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Hypoxic preconditioning facilitates acclimatization to hypobaric hypoxia in rat heart

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Abstract

Objectives Acute systemic hypoxia induces delayed cardioprotection against ischaemia-reperfusion injury in the heart. As cobalt chloride ($CoCl_2$) is known to elicit hypoxia-like –responses, it was hypothesized that this chemical would mimic the preconditioning effect and facilitate acclimatization to hypobaric hypoxia in rat heart.

Methods Male Sprague-Dawley rats treated with distilled water or cobalt chloride (12.5 mg Co/kg for 7 days) were exposed to simulated altitude at 7622 m for different time periods (1, 2, 3 and 5 days).

Key findings Hypoxic preconditioning with cobalt appreciably attenuated hypobaric hypoxia-induced oxidative damage as observed by a decrease in free radical (reactive oxygen species) generation, oxidation of lipids and proteins. Interestingly, the observed effect was due to increased expression of the antioxidant proteins hemeoxygenase and metallothionein, as no significant change was observed in antioxidant enzyme activity. Hypoxic preconditioning with cobalt increased hypoxia-inducible factor 1α (HIF-1α) expression as well as HIF-1 DNA binding activity, which further resulted in increased expression of HIF-1 regulated genes such as erythropoietin, vascular endothelial growth factor and glucose transporter. A significant decrease was observed in lactate dehydrogenase activity and lactate levels in the heart of preconditioned animals compared with non-preconditioned animals exposed to hypoxia.

Conclusions The results showed that hypoxic preconditioning with cobalt induces acclimatization by up-regulation of hemeoxygenase 1 and metallothionein 1 via HIF-1 stabilization.

Keywords cobalt; hypoxia-inducible factor 1; hypobaric hypoxia; oxidative stress

Introduction

Exposure to high altitude hypoxia (oxygen deprivation) causes an immediate reaction in the cardiovascular system, characterized by increased heart rate, stroke volume, systemic arterial pressure and cardiac output.[1] Because of the high coronary arteriovenous difference, the myocardium is not able to bring about a substantial improvement in oxygen supply by increased extraction of oxygen from the blood and thus the only way of meeting the high demands is through alterations in cellular, biochemical homeostasis, reprogramming of transcription factors and gene expression. This can be achieved by preconditioning to hypoxia, which is acquired over a period of days to weeks.

Reactive oxygen species (ROS) are generated in hypoxic heart[2] from cardiomyocytes, endothelial cells and neutrophils, and this may depress myocardial contraction through interaction with membrane lipids, proteins (by oxidation) as well as DNA, causing cell damage and death.[3] Also, under hypoxic conditions, the cellular defence system of enzymatic antioxidants such as glutathione peroxidase (GPx), the superoxide dismutases (SODs) and glutathione reductase (GR), and non-enzymatic antioxidant, namely glutathione (GSH), get disturbed.[4]

Recently, we have shown that hypoxic preconditioning with a low dose of cobalt prolonged the survival time under lethal hypoxia[5] and is advantageous in protecting the lung and brain by attenuating hypobaric hypoxia-induced oxidative injury.[6,7] Earlier studies have reported that preconditioning with cobalt could protect cardiac and renal tissue from injury through selective activation of hypoxia-inducible factor 1 (HIF-1) signalling.[8,9] Cobalt

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stabilizes HIF-1α by antagonizing Fe^{2+}, which is an essential cofactor along with oxygen for prolyl hydroxylase which degrades HIF-1α under normoxic conditions.[10] The genes controlled by HIF-1 include erythropoietin (EPO),[11] which stimulates erythropoiesis and therefore increases the oxygen carrying capacity of the blood, vascular endothelial growth factor (VEGF), which increases angiogenesis and therefore increases oxygen delivery to the hypoxic site, glucose transporter (Glut-1) and various glycolytic enzymes that are responsible for increased energy production through glycolysis during periods of reduced energy production via oxidative phosphorylation.[11] Other proteins stimulated by both hypoxia[12] and cobalt[13] are hemeoxygenase 1 (HO-1) and metallothionein (MT).

Our study on the effect of cobalt supplementation on hypobaric hypoxia induced oxidative stress in heart is the first of its kind, wherein the effect of cobalt preconditioning in ameliorating the oxidative stress of hypobaric hypoxia was studied at the molecular level. Such molecular adaptation to hypobaric hypoxia might eventually lead to functional improvement and preservation of vital organ function under high altitude conditions.

Materials and Methods

Animals

Male Sprague-Dawley rats (175 ± 25 g) were used for all experiments. Animals were maintained under a 12-h light–dark cycle at constant temperature (24°C ± 2°C) in the Institute's animal house facility. All animal procedures and experimental protocols were approved by the Institutional Animal Ethic Committee and followed the Universities Federation for Animal Welfare guidelines for animal research.

Cobalt preconditioning and hypoxic exposure

The optimum dose of cobalt chloride for hypoxia preconditioning was determined by measuring hypoxic gasping and hypoxic survival time. The optimum dose was found to be 12.5 mg $CoCl_2$/kg bodyweight, which was dissolved in sterile distilled water and fed orally by gastric cannula, once daily for 7 days prior to hypoxia exposure; this dose was used for all subsequent experiments.[6–8] Forty rats were randomly divided into four groups of 10 animals each: (i) the control group under normoxia were given distilled water; (ii) the hypoxia group; (iii) the hypoxic preconditioned group kept under normoxia (12.5 mg Co/kg for 7 days); and (iv) the hypoxic preconditioned group exposed to hypoxia. The animals were exposed to an altitude of 7622 m for different time periods (1, 2, 3 and 5 days) in an animal decompression chamber at 24°C (Decibel Instruments, Delhi, India) coupled to a mercury barometer. The airflow in the chamber was 2 l/min, with relative humidity maintained at 50–55%. The animals were taken out of the hypoxic chamber once every 24-h exposure for 15 min to replenish food and water. All the rats fed with cobalt chloride were healthy; there was no change in behaviour, food and water intake or bodyweight, and no mortality was observed.

