

Role of Oxidative Stress and NFkB in Hypoxia-Induced Pulmonary Edema

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Hypoxia is well known to increase the free radical generation in the body, leading to oxidative stress. In the present study, we have determined whether the increased oxidative stress further upregulates the nuclear transcription factor (NFkB) in the development of pulmonary edema. The rats were exposed to hypobaric hypoxia at 7620 m (280 mm Hg) for different durations, that is, 3 hrs, 6 hrs, 12 hrs, and 24 hrs at $25 \pm 1^\circ\text{C}$. The results revealed that exposure of animals to hypobaric hypoxia led to a significant increase in vascular leakage, with time up to 6 hrs (256.38 ± 61 rfu/g) as compared with control (143.63 ± 60.1 rfu/g). There was a significant increase in reactive oxygen species, lipid peroxidation, and superoxide dismutase levels, with a concurrent decrease in lung glutathione peroxidase activity. There was 13-fold increase in the expression of NFkB level in nuclear fraction of lung homogenates of hypoxic animals over control rats. The DNA binding activity of NFkB was found to be increased significantly ($P < 0.001$) in the lungs of rats exposed to hypoxia as compared with control. Further, we observed a significant increase in proinflammatory cytokines such as IL-1, IL-6, and TNF- α with concomitant upregulation of cell adhesion molecules such as ICAM-I, VCAM-I, and P-selectin in the lung of rats exposed to hypoxia as compared with control. Interestingly, pretreatment of animals with curcumin (NFkB blocker) attenuated hypoxia-induced vascular leakage in lungs with concomitant reduction of NFkB levels. The present study therefore reveals the possible involvement of NFkB in the development of pulmonary edema. *Exp Biol Med* 233:1088–1098, 2008

Key words: HAPE; NFkB; oxidative stress; proinflammatory cytokines

Introduction

High-altitude pulmonary edema (HAPE) is a non-cardiac, acute, and potentially lethal pulmonary disorder. The rapid ascent to altitudes above 2450 m can lead to high-altitude pulmonary edema (5, 23) in nonacclimatized individuals. The altitude, speed, and mode of ascent are the most important determinants for the occurrence of HAPE. This illness usually occurs 2 to 3 days after acute exposure to altitudes above 2500 to 3000 m. It is characterized by increased pulmonary arterial pressure, vasoconstriction with elevated vascular permeability, and hypoxemia (22). The incidence of subclinical HAPE may be almost 70% in individuals exposed to an altitude of 4500 m. However, a recent report suggests that HAPE may occur in individuals performing heavy exercise at altitudes as low as 2,400 m (19). In spite of intensive research, the pathophysiology of HAPE is yet to be elucidated.

Clinical investigations reported that HAPE-susceptible people show a patchy peripheral distribution of edema in lungs with normal wedge pressure (7). On the other hand, some studies reported that increased pulmonary artery pressure with precapillary vasoconstriction leads to edema formation in HAPE patients (7, 24). The pathogenesis of HAPE is attributed to hydrostatic mechanisms leading to capillary leak in pulmonary edema (24). Urano *et al.* (44) have reported that exposure to severe hypoxia resulted into uneven blood flow distribution followed by increased vascular permeability. Similar results were also reported by Hopkins *et al.* (23). However, HAPE-susceptible subjects showed a significantly higher increase of pulmonary artery systolic pressure (PASP) during hypoxia at rest compared with controls (16), and considered that PASP measurements at rest during hypoxia or exercise in normoxia are most feasible for the identification of HAPE-susceptible subjects. On the other hand, several studies have reported that the inflammation process plays an important role in the pathogenesis of HAPE (6, 26).

