Hypoxic Preconditioning with Cobalt Attenuates Hypobaric Hypoxia-Induced Oxidative Damage in Rat Lungs

Dhananjay Shukla,1 Saurabh Saxena,1 Purushotman Jayamurthy,1 Mustoori Sairam,1 Mrinalini Singh,1 Swatantra Kumar Jain,2 Anju Bansal,1 and Govindaswamy Ilavazaghan1

Abstract

Shukla, Dhananjay, Saurabh Saxena, Purushotman Jayamurthy, Mustoori Sairam, Mrinalini, Singh, Swatantra Kumar Jain, Anju Bansal, and Govindaswamy Ilavazaghan. High Alt. Med. Biol. 10:57–69, 2009.—Hypoxic preconditioning (HPC) provides robust protection against injury from subsequent prolonged hypobaric hypoxia, which is a characteristic of high altitude and is known to induce oxidative injury in lung by increasing the generation of reactive oxygen species (ROS) and decreasing the effectiveness of the antioxidant defense system. We hypothesize that HPC with cobalt might protect the lung from subsequent hypobaric hypoxia-induced lung injury. HPC with cobalt can be achieved by oral feeding of CoCl2 (12.5 mg kg−1) in rats for 7 days. Nonpreconditioned rats responded to hypobaric hypoxia (7619 m) by increased reactive oxygen species (ROS) generation and a decreased GSH/GSSG ratio. They also showed a marked increase in lipid peroxidation, heat-shock proteins (HSP32, HSP70), metallothionins (MT), levels of inflammatory cytokines (TNF-α, IFN-γ, MCP-1), and SOD, GPx, and GST enzyme activity. In contrast, rats preconditioned with cobalt were far less impaired by severe hypobaric hypoxia, as observed by decreased ROS generation, lipid peroxidation, and inflammatory cytokine release and an increased GSH/GSSG ratio. Increased expression of antioxidative proteins Nrf-1, HSP-32, and MT was also observed in cobalt-preconditioned animals. A marked increase in the protein expression and DNA binding activity of hypoxia-inducible transcriptional factor (HIF-1α) and its regulated genes, such as erythropoietin (EPO) and glucose transporter-1 (glut-1), was observed after HPC with cobalt. We conclude that HPC with cobalt enhances antioxidant status in the lung and protects from subsequent hypobaric hypoxia-induced oxidative stress.

Key words: hypobaric hypoxia, oxidative stress, CoCl2

Introduction

High altitude exposure has been considered to be an extreme physiological stress inducing a wide range of deleterious effects at the cellular and systemic level and the root cause of many high altitude ailments, such as acute mountain sickness (AMS), high altitude cerebral edema (HACE), and high altitude pulmonary edema (HAPE). Low oxygen pressure seems to be favorable to low oxidative stress, but high altitude exposure is associated with increased oxidative damage, which could be a consequence of increased activity of the reactive oxygen species (ROS) generation and decreased activity of the antioxidant defense system. Under physiological conditions, ROS produced in the course of normal metabolism are fully inactivated by an elaborate cellular and extracellular antioxidant defense system (Maxwell, 1995; Yashimata et al., 1995). During hypoxia, less oxygen is available to be reduced to H2O by cytochrome oxidase, causing an accumulation of reducing equivalents within the mitochondrial respiratory sequence. This reductive stress leads to ROS formation by the auto-oxidation of one or more mitochondrial complexes, such as the ubiquinone–ubiquinol redox couple (Mohanraj et al., 1998). In this way the ROS level is increased in hypobaric hypoxia, which causes oxidative stress (Jayalakshmi et al., 2005; Maiti et al., 2006). Also, under hypoxic stress the cellular defense

1Defence Institute of Physiology & Allied Sciences, Defence Research and Development Organization, Lucknow Road, Timarpur, Delhi 110054, India.
2Department of Biotechnology, Jamia Hamdard University, New Delhi 110062, India.
CoCl₂ (Co₄₂ normoxic conditions (Xi et al., 2004). Administration of /H9251 HIF-1α by antagonizing Fe²⁺, which is an essential cofactor along with oxygen for prolyl hydroxylases that degrade HIF-1α in normoxic conditions (Xi et al., 2004). Administration of CoCl₂ (Co²⁺) in 7-day-old rats was shown to provide protection against ischemia–reperfusion injury in the brain (Bergeron et al., 2000; Miller et al., 2001). It has recently been reported that pretreatment with a low dose of cobalt in mice induced cardiac preconditioning, and this protective effect of CoCl₂ is achieved through selective activation of HIF-1α signaling (Xi et al., 2004). Matsumoto et al. (2003) reported that administration of cobalt resulted in a marked protection against ischemic renal injury. Therefore, it is reasonable to anticipate that preconditioning with cobalt might be protective against hypobaric hypoxia-induced oxidative injury in lungs. Previously, our studies with rats acclimated to hypoxia by cobalt preconditioning have shown consistent and prolonged survival during subsequent hypobaric hypoxia exposure (Shrivastava et al., 2008) and that this acclimation is advantageous in protecting the brain by attenuating hypobariic hypoxia-induced oxidative injury (Shrivastava et al., 2007). Protection by cobalt preconditioning against hypobaric hypoxia-induced oxidative stress in lungs has not been described previously. In the present study, hypoxic preconditioning with cobalt was protective against hypobaric hypoxia-induced lung oxidative injury.