Biochemical analysis

After hypoxic exposure, the rats were killed; the heart was dissected out (after perfusion with 20 ml sterile phosphate-buffered saline (PBS; pH 7.4) to remove the blood) and stored at −80°C for further analysis. Later, the heart was homogenized in cold 0.154 M KCl fortified with a protease inhibitors cocktail (Sigma Co., St Louis, MO, USA), to obtain 10% homogenate (w/v). One part of the homogenate was then centrifuged (Sigma Co., Munich, Germany) at 2900*g* for 10 min at 4°C. The pellet containing tissue/cell debris was discarded and the supernatant was used to determine ROS, GSH, lipid peroxidation and the protein oxidation. The other part of the homogenate was centrifuged at 14 000*g* for 20 min at 4°C and the supernatant was used for estimation of antioxidant enzyme activity such as glutathione-S-transferase (GST), GPx and SOD levels. The protein content in the homogenate was determined by the method of Lowry *et al*.[14]

Reactive oxygen species

ROS generation was measured in supernatant with 2,7-dichlorofluorescein-diacetate by the method of LeBel and Bondy,[15] as modified by Kim *et al*. (1996).[16] In brief, 50 μl of supernatant was added to a tube containing 2938 μl of 0.1 M PBS (pH 7.4) and 12 μl of 1.25 mM, 2,7-dichlorofluorescein-diacetate prepared in methanol. The assay mixture was incubated for 15 min at 37°C and 2,7-dichlorofluorescein formation was determined fluorimetrically using a spectrofluorimeter (Varian, Inc., Walnut Creek, CA, USA). The fluorescent intensity represents the amount of ROS formed in the individual sample. The results were expressed as relative fluorescent units.

Lipid peroxidation

Lipid peroxidation was measured by estimating the level of thiobarbituric acid reactive substance (TBARS) absorbance at 531 nm.[17] Results were calculated from the molar extinction coefficient of TBARS as 1.56×10^5 M^{-1} cm^{-1} and were expressed as nmoles of TBARS formed per mg protein.

Protein oxidation

Protein oxidation was measured by determining the carbonyl groups after derivatization with 2,4-dinitrophenyl hydrazine (DNPH).[18] Carbonyl content was calculated from its molar absorption coefficient as 22 000 M^{-1} cm^{-1} and results were expressed as nmol protein carbonyl per mg protein. Briefly, equal volumes of supernatant and 10 mM DNPH/2 M HCl were incubated for 60 min at 50°C. Protein was then precipitated with 20% trichloroacetic acid (TCA), and unreacted DNPH was removed by centrifugation at 1400*g* for 10 min. The precipitate was washed three times with a cold ethanol/ethyl acetate (1 : 1) mixture, and finally the precipitate was dissolved in 1 M NaOH. The absorbance was measured at 450 nm and the carbonyl content was obtained as nmol/mg protein.

Lactate and lactate dehydrogenase

Lactate dehydrogenase (LDH) (EC 1.1.1.27) activity and lactate levels were determined using Randox kits (Randox Laboratory Ltd, Crumlin, Antrim, UK) following the manufacturer's instructions.

Table 1 RT-PCR primer sequences, product size and annealing temperature

Sequence no.	Gene	Primer sequence	Product size (bp)	Annealing temperature (°C)
1	Actin	F: 5′ AAC CGT GAA AAG ATG ACC CAG ATC ATG TTT 3′ R: 5′ ATG CGG CAG TGG CCA TCT CTT GCT CGA AGT C 3′	352	58
2	HIF-1α	F: 5′ TGC TTG GTG CTG ATT TGT GA 3′ R: 5′ GGT CAG ATG ATC AGA GTC CA 3′	210	48
3	HO-1	F: 5′ CAC GCA TAT ACC CGC TAC CT 3′ R: 5′ AAG GCG GTC TTA GCC TCT TC 3′	227	52
4	MT-1	F: 5′ ACT CTG AGT TGG TCC GGA AA 3′ R: 5′ GCC TTC TTG TCG CTT ACA CC 3′	290	54
5	EPO	F: 5′ AGG CGC GGA GAT GGG GGT GC 3′ R: 5′ GTT GGG AGT GGT GGA GGG TTG G 3′	660	56
6	Glut-1	F: 5′ CTT TGT GCT TGC CGT GCT TA 3′ R: 5′ CAC ATA CAT GGG CAC AAA GC 3′	127	52
7	VEGF	F: 5′ ATG AAC TTT CTG CTC TCT TG 3′ R: 5′ GCA GGA ACA TTT ACA CGT CTG C3′	574	55

EPO, erythropoietin; F, forward primer; Glut-1, glucose transporter; HIF-1α, hypoxia-inducible factor 1α; HO-1, hemeoxygenase 1; MT-1, metallothionein 1; R, reverse primer; VEGF, vascular endothelial growth factor.

Nitrite levels

Nitrite, a biological metabolite of nitric oxide (NO), was measured in heart tissue homogenate using Griess reagent.[19] Briefly, 100 mg heart tissue was homogenized in 1 ml PBS and centrifuged at 2500*g* for 10 min. Supernatant (100 μl) was mixed with an equal volume of Griess reagent (Sigma, USA) and incubated for 30 min in darkness. A chromophore azo-derivative thus formed was measured at 540 nm in a microplate reader (Molecular Devices, Sunnyvale, CA, USA). A standard curve was prepared by using different dilutions of sodium nitrite with each assay.

Enzymatic and non-enzymatic antioxidants

Reduced GSH levels were measured fluorimetrically by the method of Hissin and Hilf.[20] The activities of GPx (EC 1.11.19) and total SOD (EC 1.15.1.1) were determined using commercial kits (Randox) as per the manufacturer's instructions. GST (EC 2.5.1.1.8) was determined using the protocol described by Habig *et al.*[21] Briefly, a 100-μl sample was mixed with 2.790 ml 0.1 M potassium phosphate buffer (pH 6.5) and 100 μl of 100 mM GSH. The reaction was initiated by adding 1-chloro 2, 4-dinitrobenzene (40 mg/ml in ethanol) and the optical density was recorded at 340 nm.

