXIA INDUCED OXIDATIVE STRESS.

High altitude is characterized by hypobaric hypoxia, which is considered as an acute physiological stress often leading to oxidative stress, causing potential damage to proteins, lipids and DNA. The decrease in cellular oxygen levels leads to increase in free electrons in the cell resulting in formation of superoxide anion, H_2O_2 and hydroxyl radicals ($OH^{\cdot}$-). The disturbances in oxygen availability have been implicated in number of disorders including stroke, head trauma, neoplasia, vascular malformations, neurodegenerative disorders and in high altitude ailments. In this study we determined the effect of cobalt on hypobaric hypoxia induced oxidative stress.

The rats were randomly divided into 4 (n=6) groups as described earlier: (i) Control (normoxia), (ii) Hypoxic group, (iii) $CoCl_2$ supplemented group under normoxia, (iv) $CoCl_2$ supplemented group (12.5 mg cobalt/kg bw for 7 days) exposed to a simulated altitude of 7619 m for 48h. After hypoxic exposure, the rats were sacrificed and brain/lung were dissected out after thorough perfusion with normal saline and various parameters viz; free radical production, lipid peroxidation, protein oxidation, antioxidant enzymes (SOD, GPx, G-6-PDH, GST) were estimated in tissue homogenate.

To assess the effect of hypoxia induced oxidative stress in brain/lung, ROS levels were determined using a fluorescent probe DCFH-DA (2'7'dichlorofluorescein acetate). There was an appreciable increase ($P<0.05$) in ROS levels in animals exposed to hypoxia when compared to the control animals. Administration of cobalt significantly inhibited hypoxia induced ROS generation and maintained their levels similar to that of control values. A marked increase in lipid peroxidation (MDA levels) was noticed in animals exposed to hypoxia. However, $CoCl_2$ supplementation significantly attenuated hypoxia induced lipid peroxidation in brain/lung ($P<0.001$). We also determined formation of 4-HNE adducts by immunoblotting using anti-4 HNE antibodies. A significant increase in 4–HNE adduct levels were noticed in animals exposed to hypoxia (~40%). Supplementation

of $CoCl_2$ marginally reduced 4-HNE adducts formation induced by hypoxia. The protein oxidation was measured by determining the carsbony[1] groups after derivitization of proteins with dinitrophenylhydrazine (DNPH). The results showed a considerable increase ($P<0.001$) in protein oxidation in animals exposed to hypoxia as compared to the control. Administration of $CoCl_2$ appreciably inhibited the formation of protein carbonyls levels.

Since, $CoCl_2$ supplementation significantly inhibited ROS levels and oxidation of cellular proteins and lipids, the effect of cobalt administration on endogenous antioxidant levels was determined during hypoxic exposure. The results showed a considerable fall in GSH levels with a concomitant increase in GSSG levels during hypoxic exposure. But administration of cobalt had no effect on hypoxia induced decrease in GSH levels. However, cobalt supplementation maintained the antioxidant enzymes levels similar to that of control values. Since cobalt administration attenuated oxidative stress induced by hypoxia, it was suggested that cobalt acts via a different pathway (Non-GSH mediated) in reducing hypoxia induced oxidative stress.

It was therefore sought whether anti-oxidant activity of cobalt is mediated by HO-1 (hemoxygenase) which is known to possess anti-oxidant and anti-apoptotic activity. In the present study too, decreased GSH and enhanced HO-1 levels were seen in brain following hypoxic exposure after cobalt supplementation. HO-1 has been shown to over-express following heat shock and oxidative stress. Several reports have proposed that HO-1 induction represents an antioxidant defense, operating by decreasing the levels of potential pro-oxidants and increasing the concentration of active bile pigments, such as bilirubin, capable of acting as antioxidants. Hence one of the possible reasons for the observed reduction in oxidative stress might be increase in HO-1 levels.