It has been reported earlier that high-altitude exposure results in increased reactive oxygen species (ROS) generation, leading to enhanced oxidative damage to lipids,

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proteins, and DNA (42). However, there is no direct evidence on the role of ROS in causing acute mountain sickness (AMS), HAPE, and high-altitude cerebral edema (HACE). Numerous oxidative stress-sensitive transcription factors such as nuclear factor κ B (NF κ B) and activator protein 1 (AP-1) can mediate an inflammatory response caused by oxidative stress by inducing gene transcription of cytokines such as IL-1, IL-6, TNF- α , and adhesion molecules ICAM-1 (intracellular cell adhesion molecule) and VCAM-1 (vascular cell adhesion molecule) (21). Furthermore, Carol and Brain (10) reported that the cell adhesion molecules like ICAM-I and VCAM-I genes contain a NF κ B site on the promoter region and are known to be regulated by NF κ B. However, no direct evidence on the role of inflammatory cytokines and cell adhesion molecules in HAPE is known.

We were particularly interested in exploring the molecular mechanism involved in hypoxia-induced pulmonary edema. Therefore, the present study was designed to determine whether hypobaric hypoxia-induced oxidative stress leads to activation of NF κ B (in HAPE) in the lungs of rats. The study was also proposed to determine the levels of proinflammatory cytokines and cell adhesion molecules in the lungs of rats exposed to hypoxia. Our hypothesis in this study is that oxidative stress and NF κ B signaling would certainly contribute to hypoxia-induced pulmonary vascular leakage. Further, we reasoned that if this hypothesis were true, then treatment with NF κ B blocker (curcumin) during hypoxia would result in reduced transvascular leakage in the lungs of rats.

Materials and Methods

Experimental Design. Animals. The experiments were carried out using male Sprague-Dawley rats, weighing 150–200 g. Rats were maintained at $25 \pm 1^\circ\text{C}$ with 12:12-hr light to dark cycles each and given food and water *ad libitum*. This study had the approval of the Institute's Ethic Committee and followed the guidelines of Universities Federation for Animal Welfare (UFAW) guidelines for animal research.

Hypoxic Exposure. The experiment was conducted in two phases, that is, phase I and phase II.

Phase I experiment. A total of 60 rats were used in the phase I experiment; rats were divided into five groups of 12 rats each. Group 1 served as control or normoxia (0 hrs); Group 2 was exposed to hypoxia for a 3-hr duration; Group 3 was exposed to hypoxia for a 6-hr duration; Group 4 was exposed to hypoxia for a 12-hr duration; and Group 5 was exposed to hypoxia for a 24-hr duration.

Phase II experiment. In phase II, the NF κ B blockade study was carried out using curcumin in 48 rats, which were divided into four groups of 12 rats each. Group 1 served as control or normoxia (0 hrs) receiving only vehicle; Group 2 (hypoxia) received only vehicle and was exposed to hypoxia for 6 hrs; Group 3 was supplemented with curcumin 50 mg/

kg body weight (BW); and Group 4 (hypoxia + curcumin) was supplemented orally with curcumin 50 mg/kg BW and was exposed to hypoxic stress for 6 hrs.

The rats were exposed to a simulated altitude of 7620 m (25,000 ft) in a hypobaric chamber (Decibel Instruments India Limited, Delhi, India) for different periods of time, namely 3, 6, 12, and 24 hrs. The temperature of the hypobaric chamber was maintained at $25 \pm 1^\circ\text{C}$ with an air flow rate of 4 liter/hr and barometric pressure of 280 mm Hg. The partial pressure of arterial oxygen in control rats was found to be 95 ± 2 mm Hg, and in hypobaric rats it was found to be 38 ± 2 mm Hg, indicating that the rats were exposed to reduced levels of partial pressure of oxygen in the hypobaric chamber. The animals were provided with adequate quantities of food and water during exposure to hypoxia. We have exposed the rats to hypobaric hypoxia (280 mm Hg) because the smaller animals have higher capillary density in tissues, making them more resistant to hypoxia than humans are.