**Experimental procedures**

**Animals**

Male Sprague–Dawley rats (200 to 250 g) were used in all studies. The animals were maintained in the institute’s animal house at 24 ± 2°C with a 12-h to 12-h light–dark cycle. They were fed a standard pelleted diet and sterile tap water ad libitum. The experimental protocol was carried out in accordance with the guidelines of the Ethics Committee of the Institute and in compliance with the Committee for the Purpose of Control and Supervision of Experiments on Animals. Efforts were made to minimize animal suffering and the number of animals used for experimentations. All chemicals were purchased from Sigma (St. Louis, Missouri, USA). Manufacturers of the kits and antibodies used are given in the following sections where appropriate.

**CoCl₂ preconditioning and hypoxic exposure**

The optimum dose of cobalt was determined by feeding rats different concentrations of cobalt chloride (CoCl₂ · 6H₂O) dissolved in sterile water for different time periods, and the gasping times were recorded. The optimum dose was 12.5 mg Co/kg body weight for 7 days, which was used for all subsequent experiments (Shrivastava et al., 2008). The rats were randomly subdivided into four experimental groups of 6 animals each: (i) control, (ii) hypoxic group, (iii) CoCl₂-preconditioned group kept under normoxia, and (iv) CoCl₂-preconditioned group exposed to hypoxia. The rats were exposed to a simulated altitude of 7619 m for 48 h in an animal decompression chamber at 28 ± 2°C (Decibel Instruments, Delhi, India). The airflow in the chamber was 2 L/min, with relative humidity maintained at 55% to 60%. The rats were taken out of the hypoxic chamber once after 24-h exposure for 15 min for replenishing food and water. We exposed rats to a simulated altitude of 7619 m for 48 h for the following reasons: (1) smaller animals have higher capillary density in tissue, making them more resistant to hypoxia than humans and (2) the occurrence of oxidative stress by hypoxia normally takes 12 to 24 h (Moolman et al., 1994; Barone et al., 1998). Most high altitude-induced problems, such as HAPE and HACE (where ROS are thought to play a role), occur during the 24 to 48 h of ascent to high altitude.

**Biochemical analysis**

After exposure to hypoxia, the animals were anesthetized using ketamine (80 mg/kg i.p.) and xylazine (20 mg/kg i.p.) and lungs were dissected out en bloc after perfusion with cold phosphate buffer saline to remove blood and snap frozen at −80°C for further analysis. Later, the whole lung was homogenized in cold 0.154 M KCl fortified with protease inhibitor cocktail (Sigma) to obtain 10% homogenate (w/v). A part of the homogenate was then centrifuged at 2900 g for 10 min at 4°C. The supernatant was used to determine glutathione levels (GSH and GSGG), free-radical production, and lipid peroxidation. The protein content in the homogenate was determined by Lowery’s method (Lowery et al., 1951).

2’, 7’-dihydrochlorofluorescein (DCFH) oxidation. ROS generation was accessed according to LeBel and Bondy (1990), as modified by Kim et al. (1996). Briefly, 50 μL of homogenate was added to a cuvette containing 2938 μL of 0.1 mol/L phosphate buffer (pH 7.4), and 12 L of 1.25 mmol/L 2’7’- dichlorofluorescein diacetate (DCF-DA) prepared in methanol was added. The assay mixture was incubated for 15 min at 37°C, and 2’7’ dichlorofluorescein formation was determined fluorimetrically using a spectrofluorimeter (Varion, Palo Alto, California, USA) at an excitation of 488 nm and emission at 525 nm. The fluorescent intensity parallels the amount of ROS formed.

**Lipid peroxidation**. Lipid peroxidation was assessed by measuring malondialdehyde (MDA) formed by thiobarbituric acid reactive substances (TBARS), using the method of Ohkawa et al. (1979). Thiobarbiturate was used as the standard, and the level of lipid peroxides was expressed as nmol MDA/mg protein. HNE-
protein adducts in the lung were determined by western blotting with rabbit polyclonal anti-4-HNE antibody (Alexis, Farmingdale, NY, USA).

Enzymatic and nonenzymatic antioxidants. Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were measured fluorometrically by the method of Hissin and Hill (1976). The activities of glutathione peroxidase (GPx) (EC 1.11.1.9) and superoxide dismutase (SOD) (EC 1.15.1.1) were determined using commercially available kits (Randox, Antrim, UK), per manufacturer’s instructions. Glutathione-S-transferase (GST) (EC 2.5.1.18) was determined using the protocol described by Habig et al. (1974). Briefly, a 100-μL sample was mixed with 2,790 μL of 0.1 mol/L potassium phosphate buffer (pH 6.5), 100 mM GSH. Reaction was initiated by adding 1-chloro 2, 4-dinitrobenzene (40 mg/mL in ethanol), and the optical density was recorded at 340 nm.