RNA isolation and reverse transcription polymerase chain reaction

Total RNA was extracted from heart homogenate using a commercially available RNA extraction kit (Qiagen, Valencia, CA, USA). The RNA quality and quantity was checked by both spectrophotometer and agarose gel electrophoresis. c-DNA was prepared by using a first-strand c-DNA synthesis kit (Fermentas, Burlington, ON, Canada) as per the manufacturer's instructions. Polymerase chain reaction (PCR) primers for various genes were purchased from Integrated DNA Technologies, Inc. (Coralville, Iowa, USA). β-Actin was used as the standard of the constitutive expression. Details of sequences, annealing temperature and amplicon size are given in Table 1. All PCR was performed in a final volume of 20 μl using master mix (Qiagen) with 1 μl of cDNA template. The PCR was conducted using a thermal cycler (MJ Research, Waltham, MA, USA). Initial denaturation was carried out at 94°C for 3 min, followed by denaturation at 94°C for 1 min, annealing for 1 min and extension at 72°C for 1 min for a total of 30 cycles, followed by final extension at 72°C for 10 min. The PCR products were electrophoresed on 2% agarose gel and visualized by UV illumination. The images of the PCR products were captured and the optical density of the band was quantified by using Labworks software (UVP BioImaging Systems, Cambridge, UK).

Protein expression studies

Preparation of nuclear and cytosolic extract

For nuclear and cytosolic fractionation, frozen heart tissue was homogenized in an ice-cold buffer A (0.5 M sucrose, 10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenyl methyl sulfonyl fluoride (PMSF) fortified with protease inhibitors (Sigma, USA)). Homogenates were kept on ice for 15 min and 0.6% nonidet P-40 was added, and then centrifuged for 10 min at 2000*g* at 4°C. The supernatant with cytosolic fraction was collected, stored and the pellet was dissolved in cold buffer B (20 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM NaCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, 0.5 mM PMSF and cocktail of protease inhibitors) for nuclear fraction. It was incubated for 30 min on ice followed by centrifugation at 20 000*g* at 4°C for 15 min. The supernatant containing the nuclear fraction was aliquoted and stored at −80°C for further analysis.

Western blotting

Protein expression of VEGF, EPO, Glut-1, HO-1 and MT-1 were quantified in cytosolic extract, whereas HIF-1α was analysed in the nuclear extract by Western blot analysis. Protein (40 μg) was separated by SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose

Table 2 Effect of hypoxic preconditioning with cobalt on generation of reactive oxygen species

	ROS (relative fluorescent units/min per mg protein)		NO (μmol/100 mg tissue)	
	Hypoxia	$CoCl_2$	Hypoxia	$CoCl_2$
Control	115.95 ± 0.32	117.76 ± 0.14	44.77 ± 0.10	38.93 ± 0.06
1 day	201.94 ± 0.76*	143.11 ± 0.16$^{\#}$	78.68 ± 0.14*	50.87 ± 0.10$^{\#}$
2 days	207.32 ± 0.61*	150.97 ± 0.65$^{\#}$	86.90 ± 0.18*	74.57 ± 0.09$^{\#}$
3 days	217.45 ± 0.53*	153.50 ± 0.63$^{\#}$	94.99 ± 0.10*	77.73 ± 0.03$^{\#}$
5 days	192.63 ± 0.60*	160.77 ± 0.33$^{\#}$	107.25 ± 0.13*	82.95 ± 0.02$^{\#}$

NO, nitric oxide; ROS, reactive oxygen species. ROS generation increased significantly after 1 day and decreased after 5 days of exposure to hypobaric hypoxia at 7622 m; the effect was nearly neutralized by cobalt supplementation (12.5 mg Co/kg for 7 days). Nitrite as an indicator of NO was measured using Griess reagent in fresh heart homogenates using dichlorofluoroscein as a probe. There was a significant increase after hypobaric hypoxia and this was attenuated after hypoxic preconditioning with cobalt. Experiments were done in triplicate. Values are mean ± SD analysed by analysis of variance followed by post-hoc Newman Keul's test. *$P < 0.05$ control vs hypoxia. $^{\#}P < 0.05$ hypoxia vs cobalt.

membrane (Hybond; Millipore, Billerica, USA). The membranes were blocked with 3% bovine serum albumin in PBS containing 0.1% Tween 20 (Sigma, USA), washed and probed with respective mouse/rabbit polyclonal antibodies (Santa-Cruz Biotechnology, Santa-Cruz, CA, USA). The membranes were then incubated with anti-mouse/rabbit-IgG HRP conjugate (Santa-Cruz Biotechnology). The membrane was washed and incubated with chemiluminescent substrate (Sigma, USA) and the bands were developed using X-ray films (Kodak, Rochester, NY, USA). Quantification was performed by densitometry using Labworks software (UVP BioImaging Systems).

DNA binding studies

Nuclear extracts were prepared from whole heart using a nuclear extraction kit (Biovision, Mountain View, CA, USA) following the manufacturer's instructions. 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 were incubated in binding buffer (10 mM Tris-HCl pH-7.4, 50 mM NaCl, 50 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA and 5 mM DTT) on ice for 10 min. Later, 10 pmol of biotinylated HIF-1 probe (Operon, Alameda, CA, USA) with the following sequences were added and incubated at room temperature for an additional 30 min: HIF-1 forward 5′-GCCCTACGTGCTGTCTCA-3′, HIF-1 reverse 5′-TGAGACAGCAC-GTAGGGC-3′, HIF-1 mutant forward 5′CCTAAAAGCTGTCTCA 3′, HIF-1 mutant reverse 5'TGAGACAGCTTTTAGG 3′. The samples were separated on a 6% native polyacrylamide DNA retardation gel and then electroblotted onto a positively charged nylon membrane. Biotinylated DNA–protein complex was detected with peroxidase-conjugated streptavidin and a chemiluminescent substrate kit (Peirce, Rockford, IL, USA).

Statistical analysis

All the experiments were performed at least three times and the data are presented as mean ± SD. Statistical analysis was carried out by analysis of variance followed by post-hoc Newman Keul's test using SPSS version 14.0 statistical software (SPSS Inc, Chicago, IL, USA). $P < 0.05$ was considered as significant.