Sample Preparation and Measurement of Oxidative Stress. After hypoxic exposure, animals were sacrificed and the lungs were perfused with cold phosphate-buffered saline (PBS). The lung tissue was collected and then washed with cold saline (0.9% NaCl) and a 10% homogenate in 0.154 M KCl was prepared at 4°C for estimating various biochemical parameters.

Determination of Biochemical Parameters. The production of free radicals (ROS) in the lung homogenate was determined time dependently, that is, at 0 hrs, 1.5 hrs (90 mins), 3 hrs, 6 hrs, 12 hrs, and 24 hrs by using DCFH-DA (2,7-dichlorofluorescein diacetate), and the fluorescence was measured by spectrofluorometer (Varian, Walnut Creek, CA) with an excitation at 485 nm and emission at 530 nm (13). Malondialdehyde (MDA) levels were also determined time dependently, that is, at 0 hrs, 1.5 hrs (90 mins), 3 hrs, 6 hrs, 12 hrs, and 24 hrs in the lungs (34) by 2-thiobarbuturic acid assay and measuring the absorbance at 532 nm using 1,1,3,3-tetra-ethoxy propane as standard. The reduced glutathione (GSH) was determined in the lungs by the method of Kum-Talt and Tan (27) using dithionitrobenzoic acid (DTNB) reagent and measuring the absorbance at 412 nm. Glutathione peroxidase (GPx) and superoxide dismutase (SOD) levels in lungs were measured using commercial kit (Randox, Crumlin, UK) as per manufacturer's instructions. The protein concentration was estimated by the method of Lowry *et al.* (28).

Determination of Pulmonary Edema. Determination of vascular permeability. The vascular permeability of lungs was determined following the method of Baba *et al.* (3), with some modifications. In brief, half an hour before the completion of hypoxic exposure, the rats were taken out of the hypobaric chamber, and 200 μl of sodium fluorescein (5 mg/kg BW in PBS; Sigma Chemical Co., St. Louis, MO) was injected through the tail vein. Later the rats were placed back in the hypoxia chamber and exposed again to hypoxia for additional 30 mins. Later the animals were taken out of

the hypoxia chamber, anesthetized, and perfused with PBS through the left heart ventricle to remove the fluorescent tracer from the vascular bed. The lungs were removed, washed with cold saline, and divided into two equal parts. One part of the lung was kept in 3% formamide for about 18 hrs at room temperature (RT). Later, the tissues were centrifuged for 10 mins at 3,000 rpm, and the fluorescence in the supernatant was measured using a spectrofluorometer (Varian) with 485 nm excitation and emissions at 530 nm. The other part of the lung was weighed and kept in an oven at 80°C for 72 hrs. Later, the dry tissues were collected and weighed again to determine the dry weight. The results were presented as relative fluorescence units per gram (rfu/g) dry weight.

Determination of lung water content. To quantify the lung water content in lungs from both normoxic and hypoxic animals, the wet weight of the lungs was determined immediately after removal. The samples were rinsed with the cold PBS and dried at 80°C for 72 hrs, and the edema index was expressed as wet-to-dry weight ratio (W/D ratio) (46).

Protein Expression Studies. Sample preparation. After hypoxic exposure, the animals were sacrificed using ketamine hydrochloride (80 mg/kg) and xylazine (20 mg/kg) anesthesia and the lungs were perfused with cold PBS. The whole lung was removed and washed with cold saline and then homogenized in a buffer containing 0.01 M Tris HCl, pH 7.6, 0.1 M NaCl, 0.1 mM dithiothreitol, 0.001 M EDTA, 100 µg/ml phenylmethylsulfonyl fluoride (PMSF) and 10 µl/ml protease inhibitor cocktail (Sigma). The contents were centrifuged at 3000 rpm for 15 mins at 4°C, and the supernatant was collected and stored at -80°C.