Inflammatory cytokines in BALF

The levels of inflammatory cytokines reflected in the airspace of the lungs were quantitated in bronchoalveolar lavage fluid (BALF) collected from different groups of animals. The animals were anesthetized and the lungs removed by cutting the trachea. Ten milliliters of ice-cold PBS was gently instilled into the lungs through the trachea, withdrawn, and re-instilled four times. Lung lavage fluid was pooled and centrifuged at 2000 rpm for 10 min at 4°C for removing cells and debris, and the supernatant was aliquoted and frozen at −80°C until assayed. The TNF-α, IFN-γ, and MCP-1 in the BALF were measured by using commercially available optEIA ELISA kit (BD Biosciences, San Jose, CA, USA). Absorbance was read at 450 nm using a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

mRNA expression studies

Total RNA was extracted from lung using a commercially available RNA extraction kit (Qiagen, Valencia, CA, USA). cDNA was prepared by using a first-strand cDNA synthesis kit (Fermentas, Burlington, ON, CA, USA), per manufacturer’s instructions. PCR primers for various genes were purchased from Integrated DNA Technologies, Inc., USA, and contained the following sequences: (1) Actin sense, 5’ AAG CGT AAG ATG ACC CAG ATC ATG TTT 3’ antisense, 5’ ATG CGG CAG TGG CCA TCT CCT GCT CGA AGT C 3’; (2) HSP-32 sense, 5’ CAC GCA TAT ACC CGC TAC CT 3’, antisense, 5’ AAG CGG GTC TTA GCC TCT TC 3’; (3) HSP-70 sense, 5’ ACC AAG CAG ACG ACC TTC ACC ACC TAC 3’, antisense 5’ GTT TGT AGG GAT CCA AGG AAA AA 3’; (4) MT-1 sense, 5’ ACT CTG AGT TGG TCC GGA AA 3’, antisense, 5’ GCC TTC TTT CCC GTC TTT CCT ACA CC 3’; (5) MT-2 sense 5’ CAC TTG TCC GAA GCC TCT TT 3’, antisense, 5’ ACA GAT GCA TCC TGC TCC TG 3’; (6) MT-3 sense, 5’ GGA CAC GCA GCA CTA TTC AC 3’, antisense, 5’ CCT GGA TAT GGA CCC TGA GA 3’; (7) EPO sense, 5’ AGG CGG GCA GAT GGG GTG GC 3’, antisense 5’ GTT GGG AGT GGA GGG TTG G 3’; (8) Glut-1 sense 5’ CAC ATA CAT CGG CAC AAA GC 3’, antisense 5’ CAC CAT CGG CAC AAA GC 3’; (9) VEGF sense 5’ ATG AAC TTT CTG CTC TGT TG 3’, antisense 5’ GCA GGA ACA TTT ACA CGT CTG C 3’.

The amplified products (in bp) for actin, HSP-32, HSP-70, MT-1, MT-2, MT-3, EPO, Glut-1, and VEGF are 352, 227, 900, 290, 146, 247, 660, 127, and 574, respectively. The PCR was conducted using a thermal cycler (MJ Research Ramsey, MN, USA) with the following conditions: Initial denaturation was carried out at 90°C for 3 min, followed by denaturation at 94°C for 1 min; annealing at 58, 52, 60, 54, 55, 56, 59, and 57°C (actin, HSP-32, HSP-70, MT-1, MT-2, MT-3, EPO, Glut-1, and VEGF, respectively) for 1 min; and extension at 72°C for 1 min, for a total of 35 cycles, followed by a final extension at 72°C for 10 min. The PCR products were electrophoresed on 2% agarose gel and visualized by UV transillumination. The images of the PCR products were acquired and the optical density of the band was quantified by using Labworks software (UVP Bio-imaging systems, Cambridge, UK).

Protein expression studies

Whole lung was homogenized in 0.154 mol/L KCl in the presence of protease inhibitor cocktail (Sigma) to obtain 10% homogenate (w/v). Fifty micrograms of protein from each sample was subjected to SDS-PAGE and electroblotted onto nitrocellulose membrane. The membranes were blocked with 1X western blocking buffer (Sigma), washed, and probed with respective rabbit polyclonal antibodies, such as HSP-32, HSP-70, MT, EPO, Glut-1, VEGF, HIF-1α, and β-actin (Santa Cruz, CA, USA). The membranes were washed extensively with phosphate-buffered saline-tween (PBST, 0.1%) and incubated with respective HRP conjugated secondary antibody (1:40,000) for 2 h. The membranes were then incubated with chemiluminescent substrate (Sigma), and the bands were developed using x-ray films (Kodak, UK). The optical density of the band was quantified by using Labworks software (UVP Bio-imaging systems).