Results

ROS generation

To assess the effect of hypoxia-induced oxidative stress in heart, ROS generation was determined using a 2,7-dichlorofluorescein-diacetate probe. Exposure to hypoxia for 1 day resulted in a rapid increase in ROS generation that was maintained for the next 3 days when compared with normoxic control animals (Table 2; $P < 0.05$). However, the ROS level was decreased significantly after 5 days hypoxia exposure when compared with 3 days exposure (Table 2; $P < 0.05$). Hypoxic preconditioning (HPC) with cobalt significantly inhibited hypobaric hypoxia=induced ROS generation after 1 day of hypoxia; lower values were maintained in animals exposed for 2, 3 and 5 days when compared with the respective hypoxia exposed animals (Table 2; $P < 0.05$). No significant difference was observed in the normoxic control group and cobalt preconditioned animals kept in normoxic conditions (Table 2). Our data suggest that preconditioning with cobalt reduces hypobaric hypoxia-induced oxidative stress in heart.

Nitrite production

NO, an important regulatory factor in the cardiovascular system,[22] was determined by measuring nitrite levels. Exposure of animals to hypoxia resulted in a gradual increase in nitrite production from Day 1, reaching a peak on Day 5 relative to normoxic control animals (Table 2; $P < 0.05$). However, animals preconditioned with cobalt showed a decrease in nitrite levels from Day 1 to Day 5 of hypoxia exposure as compared with the respective non-preconditioned animals exposed to hypoxia (Table 2; $P < 0.05$). No significant change was observed in nitrite levels in cobalt pretreated animals kept in normoxic conditions relative to normoxic control animals (Table 2).

Lipid peroxidation

Since cobalt preconditioning led to reduced oxidative stress, we determined the levels of lipid peroxidation. Exposure to hypoxia caused a marked increase in lipid peroxidation after 1 day as observed by an increase in MDA levels. Further exposure to hypoxia for 2 and 3 days resulted in a gradual increase in MDA levels, with a peak at 5 days when compared with

Table 3 Lipid peroxidation and protein oxidation in rat heart after 1–5 days of continuous hypoxia and hypoxic preconditioning with cobalt

	TBARS (nmol/mg protein)		Protein oxidation (nmol/mg protein)	
	Hypoxia	$CoCl_2$	Hypoxia	$CoCl_2$
Control	2.75 ± 0.04	2.686 ± 0.04	113.95 ± 0.34	107.76 ± 0.74
1 day	5.02 ± 0.14*	3.097 ± 0.10#	132.94 ± 0.77*	115.11 ± 0.26#
2 days	6.16 ± 0.08*	4.519 ± 0.09#	139.32 ± 0.66*	123.97 ± 0.55#
3 days	7.99 ± 0.18*	5.056 ± 0.03#	144.45 ± 0.57*	124.50 ± 0.63#
5 days	9.322 ± 0.11*	6.857 ± 0.02#	155.63 ± 0.63*	131.77 ± 0.23#

TBARS, thiobarbituric acid reactive substance. Lipid peroxidation was determined by spectrophotometric measurement of TBARS products and protein oxidation by measurement of the carbonyl groups after derivatization of proteins with dinitrophenylhydrazine. Exposure led to a significant increase in both lipid and protein oxidation and this was attenuated after hypoxic preconditioning with cobalt (12.5 mg Co/kg for 7 days). Experiments were done in triplicate. Values are mean ± SD analysed by analysis of variance followed by post-hoc Newman Keul's test. *$P < 0.05$ control vs hypoxia. #$P < 0.05$ hypoxia vs cobalt.

Table 4 Antioxidant status of antioxidant enzymes in rat heart after hypoxia exposure and cobalt preconditioning

	GSH (μg/mg protein)		SOD (U/mg protein)		GST (μmol/min per mg protein)		GPx (U/mg protein)	
	Hypoxia	$CoCl_2$	Hypoxia	$CoCl_2$	Hypoxia	$CoCl_2$	Hypoxia	$CoCl_2$
Control	147.83 ± 0.44	142.23 ± 0.83	0.727 ± 0.03	0.73 ± 0.003	169.78 ± 1.23	170.98 ± 1.03	1.80 ± 0.11	1.86 ± 0.002
1 day	129.43 ± 0.54*	131.01 ± 0.49	0.979 ± 0.003*	0.986 ± 0.04	170.38 ± 0.20	171.89 ± 0.77	1.921 ± 0.01*	1.92 ± 0.01
2 days	123.27 ± 0.51*	124.49 ± 0.39	1.074 ± 0.01*	1.053 ± 0.003	178.70 ± 0.33*	179.77 ± 0.55	3.128 ± 0.03*	3.22 ± 0.06
3 days	117.52 ± 0.44*	118.19 ± 0.66	1.132 ± 0.01*	1.137 ± 0.005	187.957 ± 0.73*	191.49 ± 0.36	3.88 ± 0.06*	3.66 ± 0.01
5 days	103.32 ± 0.65*	110.81 ± 0.66#	1.295 ± 0.004*	1.201 ± 0.08#	200.15 ± 0.38*	196.08 ± 1.10	5.309 ± 0.01*	5.37 ± 0.01

GSH, reduced glutathione; GST, glutathione-S-transferase; GPx, glutathione peroxidase; SOD, superoxide dismutase. A significant decrease in GSH level was observed after hypoxia exposure (7622 m), however antioxidant enzyme levels increased. Hypoxic preconditioning with cobalt (12.5 mg Co/kg for 7 days) had no effect on the GSH level or on antioxidant enzymes. Experiments were done in triplicate. Values are mean ± SD analysed by analysis of variance followed by post-hoc Newman Keul's test. *$P < 0.05$ control vs hypoxia. #$P < 0.05$ hypoxia vs cobalt.

normoxic control animals (Table 3; $P < 0.05$). HPC with cobalt significantly inhibited the hypoxia-induced lipid peroxidation as compared with the respective hypoxia exposed animals (Table 3; $P < 0.05$). No significant difference was observed in the normoxic control group and cobalt preconditioned animals kept in normoxic conditions.