Many studies showed that exposure of cells or animals to oxidative stress can induce expression of heat shock proteins (HSPs). Hsps are enhanced after traumatic brain injury and in neurodegenerative diseases. The inducible form of HSP70 serves as a useful marker of cellular response to hypoxic insult. Therefore the m-RNA and protein levels of HSP-70 were measured in brain/lung of rats exposed to hypoxia. A marked increase in HSP70 levels was seen in animals exposed to hypoxia while cobalt supplementation markedly inhibited HSP70 expression possibly due to reduction in hypoxia induced oxidative stress in cobalt administered animals.

Metallothioneins constitute a family of metalloproteins involved in cytoprotection during oxidative stress. ROS and oxidative stress increase expression of MT which are highly efficient free radical scavengers. Therefore the relative m-RNA levels of MT in control and cobalt fed animals was determined by RT-PCR following hypoxic exposure. A significant increase in MT- m-RNA levels was seen in brain/lung of animals exposed to hypoxia and cobalt + hypoxia groups. The results of protein expression showed significant increase in MT levels in cobalt supplemented group as compared to control and hypoxic groups. Regulation of MT gene by cobalt was reported to be mediated by activation of metal response element/ metal transcription factor-1 which activates HIF-1.

Since, HO-1 and MT genes are known to be regulated by a single transcriptional factor Hif-1α, Hif-1α protein levels were determined by immunoblotting. A significant increase in Hif-1α levels was found in brain/lung of animals exposed to hypoxia. Supplementation of cobalt during both normoxia and hypoxia also resulted in an appreciable increase in Hif-1α levels. Thus the observed anti-oxidant activity of cobalt through HO-1 and MT was found to be mediated via Hif-1α signaling mechanisms.

To summarize, this part of the study showed that administration of cobalt attenuated hypoxia induced oxidative stress by preventing ROS generation, oxidation of cellular proteins and lipids. Interestingly, cobalt supplementation had no effect on endogenous GSH levels. Cobalt administration attenuated the oxidative stress induced by hypobaric hypoxia by maintaining higher HO-1 and MT levels in brain/lung. The findings of the study reveal the possibility of using cobalt either as drug or neutraceutical for prevention of high altitude induced oxidative stress. In the further studies the possibility of use of cobalt in reducing the incidence of HACE/HAPE was observed.

5 COBALT CHLORIDE PREVENTS HYPOBARIC HYPOXIA- INDUCED CEREBRAL/PULMONARY EDEMA IN RATS

To determine whether chemical preconditioning by cobalt chloride prevents hypoxia induced cerebral edema, the rats were fed orally with cobalt (12.5 mg Co/Kg BW) for 7 days and exposed to simulated hypoxia of 9142 m, for 5 hrs.

The formation of cerebral edema was measured by a direct method using a fluorescent probe sodium fluoroscein that can be measured in brain and lung to quantify the vascular leakage. A marked increase in vascular leakage was observed in brain/lung of rats exposed to hypoxia. Interestingly, pre-conditioning of animals with cobalt for 7 days markedly attenuated hypoxia induced transvascular leakage in brain and lung. The present study is first of its kind in revealing the efficacy of cobalt in attenuating the vascular leakage induced by hypobaric hypoxia. The endothelial cells of cerebral vasculature form the blood brain barrier (BBB), which protects neurons and glia from peripheral circulating mediators and toxic substances. It has been reported that the HACE predominantly is vasogenic and damages BBB and this has been attributed to increased NO levels produced by iNOS. Further, it has also been demonstrated that blocking VEGF expression significantly prevented hypoxia induced vascular leakage in brain. Therefore, the NO and VEGF levels were measured in brain/lung of rats exposed to hypoxia. A significant increase in NO and VEGF levels was noticed in animals exposed to hypoxia as compared to control animals. Supplementation of cobalt

markedly inhibited the NO and VEGF levels which in-turn could be responsible for the observed fall in vascular leakage. There was a significant increase in generation of ROS levels in brain/lung of animals exposed to hypoxia as revealed by increased DCF fluorescence when compared to the control animals. Administration of cobalt significantly inhibited hypoxia induced ROS generation.