DNA binding activity

Nuclear extracts were prepared from whole lung using a nuclear–cytoplasmic extraction kit (Biovision, Mountain-view, CA, USA), following the manufacturer’s instructions, and stored at −80°C until further analysis. To assess nuclear HIF-1 binding activity, gel mobility shift assays were carried out. The binding mixture (25 μL) containing 5 μg protein of nuclear extract and 1 μg of poly dI–dC was incubated in a binding buffer (10 mmol/L tris-HCl pH 7.4, 50 mmol/L NaCl, 50 mmol/L KCl, 1 mmol/L MgCl2, 1 mmol/L EDTA, 5 mmol/L DTT) on ice for 10 min. Later 10 pmol of biotinylated HIF-1 probe (Operon, Germany) with the following sequences was added and incubated at room temperature for an additional 30 min: HIF-1 F 5’-GCCCTACGTGCT-GTCTCA-3’, HIF-1 R 5’-TAGACACAGC-ATAGGCCG-3’, HIF-1 mutant F 5’ CCTAAACGCTGCTCA 3’, HIF-1 mutant R 5’ TAGACACACGTTCAGGG 3’). The samples were separated on a 6% native polyacrylamide DNA retardation gel and then electrophorbed onto positively charged nylon membranes. Biotinylated DNA–protein complex was detected with peroxidase-conjugated streptavidin and a chemiluminescent substrate kit (Perice, Rockford, IL, USA).

Immunohistochemical studies

VEGF expression in lung was also determined by immunohistochemical studies. The lungs were inflated, and the
pulmonary artery was perfused with 4% paraformaldehyde. Excised lungs were fixed in 4% paraformaldehyde overnight at 4°C and cryosectioned (Leica, Nusslock, Germany). Frozen sections (60 μm) were used for immunostaining with VEGF-specific antibodies. Sections were treated with 0.3% hydrogen peroxide in methanol for 20 min, preincubated with 5% goat serum, and treated with anti-VEGF antibody (1:200, Santacruz, CA, USA) for 1 h at 37°C. The sections were then incubated with a biotinylated goat antirabbit secondary antibody, treated with the avidin–biotin complex (ABC kit, Santacruz, CA USA), and stained with diaminobenzidine tetrahydrochloride and hydrogen peroxide.

Data analysis

All experiments were performed three times. Cytokines ELISA contained four to six replicates with six different animals. Each data point in the graph represents mean ± SD. Analysis of variance (ANOVA) with post hoc Bonferroni analysis was used to determine statistical significance between groups as applicable. Student’s t-test was performed on the means of two sets of sample data and considered significant if the p value was ≤0.05.

Results

ROS generation

As illustrated in Fig. 1, exposure of nonpreconditioned animals to hypobaric hypoxia caused an increase in lung ROS generation (55.6 ± 5.1 vs. 23.8 ± 1.4 nmol/min/mg protein, p < 0.001) relative to control animals as observed by an increase in DCF fluorescence. Hypoxic preconditioning by cobalt significantly attenuated hypoxic-induced ROS generation (37.8 ± 4.0 nmol/min/mg protein, p < 0.05), indicating reduced oxidative stress. No significant difference was observed in control and cobalt-preconditioned animals kept in normoxic condition.

Lipid peroxidation

Exposure of nonpreconditioned animals to hypoxia causes a marked increase in mean tissue MDA levels (38.2 ± 1.8 vs. 15.7 ± 0.7 nmol /mg protein, p < 0.05) relative to control animals. Hypoxic preconditioning with cobalt reduced mean tissue MDA levels significantly (24.9 ± 1.2 nmol/mg protein, p < 0.01) after exposure to hypoxia when compared to non-preconditioned animals (Fig. 2A). No significant change was observed in MDA levels of cobalt-preconditioned animals kept under normoxic conditions relative to control animals. We also determined HNE-adducts in lung by immunoblotting using anti-4-HNE- adduct antibody. A significant increase in 4-HNE adducts levels was noticed in lung of animals exposed to hypoxia. Supplementation of cobalt markedly inhibited 4-HNE adducts formation induced by hypoxia (Fig. 2B).

Glutathione status and antioxidant enzyme activity

Because cobalt preconditioning significantly inhibited ROS levels and oxidation of lipids, we determined the en-
Free radicals generated in the lungs during hypoxia exposure are neutralized by endogenous antioxidant levels in cobalt-preconditioned animals after hypoxia exposure. Exposure to hypoxia resulted in a significant increase in SOD, GPx, and GST levels in lungs of nonpreconditioned rats relative to control animals ($p < 0.05$). However, hypoxic preconditioning by cobalt significantly ($p < 0.01$) attenuates an increase in the levels of the antioxidant enzymes observed in hypoxia (Table 1), indicating reduced oxidative stress.