Protein oxidation

The effect of hypoxia on protein oxidation was measured by determining the protein carbonyl content in heart homogenate after derivatization with DNPH. The results showed a gradual increase in protein oxidation in rats exposed to hypoxia for 1, 2, 3 and 5 days as compared with normoxic control animals (Table 3; $P < 0.05$). HPC with cobalt significantly reduced the hypobaric hypoxia-induced protein oxidation relative to the respective hypoxia exposed control animals (Table 3; $P < 0.05$). No significant change was observed in protein oxidation in cobalt treated animals kept in normoxic conditions relative to normoxic control animals.

GSH status

The GSH level was determined to check the effect of HPC on endogenous non-enzymatic antioxidant. Exposure of animals to hypobaric hypoxia resulted in a gradual fall in mean tissue GSH levels from Day 1 to Day 5 as compared with normoxic control animals (Table 4; $P < 0.05$). Administration of cobalt did not have any effect on GSH levels as no significant difference was observed between hypoxia exposed animals and cobalt preconditioned animals exposed to hypoxia (Table 4).

Antioxidant enzyme activity

Considering that cobalt led to a significant decrease in ROS generation, lipid and protein oxidation, we investigated the antioxidant enzyme activity in cobalt supplemented rats after hypoxia exposure. As shown in Table 4, SOD activity significantly increased after 1 day of hypoxic exposure and it increased further up to 5 days as compared with normoxic control animals ($P < 0.05$). Animals preconditioned with cobalt showed lower SOD activity when compared with the respective hypoxic control groups (Table 4). No difference was observed between normoxic control and cobalt pretreated animals kept in normoxic conditions. A gradual increase in GPx and GST levels (Table 4; $P < 0.05$) was also observed up to 5 days of hypoxia exposure. However, animals preconditioned with cobalt showed no significant difference in GPx and GST activities as compared with the respective hypoxic control groups.

LDH and lactate levels

LDH activity and lactate levels showed a significant increase from Day 1 and increased gradually up to 5 days of hypoxia exposure relative to normoxic control animals (Table 5; $P < 0.05$). HPC with cobalt significantly reduced the heart LDH activity and lactate levels as compared with the respective hypoxia exposed animals (Table 5; $P < 0.05$). No

Table 5 Lactate dehydrogenase activity and lactate levels

	LDH (nmol/mg protein)		Lactate (nmol/mg protein)	
	Hypoxia	$CoCl_2$	Hypoxia	$CoCl_2$
Control	8.50 ± 0.04	8.84 ± 0.04	62.694 ± 0.34	66.39 ± 0.74
1 day	9.92 ± 0.14	10.04 ± 0.10	77.42 ± 0.77*	72.58 ± 0.26#
2 days	11.37 ± 0.08*	10.24 ± 0.09#	83.45 ± 0.66*	74.79 ± 0.55#
3 days	13.33 ± 0.18*	10.713 ± 0.03#	86.55 ± 0.57*	80.54 ± 0.63#
5 days	14.18 ± 0.11*	10.80 ± 0.02#	93.03 ± 0.63*	82.66 ± 0.23#

LDH, lactate dehydrogenase. LDH and lactate levels (markers of anaerobic glycolysis) increased significantly after hypoxia (7622 m), indicating a switch over to anaerobic metabolism. These markers were attenuated after hypoxic preconditioning with cobalt (12.5 mg Co/kg for 7 days). Experiments were done in triplicate. Values are mean ± SD analysed by analysis of variance followed by post-hoc Newman Keul's test. *$P < 0.05$ control vs hypoxia. #$P < 0.05$ hypoxia vs cobalt.

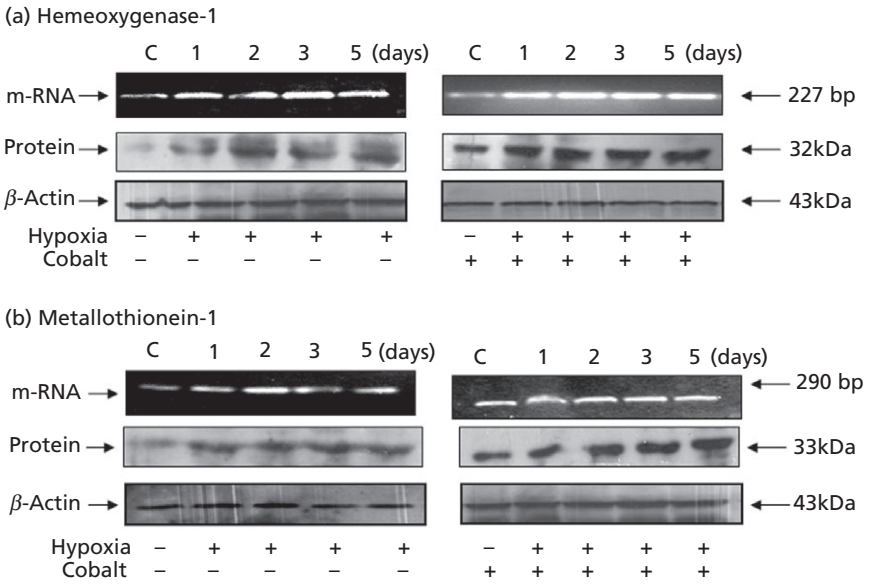

Figure 1 Effect of hypoxic preconditioning with cobalt on the expression of antioxidant proteins hemeoxygenase 1 and metallothionein 1 in rat heart homogenate. (a) Representative mRNA and immunoblot of hemeoxygenase 1. (b) Representative mRNA and immunoblot of metallothionein 1. There was a significant increase in hemeoxygenase 1 and metallothionein 1 levels after continuous hypobaric hypoxia as well as hypoxic preconditioning with cobalt (12.5 mg Co/kg for 7 days). The figures are representative of three independent experiments. These were normalized with actin to observe any change in expression.

significant change was observed in LDH activity and lactate levels in cobalt pretreated animals kept in normoxic condition relative to normoxic control animals (Table 5).