6. NUCLEAR FACTOR êB (NFêB)

NFêB, a redox sensitive protein is expressed abundantly during oxidant stress. It is a ubiquitous transcriptional factor composed of a complex of proteins which are critical regulators of a variety of responses especially inflammation. NFêB is shown to have a central role in immunological processes and is involved in other diseases as well (Baldwin, 1996). NFêB is a heterogenous collection of dimers of members of NFêB/Rel family. In the inactive state NFêB remains bound to inhibitory complex, IêB in the cytoplasm. When stimulated, IêB dissociates via phosphorylation from the complex ultimately leading to nuclear translocation of active NFêB. In the nucleus, NFêB binds to a specific DNA motif and regulates transcription of target genes containing NFêB consensus sequence in their promoter region. NFêB has been found to respond to a broad range of stimuli and conditions which includes ROS, inflammatory cytokines, growth factors, adhesion molecules, cell surface receptors, extracellular stress and intracellular oxidative stress. It also seems to be associated with certain neurodegenerative diseases such as Alzheimer's and Parkinson's.

The expression of NFêB has also been positively correlated with increases in paracellular permeability, associated with alterations in tight junction (TJ) proteins. Most of the studies on NFêB have been done on ischemia and neurodegeneration but there is paucity of data on its role in hypobaric hypoxia induced neuroinflammation and related ailments as HACE.

Hypoxia has been shown to activate NFêB in a number of studies but the signaling mechanisms leading to this are not clear. Since exposure of animals to hypoxia resulted in an appreciable increase in oxidative stress as revealed by enhanced ROS and NO levels, NFêB levels were determined in brain and lung of rats by EMSA. The results revealed a significant increase in DNA binding activity of NFêB in rats exposed to hypoxia as compared to the control animals. When NFêB is activated, the p65 subunit of the NFêB containing trans-activation domain is translocated to the nucleus. In the inactive state, the p65 subunit of NFêB is retained in the cytoplasm. The results of immunoblotting also revealed a significant increase in nuclear p65 levels in rats exposed to hypoxia relative to control animals. Interestingly, chemical preconditioning of animals with cobalt significantly attenuated the NFêB DNA binding activity and p65 nuclear translocation. Inhibition of NFêB had been shown to result in down-regulation of VEGF.

7. PRO-INFLAMMATORY MEDIATORS

Since, a number of inflammatory mediators such as interleukins and cell adhesion molecules are regulated by NFêB, their relative levels were measured by ELISA and immunoblotting in brain and lung. The results revealed a significant increase in inflammatory interleukins such as MCP-1, IFN- ã, TNF-a, IL-1 ß in rats exposed to hypoxia. The cell adhesion molecules (VCAM-1, P-selectin) that are responsible for the adhesion and migration of leukocytes to inflamed site were also enhanced in hypoxic rats as compared to the control animals. It has been reported that MCP-1 induced prolonged brain vascular permeability by directly activating brain endothelial cells. MCP-1 not only attracts leukocytes but also has a role in 'opening' the BBB during leukocyte extravasation by acting directly on brain ECs. Similarly, IL-1 is also shown to directly regulate expression of pro-inflammatory molecules such as TNF-a, Cox-2, NO and MCP-1. IL-1 has also been shown to induce expression of phospholipase A and cyclooxegenase-2, which increase the levels of arachidonic acid and prostaglandins respectively and can induce edema with production of ROS. Moreover, inhibition of IL-1 signaling has been shown protective in neuroinflammation and subsequent neurodegeneration. Interestingly, in the present study cobalt preconditioning attenuated hypoxia induced increase in pro-inflammatory cytokines.