**Inflammatory cytokines**

Since various inflammatory cytokines have been reported to be involved in high altitude-induced lung injury, we determined their levels in BALF of rats after exposure to hypoxia. Exposure of nonpreconditioned animals to hypoxia resulted in a significant increase in TNF-$\alpha$, IFN-$\gamma$, and MCP-1 secretion relative to control animals (Fig. 3, $p < 0.002$). The levels of TNF-$\alpha$ and MCP-1 were decreased after HPC with cobalt relative to nonpreconditioned animals exposed to hypoxia (Figs. 3A and C, $p < 0.01$). No change was observed in IFN-$\gamma$ levels after HPC with cobalt relative to hypoxia (Fig. 3B).

**Expression of heat-shock proteins**

Heat-shock proteins (HSPs) are stress-responsive proteins that serve as useful markers of cellular response to hypoxic insult. HSP-32, also known as hemeeoxygenase-1 (HO-1), and HSP-70 are the members induced by oxidative stress. Therefore, we measured their expression in mRNA and protein levels in lung of animals exposed to hypoxia with and without cobalt preconditioning. Exposure of nonpreconditioned animals to hypoxia resulted in a significant increase in lung HSP-32 mRNA and protein expressions relative to control animals ($p < 0.01$). Preconditioning with cobalt also increases HSP-32 mRNA and protein expression markedly in both normoxic and hypoxic conditions ($p < 0.001$) compared to nonpreconditioned animals. Hypoxia exposure of nonpreconditioned animals causes a marked increase in HSP-70 mRNA ($p < 0.03$) and protein expression, but this stress was brought back to near-basal levels in cobalt-pretreated groups (Figs. 4A an B).

**Expression of metallothionein**

The mRNA expression of metallothionein, MT-1, MT-II, and MT-III was elevated after exposure to hypoxia. Preconditioning with cobalt also increases MT mRNA expressions in both normoxic and hypoxic conditions relative to control animals (Fig. 5A, $p < 0.04$). The levels of MT protein expression increased significantly after exposure of nonpreconditioned animals to hypoxia relative to control. However, preconditioning with cobalt caused a marked increase in MT protein expression in both normoxic and hypoxic conditions relative to control animals (Fig. 5B, $p < 0.02$).

**Expression of Nrf-1**

ROS generation increases the expression of a range of cytoprotective genes that each contain an antioxidant response element (ARE, 5’ TGAnnnGC 3’) in their promoters. Such genes include GPx, GST, HO, and ferritin. Expression of antioxidant-responsive-element (ARE)-driven genes is directed by Nrf-1 and Nrf-2 cap’n’collar bZIP transcription factors. The Nrf-1 transcription factor plays a pivotal role in cellular defense against the toxic effect of ROS. We found a strong induction of Nrf-1 protein expression in response to hypobaric hypoxia in lung of nonpreconditioned animals. HPC
with cobalt causes a marked increase in Nrf1 expression in both normoxic and hypoxic conditions (Fig. 6A).

Expression of HIF-1α

Immunoblotting showed a virtually undetectable level of HIF-1α protein in control animals. However, exposure to hypoxia resulted in a significant increase in the HIF-1α protein level. HPC with cobalt also induced a significant increase in the HIF-1α protein level during both normoxia and hypoxia (Fig. 6B). To know whether increased HIF-1α levels in hypoxia and cobalt-preconditioned groups result in increased DNA binding activity of HIF-1 to hypoxia response elements (HRE), an electrophoretic mobility shift assay was carried out using a highly specific oligonucleotide probe consisting of the enhancer region of the EPO gene. A low DNA binding activity was detected in normoxic control groups. However, a much stronger DNA binding activity was observed in hypoxic and cobalt-preconditioned groups (Fig. 6C).

Expression of HIF-1α-regulated genes

Exposure to hypoxia resulted in increased EPO and Glut-1 mRNA and protein expression. Preconditioning with cobalt also induced a marked increase in both mRNA and protein expressions of EPO and Glut-1 during both normoxic and hypoxic conditions. Exposure of nonpreconditioned animals to hypoxia resulted in a marked increase in VEGF mRNA and protein expression in lung relative to control animals. However, a decrease in VEGF mRNA and protein expression was noticed in lung of cobalt-preconditioned animals in both normoxic and hypoxic conditions (Figs. 7A and B). VEGF immunohistochemistry

As seen in Fig. 8B, under the staining condition used, hypoxia-exposed animals showed a marked increase in VEGF staining of pulmonary parenchyma relative to control animals (Fig. 8A). Preconditioning with cobalt markedly attenuates hypoxia-induced VEGF staining in lung parenchyma (Fig. 8D). Animals pretreated with cobalt and kept under normoxic condition showed a VEGF staining pattern similar to control animals (Fig. 8C).

Discussion

The principal findings of the present study are as follows: (1) Preconditioning with cobalt attenuates hypobaric hypoxia-induced inflammatory cytokine release, ROS generation, and lipid peroxidation in lungs, (2) enzymatic and nonenzymatic antioxidant levels increase in lung tissue after preconditioning with cobalt, (3) cobalt pretreatment increases HSP-32, MT-1, and Nrf-1 levels and decreases VEGF and HSP-70 levels in lungs, and (4) increased expression of EPO, Glut-1, and HIF-1 DNA binding activity was observed after HPC with cobalt. Taken together, these findings suggest that hypobaric hypoxia-induced generation of oxidative stress contributes to the development of pulmonary free-radical damage and that hypoxic preconditioning with cobalt attenuates oxidative stress by enhancing HSP-32, MT, Nrf-1, EPO, Glut-1, and HIF-1α expression.