The proteins (50 µg) were separated on 10% sodium dodecylsulphate-polyacryl-amide gel electrophoresis (Bio-Rad, Hercules, CA) and electroblotted onto nitrocellulose membranes (Millipore, Billerica, MA). The membranes were blocked with 3% bovine serum albumin (BSA) for 2 hrs and thoroughly washed with PBST (phosphate-buffered saline with 0.1 % Tween-80) and were probed with primary antibodies in 1:2000 dilution (IL-1, IL-6, TNF- α , ICAM-1, VCAM-1 and P-selectin; Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hrs. The membranes were washed and incubated with secondary antibodies conjugated with horseradish peroxidase (1:50,000; Santa Cruz Biotechnology) for 1 hr at RT. Later, the membranes were thoroughly washed with PBST (five to six times), and the bands were developed on x-ray film (Kodak, Rochester, NY) using chemiluminescent peroxidase substrate (Sigma). The optical density of bands was quantified using Labworks software (UVP Bio-imaging Systems, Upland, CA).

Nuclear and Cytoplasmic Fraction. Oxidative stress is known to modulate the redox-sensitive transcriptional proteins. For determining NF κ B, nuclear and cytoplasmic fractions were isolated from lung homogenate using a commercial kit (Bio-Vision, Mountain View, CA) according to the manufacturer's instructions. The NF κ B protein levels

in both nuclear and cytoplasmic fractions were determined by Western blotting as described above.

Transcription Factor (NF κ B) Activation Studies. Electrophoretic mobility shift assay (EMSA). The EMSA for NF κ B was carried out using a commercial kit (Pierce, Rockford, IL). The binding mixture (25 µl) containing 10 µg protein of nuclear extract and 1 µg of poly dI-dC were incubated in a Tris-EDTA buffer (10 mM TrisHCl, pH 7.4, 50 mM NaCl, 50 mM KCl, 1 mM MgCl₂, 1 mM EDTA, 5 mM DTT) on ice for 15 mins. Later 10 ng of biotinylated double-stranded NF κ B probe (NF κ B oligonucleotide probe supplied by Operon [Cologne, Germany], the sequence being NF κ B, F 5'-AGT TGA GGG GAC TTT CCC AGG C-3', NF κ B R 5'-GCC TGG GAA AGT CCC CTC AAC T-3', NF κ B mutant F 5'-AGT TGA GGC GAC TTT CCC AGG C 3', NF κ B mutant R 5'-GCC TGG GAA AGT CGC CTC AAC T3') (the underlining indicates a DNA-binding site for NF κ B) was added and incubated at RT for 30 mins. Then DNA protein complexes were separated on native 6% polyacrylamide DNA retardation gel and electroblotted onto positively charged nylon membranes. Biotinylated DNA/protein complexes were detected with peroxidase-conjugated streptavidin and chemiluminescent substrate kit (Pierce).

NF κ B Blockade Study. The rats were administered with curcumin (50 mg/kg BW) orally 1 hr before the hypoxic exposure (6 hrs). Determination of pulmonary edema and NF κ B protein expression were carried out as described above.

Statistical Analysis. Statistical analysis was performed using SPSS for Windows (15.0) software (SPSS Inc., Chicago, IL). Comparisons between five experimental groups for various time points and also curcumin-treated groups were made by using one-way ANOVA with Student-Newman-Keuls test for multiple comparisons between groups. Whereas, comparisons between normoxia-exposed (0 hrs) and hypoxia-exposed (6 hrs) animals were made using Student's *t* test. Differences were considered statistically significant for *P* < 0.05. Results are expressed as mean $\pm$ SD.

Results

Determination of Pulmonary Edema. Vascular Permeability. The effect of different periods of hypoxic exposure on development of pulmonary edema is shown in Figure 1. Exposure to hypoxia resulted in a significant increase in lung wet/dry weight ratio relative to control animals. The maximum lung water content was obtained at 6 hrs and 12 hrs of hypoxic exposure (3-fold increase in both the hours of exposures). Further increase in exposure to 24 hrs resulted in a marked decrease in wet/dry ratio (Fig. 1).