Effect of cobalt supplementation on HO-1 and MT expression

Induction of the HO-1 gene by most agents is mediated at the level of gene transcription. Induction by cobalt appears to occur by the same mechanism, as cobalt preconditioning activated mRNA in a time-dependent manner. Exposure to hypoxia resulted in a gradual increase in mRNA expression from Day 1 to Day 5 when compared with normoxic control animals (Figure 1a). HPC with cobalt showed higher mRNA expression even in normoxic conditions, and a consistent increase was observed after exposure to hypoxia for 1, 2, 3 and 5 days (Figure 1a). Consequently, protein expression also increased in a time-dependent manner in both hypoxia and cobalt preconditioned animals (Figure 1a).

MT, which acts as a free radical scavenger[23] in various stress conditions, was also determined. Exposure of animals to hypobaric hypoxia resulted in a gradual increase in heart MT-1 mRNA and protein expression from Day 1 to Day 5. However, animals preconditioned with cobalt a showed marked increase in MT-1 mRNA and protein expression even in normoxic conditions and it increased further after exposure to hypoxia up to 5 days (Figure 1b). Collectively, the present results demonstrate that HPC with cobalt elevated the expression of antioxidant proteins HO-1 and MT-1.

Effect of cobalt supplementation on HIF-1α expression

A gradual increase in HIF-1α mRNA levels was observed in the heart of animals exposed to hypoxia as compared with the normoxic control animals. In contrast, animals preconditioned with cobalt showed no significant increase in HIF1α mRNA expression (Figure 2a). Immunoblotting showed

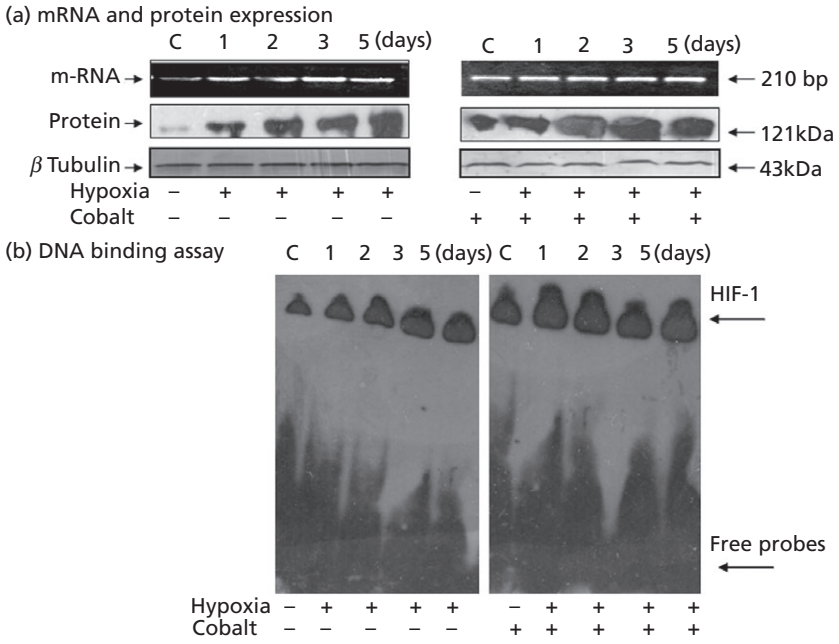

Figure 2 Expression of hypoxia inducible factor 1α. (a) Representative m-RNA and immunoblot. (b) DNA binding to the erythropoietin enhancer region. A significant increase in hypoxia inducible factor (HIF) 1α m-RNA, protein and DNA binding activity was observed after hypobaric hypoxia (7622 m), which increased further after hypoxic preconditioning with cobalt. The figures are representative of three independent experiments. These were normalized with actin to observe any change in expression.

a virtually undetectable HIF-1α protein in normoxic control animals; however exposure to hypoxia resulted in a gradual increase in HIF-1α levels up to 5 days. Interestingly, in hypoxic preconditioned animals, higher HIF-1α protein expression was observed even in the normoxic condition and it increased further after exposure to hypoxia, with a peak at Day 3 (Figure 2a).

To check the HIF-1 DNA binding activity, the electrophoretic mobility shift assay was carried out using a highly specific oligonucleotide probe consisting of an enhancer region of the EPO gene to find out whether increased HIF-1α levels in hypoxia and cobalt supplemented groups results in increased DNA binding activity. Exposure to hypoxia for 1 day caused a 2-fold increase in DNA binding activity, which increased gradually with a peak of 4-fold expression after 5 days exposure. However, a robust increase in DNA binding activity was observed in cobalt pretreated animals from Day 0 to 5 days of hypoxia exposure (Figure 2b).

Effect of cobalt supplementation on HIF-1 regulated genes

To further investigate the role of cobalt preconditioning, HIF-1 targeted genes (EPO, VEGF and Glut-1) were examined. EPO mRNA and protein expression increased following hypoxia compared with normoxia control animals in both groups (hypoxia and cobalt preconditioned), however a 2-fold increase in protein expression was observed in all the cobalt treated groups as compared with hypoxia exposed animals (Figure 3).

Exposure to hypoxia caused a gradual increase in VEGF and Glut-1 mRNA up to 5 days. HPC with cobalt also induced a marked increase in mRNA expression of VEGF and Glut-1 during both normoxic and hypoxic conditions. VEGF protein expression increased 2-fold after 1 day of hypoxia exposure and it remained elevated up to 5 days. Cobalt preconditioned animals kept under normoxia conditions showed a 3-fold increase in VEGF protein expression as compared with normoxic control animals, and higher expression was maintained in all the cobalt treated groups compared with the respective hypoxic control animals (Figure 3). Glut-1 protein expression showed no change after 1 day exposure, however it increased gradually after 2 days, reaching a peak at 5 days compared with normoxic control animals. Higher Glut-1 expression was observed in cobalt normoxia animals compared with normoxia control animals and maximum activation was observed in 2 days. Elevated Glut-1 levels were maintained in all the cobalt preconditioned animals as compared with the respective hypoxia animals (Figure 3).

Discussion

The principal findings of the present study are as follows: (i) HPC with cobalt attenuates hypobaric hypoxia-induced ROS generation, lipid peroxidation and protein oxidation; (ii) no change was observed after HPC with cobalt on enzymatic (SOD, GPx, GST) and non-enzymatic antioxidant (GSH) levels, however the expression of MT-1 and HO-1 increased markedly after preconditioning; (iii) HPC with cobalt results in increased HIF-1 DNA binding activity and expression of adaptive genes EPO, VEGF and Glut-1; and (iv) lactate levels and LDH activity were reduced after HPC with cobalt. Collectively, our data demonstrate increased hypoxic adaptation in rat heart after HPC with cobalt.