High altitude is characterized by hypobaric hypoxia, which is considered an acute physiological stress leading to oxidative stress and consequently causing potential damage to protein, lipid, and DNA (Bailey et al., 2001). Oxidative stress is the result of excessive production of oxidant species and/or depletion of intracellular antioxidant defense, leading to an imbalance in the redox status of the cell. These disturbances in the redox status have been implicated in the pathogenesis of various diseases, including chronic inflammatory lung disorders (Kinnula et al., 1995; Rahman et al., 1996), and in high altitude ailments. It was initially thought that the lung was resistant to ischemic injury because of its dual pulmonary and bronchial arterial blood supply and its independent source of oxygen available from the alveolar space (Delfebach et al., 1987). However, a study by Adkins and Taylor (1990) has described the delicate alveolar–capillary membrane network as an extremely sensitive structure that is subject to alveolar hypoxia-induced lung injury in many experimental studies and clinical conditions.

Hypoxia exposures have also been shown to increase in intracellular ROS generation and to cause pulmonary artery remodeling and pulmonary artery hypertension (Nozik-Grayck and Stenmark, 2007). In the present study, ROS generation increased in lung tissue after hypobaric hypoxia exposure, which further damages membrane lipid composition as observed by an increase in lipid peroxidation. This view

<table>
<thead>
<tr>
<th>Treatments</th>
<th>GSH (μmol/min/mg protein)</th>
<th>GSH GSSG</th>
<th>GPx (μmol/min/mg protein)</th>
<th>GST (μmol/min/mg protein)</th>
<th>SOD (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>39.9 ± 3.98</td>
<td>86.7 ± 5.98</td>
<td>0.46</td>
<td>10.13 ± 1.6</td>
<td>21.24 ± 301</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>27.0 ± 2.3a</td>
<td>135.1 ± 1.96a</td>
<td>0.20</td>
<td>21.10 ± 2.1a</td>
<td>36.38 ± 4.0a</td>
</tr>
<tr>
<td>Cobalt</td>
<td>37.0 ± 3.0</td>
<td>84.4 ± 8.46</td>
<td>0.43</td>
<td>12.09 ± 2.6</td>
<td>23.30 ± 2.2</td>
</tr>
<tr>
<td>Cob + Hyp</td>
<td>34.8 ± 3.9b</td>
<td>104.3 ± 7.95b</td>
<td>0.33</td>
<td>13.04 ± 3.10b</td>
<td>25.88 ± 2.0b</td>
</tr>
</tbody>
</table>

Animals were exposed to a simulated altitude of 7619 m for 48 h with or without cobalt preconditioning (12.5 mg kg⁻¹, 7 days), and GSH, GSSG levels, and enzyme activity were determined. Values are expressed as mean ± SD of three independent experiments performed in triplicates.

a p < 0.05 compared with control.
b p < 0.01 compared with hypoxia.
is supported by increased MDA levels in serum, heart, lung, and kidney of hypoxic rats (Nakanishi et al., 1995). Concentration of the proinflammatory mediators TNF-α, IFN-γ, and MCP-1 in BALF increases following hypobaric hypoxia-induced oxidative stress. Hypoxic preconditioning with cobalt decreases ROS generation, lipid peroxidation, and inflammatory cytokines release in lung tissue. This may represent a protective effect of cobalt preconditioning from subsequent hypobaric hypoxia-induced lung oxidative damage.

Reduced glutathione (GSH) is a tripeptide thiol that acts as an intracellular antioxidant by supplying sulphydryl groups to reduce peroxides. Hypobaric hypoxia, a characteristic of high altitude, causes a decrease in GSH levels and an increase in oxidized glutathione (GSSG) levels in vivo (Ilavazhagan et al., 2001) and increases the formation of lipid peroxidative products in cultured bovine pulmonary endothelial cells in vitro (Block, 1988). GSH was measured to show the defense mechanism against hypobaric hypoxia-induced oxidative damage. It directly scavenges ROS and protects cells or tissue from free-radical damage. The decrease in tissue levels of GSH and the increase in GSSG levels after hypoxia suggest that GSH is a defense mechanism for hypobaric hypoxia-induced lipid peroxidation. Although no statistically significant increase was observed in cobalt-preconditioned animals kept under normoxic conditions, it could be argued that preconditioning with cobalt may acti-