8. ANTI-INFLAMMATORY MEDIATORS

To address whether the observed inhibition of inflammatory mediators by cobalt is due to its anti-inflammatory activity, the anti-inflammatory mediators such as IL-6, TGF-ß, HO-1 and Metallothionein (MT) levels were measured in brain/lung of rats exposed to hypoxia. Although IL-6 is often used as a marker for systemic activation of pro-inflammatory cytokines, it also inhibits the production of proinflammatory cytokines as IFN-ã, IL-1 and TNF-á. The overall immunological effects place IL-6 among the anti-inflammatory cytokine group. A significant increase in IL-6 levels was noted during hypoxia and its levels remained elevated in cobalt + hypoxia group. Similarly, Cobalt supplementation also resulted in a marginal increase in TGF-ß levels.

Apart from increased levels of Il-6 and TGF-ß, cobalt pre-conditioning resulted in higher levels of anti-inflammaotry proteins, HO-1 and MT. HO-1 is ubiquitously induced in mammalian tissues and has anti-inflammatory properties. In a variety of patho-physiological states such as ischemia and reperfusion injury, hypertension, pulmonary hypertension, expression of HO-1 has been shown to be protective. Recently, rising interest in metallothionein's neuroprotective functions and therapeutic potential has been evident. Therefore, studies were carried out to find out whether cobalt preconditioning has any effect on these two important anti-inflammatory molecules. The results showed that cobalt administration significantly increased the expression of HO-1 and Metallothionein levels in brain of rats during hypoxic exposure. This might play some role in the observed fall in pro-inflammatory mediators after cobalt preconditioning.

Very little is known about the function of NFêB in neuroinflammation. To ascertain whether observed higher

NFêB DNA binding activity and inflammation is associated with occurrence of HACE, the rats were pre-treated with NFêB-blocker curcumin intraperitoneally 1 hour prior to hypoxic exposure. Curcumin has also been shown to downregulate the expression of cell adhesion molecules regulated by NFêB. The results indicated that curcumin administration significantly decreased transvascular leakage induced by hypoxia. To know whether observed lower levels of NFêB in cobalt preconditioned animals is due to blocking of NFêB activation or due to enhanced O_2 supply through cobalt induced Hif-1a stabilization, the animals were injected cobalt chloride in sterile water (12.5 mg Cobalt/ kg b.w., i.p) 1 h prior to hypoxic exposure. This approach would exclude the possible involvement of Hif-1α and it's down stream genes such as EPO and VEGF, as these proteins take few days to result in erythropoeisis and angiogenesis respectively. Interestingly, i.p. administration of cobalt was unable to attenuate the vascular leakage induced by hypoxia suggesting that cobalt did not attenuate NFêB activity per se. Thus the observed lower levels of NFêB activity in cobalt-preconditioned animals might be due to enhanced oxygen supply mediated by Hif-1α signaling mechanisms. This was confirmed by higher levels of Hif-1α and EPO levels in rats administered with $CoCl_2$ as compared to normoxic controls suggesting that by improving the oxygen supply cobalt attenuated NFêB activity and hence lower vascular leakage. Taken together, cobalt preconditioning inhibited hypoxia induced vascular leakage in brain inhibiting i) ROS generation and NFêB activation ii) build-up of proinflammatory cytokines and promoting higher oxygen delivery by Hif-1α and its downstream genes and maintaining higher anti-inflammatory mediators. These findings provide the basis for possible use of cobalt for reducing the incidence of HACE/HAPE and other illnesses involving hypoxia-induced inflammation.