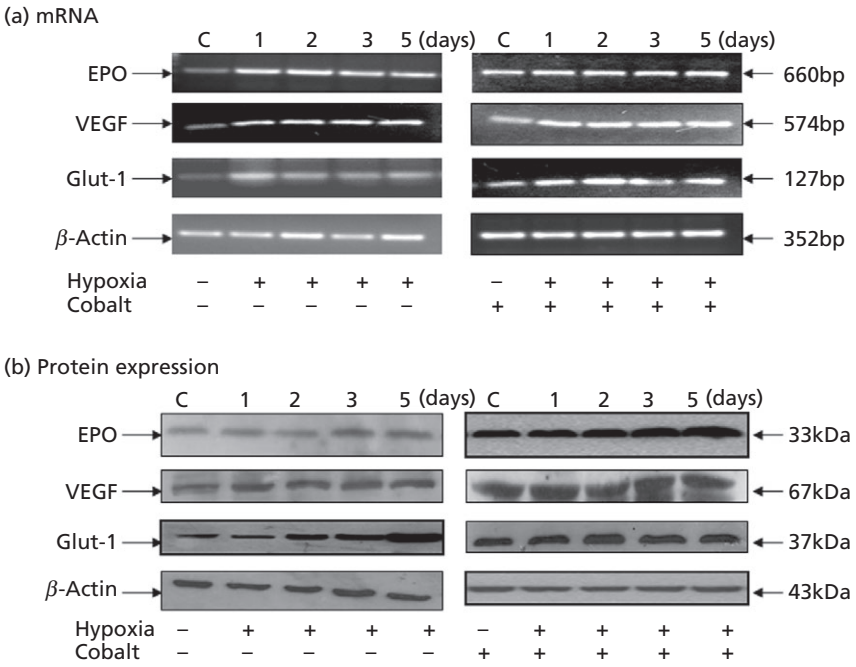

Figure 3 Effect of hypoxic preconditioning with cobalt on expression of various hypoxia responsive genes (hypoxia inducible factor 1 regulated genes). After hypoxia exposure for 1, 2, 3 and 5 days at 7622 m, the expression of erythropoietin (EPO), vascular endothelial growth factor (VEGF) and glucose transporter (Glut-1) in whole heart homogenate was determined by (a) RT-PCR and (b) Western blotting. Genes were upregulated following hypoxia as well as cobalt supplementation (12.5 mg Co/kg). The figures are representative of three independent experiments. These were normalized with actin to observe any change in expression.

Although acute systemic hypoxia induces delayed preconditioning in the heart, it is not known whether such protection can be realized by chemical induction of the hypoxic response with cobalt. In the present study, we demonstrated that HPC with cobalt promotes acclimatization to hypobaric hypoxia. Administration of cobalt was started 7 days before hypoxia exposure so that protective mechanisms were ready when the rats had to undergo hypobaric hypoxia exposure. We demonstrated that cobalt attenuated the oxidative stress by increased expression of antioxidant proteins HO-1 and MT-1 via HIF-1α. All these changes were responsible for the acclimatization of rat heart.

Hypobaric hypoxia is known to induce oxidative stress by increasing ROS generation in animals[24] and humans,[25] causing potential damage to DNA, protein and lipid. Oxidative stress is the result of excessive production of oxidant species and/or depletion of intracellular antioxidant defence, leading to imbalance in the redox status of the cell. In the present study, exposure to hypobaric hypoxia resulted in an appreciable increase in ROS generation, which was responsible for the observed increase in lipid and protein oxidation in rat heart. A decrease in ROS generation was observed after 5 days of hypoxic exposure, which might be due to the initiation of the normal process of acclimatization. However, ROS generation was attenuated from Day 1 after HPC with cobalt. Reduction in lipid peroxidation and protein oxidation after HPC further confirms reduced hypoxia-induced oxidative stress. GSH was measured to show the defence mechanism against hypobaric hypoxia-induced oxidative damage. It directly scavenges ROS and protects cells or tissue from free radical damage. No statistically significant difference was observed in cobalt preconditioned animals exposed to hypoxia relative to control animals, therefore it could be argued that preconditioning with cobalt may activate some other protective mechanisms. To correlate with the observed fall in oxidative stress, we monitored the levels of enzymatic antioxidants GPx, GST and SOD. Interestingly, no statistically significant increase was observed in GPx and GST activities in cobalt-preconditioned animals relative to non-preconditioned control animals and this result was in accordance with previous studies.[6,26] As demonstrated in earlier reports,[27] HPC with cobalt also resulted in a decrease in SOD activity. We studied NO to determine the cardioprotective effect at high altitude.[28] To our surprise, cobalt preconditioning decreased the hypobaric hypoxia-induced increased NO level in rat heart. Taken together, our data indicate that cobalt activates some other protective pathways (non-GSH and non-NO mediated) to initiate the process of acclimatization.

Our results are in contrast with earlier reports showing that cobalt supplementation resulted in increased ROS generation.[26,27] The reasons for the contradictory results are unknown, although it is possible that the results may vary according to dose, route of administration, pre-existing heart damage, age, length of exposure and nutritional status as suggested by earlier reports.[29] For example, the canine model of cobalt-induced cardiomyopathy required a daily intravenous dose of 5 mg/kg cobalt sulfate in conjunction with a low-protein, low-thiamine diet.[30]

In the present study, since no change was observed in enzymatic or non-enzymatic antioxidants; we therefore