![FIG. 3. Levels of inflammatory cytokines after HPC with cobalt. Exposure of nonpreconditioned animals to hypobaric hypoxic (7619 m, 48 h) resulted in a marked increase in TNF-α, IFN-γ, and MCP-1 levels. HPC with cobalt (12.5 mg kg⁻¹, 7 days) significantly attenuated hypoxia-induced increase in TNF-α and MCP-1 levels. Values are mean ± SD. Data shown are representative of three separate experiments in triplicate. Significant test differences between groups were determined by analysis of variance followed by Bonferroni post hoc test. *p < 0.002 compared with control. #p < 0.01 compared with hypoxia.](image-url)
vate other protective mechanisms. To correlate with the observed fall in oxidative stress, we monitored enzymatic antioxidants, such as SOD, GPx, and GST, that are responsible for scavenging ROS, but to our surprise only a marginal increase was observed in the cobalt-preconditioned groups, pointing to the possibility that, apart from antioxidants, cobalt activates different pathways in reducing oxidative stress. Our results are in apparent disagreement with certain reports showing that cobalt induces ROS generation (Llesuy and Tomaro, 1994; Clyne et al., 2001). Cobalt was also shown to be cytoprotective against tert-butyl hydroperoxide-induced oxidative stress in HepG2 cells (Piret et al., 2002). Although the reasons for these apparently contradictory results are unknown, it is possible that they may have occurred due to different experimental conditions, tissues, or cells or to the time exposure utilized in the assay to measure oxidative stress parameters.

An extensive body of literature has shown that oxidative stress can induce expression in certain heat-shock proteins (Snoecks, 2001). HSPs play a key role in cardiopulmonary protection against a variety of stressful stimuli, including ischemia (Neely and Ross, 1986), hypoxia (Howard and Geoghegan, 1986), exposure to transition metal (Low et al., 1989), and pressure overload. The inducible form of HSP-70 serves as a useful marker of cellular response to hypoxic insult (Nowak et al., 1994). We therefore analyzed the induction of HSP-70 and found a marked increase during hypoxia, which was brought down after preconditioning, with cobalt indicating a reduction in oxidative stress. Another member of the family is HSP-32, or HO-1, which is an antioxidant enzyme that regulates the intracellular level of heme and mediates an adaptive response to oxidative stress. The initial degradation of heme by microsomal HO involves the loss of iron and the formation of biliverdin, which is subsequently reduced to bilirubin by the cytosolic biliverdin reductase. Bilirubin is an efficient scavenger of ROS and has been re-
ported to attenuate free-radical-mediated damage to serum albumin (Neuzil and Stocker, 1993). Furthermore, increased bilirubin levels by HO in various tissues have been regarded as an important cellular defense mechanism against oxidative injury (Llesuy and Tomaro, 1994). Cobalt has been reported to induce HO in several in vivo and in vitro systems (Maines and Kappas, 1976; Abraham et al., 1988; Mains, 1992). Our data clearly demonstrate that preconditioning with cobalt considerably increases HO-1 mRNA and protein expression in lung tissue. This intracellular adaptation induced by cobalt promotes an increased defensive response against hypobaric hypoxia-induced oxidative stress.

Metallothionein (MT) consists of small cysteine-rich proteins that play a cytoprotective role in stress situations such as oxidative stress (Dalton et al., 1996; Giedroc et al., 2001). These proteins are also very efficient hydroxyl radical scavengers (Thornally and Vasak, 1985), and many in vivo studies indicate that MT indeed provides protection against oxidative injury in multiple organ systems, strongly implicating its antioxidant function. Therefore, we measured mRNA levels of MT-I, MT-II, and MT-III after hypoxia exposure and found a marked increase, indicating oxidative stress. Preconditioning with cobalt also increases mRNA and protein expression in both normoxic and hypoxic conditions. The primary determinant of MT protection against oxidative stress is the release of zinc sequestered by MT and its subsequent uptake by plasma membrane, because zinc protects against lipid peroxidation and thereby stabilizes membrane (Chapvil et al., 1972; Thomas et al., 1986). The presence of GSSG or any other oxidizing agent results in a release of zinc, which is required for the thermodynamic stability of MT (Cherian, 1977; Jacob et al., 1988; Maret, 1995). In the present study, increased GSSG in cobalt-pretreated animals caused zinc release from MT, which could be responsible for

FIG. 5. Induction of metallothioneins (MTs) expression after HPC with cobalt. (A) Representative mRNA. (B) Representative immunoblots with their relative optical densities (RODs). Exposure to hypoxia (7619 m, 48 h) causes a marked increase in mRNA expression of MTs. Cobalt preconditioning (12.5 mg kg\(^{-1}\), 7 days) enhanced MT mRNA and protein expressions in lung. Values are expressed as mean ± SD. Significant differences between groups were determined by analysis of variance followed by Bonferroni post hoc test.

\[^{*}p < 0.04\text{ compared with normoxia (N).}\]

\[^{#}p < 0.02\text{ compared with hypoxia (H), cobalt (Co), and cobalt + hypoxia (Co + H).}\]
the observed fall in lipid peroxidation. The expression of antioxidant enzyme and phase-2 metabolite genes is regulated through a family of basic leucine zipper proteins that includes Nrf-1 and Nrf-2. Nrf-1s are expressed at high levels in most tissue, including the lung, kidney, muscles, and heart (Linyun et al., 2003) and play a potential role in modulating oxidative stress (Wang et al., 2007). Nrf-1 is also a potent inducer of various cytoprotective proteins, such as HO-1, ferritin-H, and MT-1 (Laura et al., 2003). In the present study, both hypoxia and cobalt preconditioning increases Nrf-1 protein, which might be responsible for the increased HO-1 and MTs expression in lung tissue.