9. TOXICITY STUDIES

The possibility of use of cobalt either in the form of a drug or neutraceutical prompted us to evaluate the safety of this standardized dose (12.5 mg cobalt kg-1 BW, 7days). Though there is a lot of ambiguity on the toxicity of cobalt, the toxicity of cobalt was dependent on route of administration, pre-existing cardiac problems, age, period of exposure, nutritional status etc. Recently there have been reports indicating no severe systemic toxic effects or mortality with cobalt. Cobalt was traditionally used to treat anemia in pregnant women and infants and used to treat refractory anemia in patients undergoing long-term hemodialysis. It has been reported that responsiveness of HIF-1 to hypoxia and not $CoCl_2$ wanes with age, which may be further helpful in age related brain disorders involving oxygen deprivation. There have been reports that chronic feeding of cobalt, 75 mg/Kg, 3 times /week for 5 weeks resulted in enhanced angiogenesis in rat myocardium. Pretreatment with cobalt protoporphyrin significantly reduced the apoptosis in myocytes /endothelial cells after cold ischemia reperfusion (I/R) injury. The studies conducted in our laboratory also revealed no mortality or any significant systemic toxicity when rats were fed with 12.5 mg cobalt /Kg BW for 7 days based on various hematological and biochemical parameters in rats. The metal content was also assessed in various tissues indicating negligible adverse effect of any kind. Most of the effects that were observed in the study can be explained in the terms of the predictable physiological adaptive response to cobalt supplementation. Cobalt is known to increase erythropoeisis, therefore increased RBC, Hb and Hct values in blood of cobalt fed animals were normalized after a week of cobalt supplementation. No significant change was observed in SGPT, SGOT, LDH levels in blood of animals indicating normal liver function. These results are in accordance with previous reports showing no morphological or enzymatic changes in livers of rats exposed to 2.5-30.2 mg Co/Kg for 3-7 months. Other biochemical parameters estimated showed no significant change as creatinine (kidney function test), creatine kinase (marker for skeletal muscle disease, myocardial infarction and cerebrovascular accidents), uric acid, protein. Cobalt concentrations as estimated in various tissues was well within range i.e. <1mg/kg DM. Thus the dose of cobalt i.e. 12.5 mg Co/Kg bw is nontoxic and therefore may be considered for developing a suitable neutraceutical.

10. MICROARRAY STUDIES

Acclimatization to high altitude is very complex and involves interplay of several genes. Therefore, microarray studies were carried out which gives a global view of gene expression and provides a snapshot of the transcriptome in healthy and disease states. DNA microarray technology has become an important tool in biological investigations by allowing researchers to measure the expression levels of thousands of genes simultaneously. This information is highly useful as it uncovers gene families or more specifically pathways that are affected, but also reveals those that are unaffected. Hypotheses about genes with unknown function can also be formed by comparison of their expression levels with genes of known functions.

The present study reveals the global gene expression of brain following hypobaric hypoxia (9142m, 5h) for the first time. A very similar expression profiles were observed for both hypoxia and cobalt under given conditions as nearly same number of genes were up- or-downregulated indicating that cobalt supplementation and hypoxia may involve a similar mechanism of action. Moreover, the pathway analysis showed many common genes in any of the pathway after both the treatments. Many other genes were identified which were earlier not known to be associated with either hypobaric hypoxia or cobalt preconditioning. When cobalt preconditioned rats were exposed to such an extreme altitude they did not show much change in differential gene expression, moreover the genes which were previously affected were also brought back to nearby basal level. The study provides evidential support of many genes and pathways which may be further targeted as potential drug targets and as

sources for understanding the progression of hypoxia associated syndromes and their pathophysiology. This study also supports the beneficial role of hypoxic mimetic cobalt as a preconditioning agent for attenuating the deleterious effects hypobaric hypoxia.

11. MAJOR FINDINGS

- Cobalt (12mg/kg bw) enhances hypoxic tolerance (gasping time).
- Prevents trans-vascular leakage in brain and lungs and downregulates expression of pro-inflammatory interleukins.
- Decreases lipid peroxidation, ROS levels and maintains antioxidant status.
- Stimulates the expression of HIF-1*a* resulting in increased expression of its target genes, thus facilitating acclimatization to HA acclimatization.
- Toxicity studies indicate no adverse effect of $CoCl_2$ supplementation (biochemical, haematological, histo-pathological parameters)

Cobalt chloride, thus has potential to be used in rapid acclimatization to hypoxia. Most importantly, supplementation of hypoxia mimetics is practical and feasible way of inducing hypoxia.