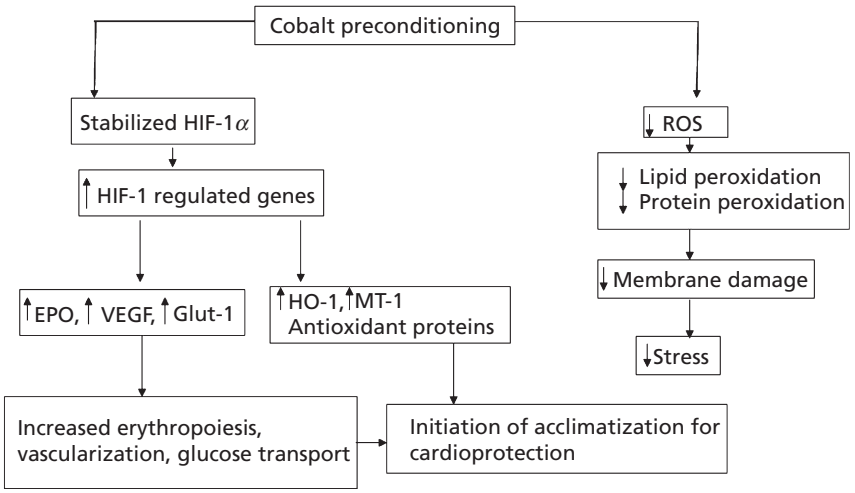

Figure 4 Putative correlation among various factors and mechanism of induction of cardioprotection by hypoxic preconditioning with cobalt. EPO, erythropoietin; Glut-1, glucose transporter; HIF-1α, hypoxia inducible factor 1α; HO-1, hemeoxygenase 1; ROS, reactive oxygen species; MT-1, metallothionein 1; VEGF, vascular endothelial growth factor.

measured antioxidant proteins such as HO-1 and MT-1, which are known to provide cardiopulmonary protection against a variety of stimuli, including ischaemia[31] and in cellular anti-oxidative reactions.[32] Moreover, a study by Ewing and Maines[33] correlates the induction of HO-1 m-RNA with depletion in endogenous GSH. The present study showed a considerable increase in HO-1 levels in the heart of rats exposed to hypoxia, which was further enhanced following cobalt supplementation. Several reports have proposed that HO is involved in the formation of bilirubin, which is an efficient scavenger of ROS and has been reported to attenuate free radical-mediated damage to serum albumin.[34] Furthermore, increased bilirubin levels by HO in various tissues have been regarded as an important cellular defence mechanism against oxidative injury.[35] MT is another family of proteins that act as free radical scavengers[23] regulated by metals and prevent metal toxicity under metal overload.[36] Our results clearly demonstrated that HPC with cobalt considerably increased MT-1 mRNA and protein expression in rat heart tissue. Moreover, cobalt is known to activate expression of HO-1 and MT-1.[37] Hence, one of the possible reasons for the observed reduction in oxidative stress might be due to increased HO-1 and MT-1 levels.

Cobalt chloride has been widely used as a hypoxia mimetic in both in-vivo and in-vitro studies.[38] Previous work has shown that on a global gene expression level, both cobalt and hypoxia regulate a similar group of genes.[39] The observed similarity in gene expression appears to be dependent on a functional HIF-1α protein.[39,40] Therefore, we determined the HIF-1α levels during hypoxia exposure and cobalt preconditioning by RT-PCR, immunoblotting and the DNA binding activity of HIF-1 by gel shift assay. HPC with cobalt for 7 days resulted in a significant increase in HIF-1α expression. The absence of major changes in the steady state levels of HIF-1α mRNA in control and cobalt pretreated animals kept under normoxic conditions suggests that cobalt-mediated HIF-1α induction results from a change in the rate of HIF-1α protein synthesis or degradation, not from increased mRNA synthesis.

As we observed stabilization of HIF-1α by hypoxia and cobalt, we determined the levels of its target genes EPO, Glut-1 and VEGF, which are responsible for erythropoiesis, glucose transport and angiogenesis, respectively. EPO has been shown to have cardioprotective[41] and antiapoptotic activity.[41,42] A noticeable increase in HIF-1 DNA binding activity to the EPO enhancer region was observed in cobalt preconditioned animals both in cobalt normoxic and cobalt hypoxic groups, resulting in higher EPO levels and, hence, increased erythropoiesis. Our results are in accordance with in-vitro[43] and in-vivo[44] data stating that prolonged and sustained exposure to $CoCl_2$ increases erythropoietin in normoxia. Cobalt was earlier given to human patients for the treatment of anaemia.[45] Preconditioning with cobalt increased EPO mRNA and the protein provides a potential molecular basis for the observed hypoxic acclimatization in rats.

In the in-vivo heart and cultured cardiac cells, both hypoxia and cobalt quickly induced mRNA for Glut-1.[46] In our study, preconditioning with cobalt also increased Glut-1 expression both in cobalt normoxic and cobalt hypoxic rats, indicating enhanced glucose uptake for continued energy generation in the hypoxic environment. To further confirm whether hypoxic preconditioning with cobalt results in better oxygen delivery, LDH activity and lactate levels were measured in heart. Interestingly, cobalt administration attenuated the hypoxia-induced increase in LDH activity and lactate levels compared with control animals. This indicates better oxygen availability in hearts during hypoxia after cobalt preconditioning.

VEGF is the most potent endothelial-specific mitogen and it directly participates in angiogenesis by recruiting endothelial cells into hypoxic and avascular areas, and stimulates their proliferation.[47] The present study demonstrates in-vivo

induction of VEGF mRNA and protein in cardiac tissue similar to the previous studies.[48] Upregulation of VEGF may facilitate tissue perfusion and oxygenation in hypoxia, an important adaptation to hypoxia. Ladoux and Frelin[49] reported that cobalt stimulates the expression of VEGF mRNA in rat cardiomyocytes. Moreover, Endoh *et al*.[50] found an in-vivo induction of VEGF mRNA in the cardiac tissue of rats chronically treated with a low concentration of cobalt. Accordingly, our results also showed that the heart of rats preconditioned with cobalt exhibited improved mRNA and protein expression as a sign of increased acclimatization and hypoxic tolerance. Our results suggested that preconditioning with cobalt induces HIF-1 and its targeted genes, and may have a noticeable protective effect in rat heart against hypobaric hypoxia.

Conclusions

We have demonstrated for the first time that an intrinsic protective mechanism against hypoxia can be rapidly induced in unacclimatized rats by cobalt preconditioning. This novel mechanism exerts immediate and potent protection to rat heart by increased expression of HIF-1 and its target genes (Figure 4).

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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