Hypoxic preconditioning is known to elicit protective responses in liver, heart, and renal injuries (Kato et al., 2001; Katori et al., 2002; Tullius et al., 2002) through the activation of HIF-1α and its regulated genes. Therefore, we measured HIF-1α and its target gene expression by immunoblotting and the DNA binding activity of HIF-1 by gel shift assay.

FIG. 6. Effect of HPC by cobalt on Nrf1 and HIF-1α expression in lung. Exposure of nonpreconditioned animals to hypobaric hypoxia (7619 m, 48 h) caused a marked increase in (A) Nrf1 expression, (B) HIF-1α expression, and (D) HIF-1α DNA binding to EPO enhancer region, which was further enhanced after HPC by cobalt (12.5 mg kg⁻¹, 7 days). The figures are representative of three separate experiments: N, normoxia; H, hypoxia; Co, cobalt; Co + H, cobalt + hypoxia.

FIG. 7. Effect of HPC by cobalt on HIF-1-regulated genes. After hypoxic exposure for 48 h at 7619 m, the expression of EPO, VEGF, and Glut1 in whole lung was determined by (A) RT-PCR and (B) western blotting. The figures are representative of three different experiment: N, normoxia; H, hypoxia; Co, cobalt; Co + H, cobalt + hypoxia.
The results from the present study indicate that HPC with cobalt markedly increases HIF-1α protein levels in lungs, which in turn upregulates the expression of its target genes EPO and Glut-1, which are responsible for erythropoiesis and glucose transport. Besides increasing the oxygen-carrying capacity of the blood by erythropoiesis, EPO has been shown to function as a cardioprotective cytokine (Cai et al., 2003; Cai and Semenza, 2004) by playing a role in energy metabolism (Wright et al., 2004) and antiapoptotic pathways (Cai et al., 2003; Tramontano et al., 2003; Cai and Semenza, 2004). EPO has also been shown to have a protective effect against ischemia-reperfusion injury (Cai et al., 2003; Wright et al., 2004). In rat alveolar epithelial cells, Ouiddir et al. (1999) showed that both the mRNA and protein levels of glucose transporter-1 (Glut-1) were increased after hypoxia and cobalt treatment. In our study, too, HPC with cobalt increases Glut-1 receptor expression in both normoxic and hypoxic conditions, indicating enhanced glucose uptake for continued energy generation in hypoxic environments. Our results are in accordance with in vitro data (Ebert et al., 1996; Zaman et al., 1999), indicating that prolonged and sustained exposure to CoCl2 is required to increase HIF-1 target gene expression.

The most important factor reported to be responsible for the occurrence of lung injury at high altitude is VEGF, an angiogenic/permeability factor. We therefore measured the VEGF levels in lung of rats exposed to hypoxia. Since cobalt administration markedly elevated HIF-1α, we expected higher VEGF levels in these animals. In fact, we found a significant decrease in VEGF levels in cobalt-pretreated animals during both normoxic and hypoxic conditions. Recently, Peters et al. (2005) reported that cobalt supplementation inhibited VEGF production induced by hypoxia in human endothelial cells. Our immunostaining results also demonstrated decreased VEGF staining to pulmonary vasculature. Several studies have demonstrated that blocking VEGF expression by using neutralizing antibody (Schoch et al., 2002) significantly prevented vascular leakage in lung injury by hypoxic insult. Based on these data, it can be said that HPC with cobalt induces HIF-1α and its target genes and may have a noticeable protective effect on hypobaric hypoxia-induced pulmonary oxidative damage in rat lung.

**FIG. 8.** Immunohistochemical staining with VEGF antibody. (A) Staining is minimal in controls and (C) cobalt-preconditioned animals kept under normoxic condition. (D) HPC by cobalt reduces hypobaric hypoxia-induced VEGF staining. The photographs are representative of three animals examined under each condition. Original magnification: ×200.
Disclosures

Messrs. Shukla, Saxena, Jayamurthy, and Ilavazaghan and Drs. Sairam, Singh, Jain, and Bansal and have no conflicts of interest or financial ties to disclose.

References


**CoCl2 PROTECTS LUNG OXIDATIVE INJURY**

Shrivastava K., SaiRam M., Bansal A., Singh S.S., and Ilavazha

Address correspondence to:
Dr. Anju Bansal
Defence Institute of Physiology and Allied Sciences (DRDO)
Lucknow Road, Timarpur,
Delhi 110 054
India

E-mail: anjubansalsal dipas@gmail.com

Received June 25, 2008; accepted in final form July 5, 2008.