Alternatively, the vascular leakage was determined by measuring the relative fluorescence intensity in the lungs of rats exposed to hypoxia. There was a significant increase in

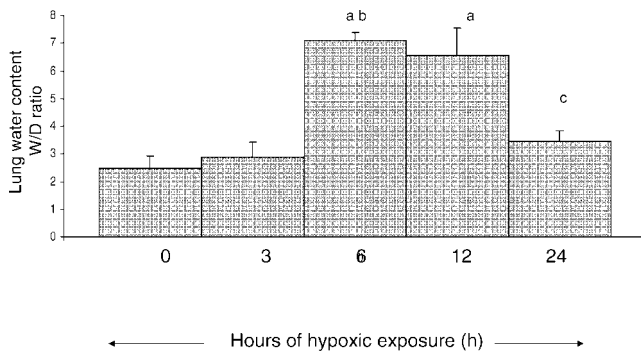


Figure 1. Effect of hypobaric hypoxia (7620 m) on lung water content in rats. The rats were exposed to simulated hypobaric hypoxia (7620 m) for different time points at $25 \pm 1^\circ\text{C}$. The lung water content was determined by taking the wet-to-dry (W/D ratio) weight of the lung tissue to give *in vivo* water content. The maximum lung water content was obtained at 6 hrs and 12 hrs of hypoxic exposure indicated that pulmonary edema was induced during this period. Values are mean $\pm$ SD ($n = 12$). Significant differences between groups were determined by analysis of variance followed by Student-Newman-Keuls test. ^a $P < 0.001$ compared with normoxia group (0 hrs). ^b $P < 0.05$ compared with hypoxia group (12 hrs). ^c $P < 0.01$ compared with hypoxia group (3 hrs, 6 hrs, and 12 hrs).

relative fluorescence intensity (40%) in the lungs of rats exposed to 7620 m for 3 hrs as compared with control animals. Maximum increase in relative fluorescence intensity (60%) was observed in animals exposed to 6 hrs of hypoxia. However, further increase in exposure time (i.e., 12 hrs and 24 hrs) resulted in a considerable fall in vascular permeability (Fig. 2). Because maximum vascular leakage was obtained at 6 hrs of hypoxic exposure, further experiments were conducted by exposing the rats to hypoxic stress for 6 hrs.

ROS, Lipid Peroxidation, and Antioxidant Levels. A gradual but significant increase in ROS generation (as revealed by increase in DCF fluorescence) was observed in lungs of rats exposed to hypoxia with time. To determine whether increased ROS levels led to increased membrane peroxidation, we determined the malondialdehyde (a volatile carbonyl) levels, an important biomarker released from the oxidation and decomposition of PUFA (polyunsaturated fatty acids). The results revealed a marked increase in MDA levels in the lungs of rats exposed to hypoxia up to 6 hrs relative to control values (Table 1). Further increase in

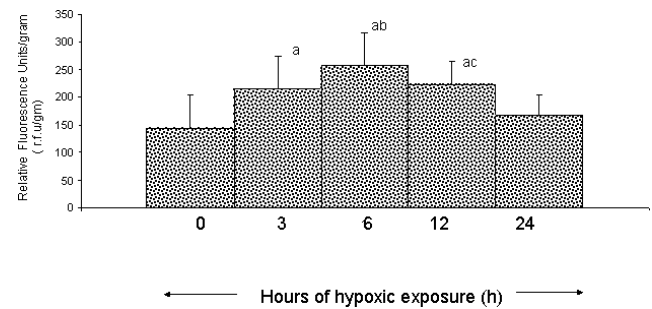


Figure 2. Effect of hypobaric hypoxia (7620 m) on pulmonary vascular leakage in rats. The rats were exposed to simulated hypoxia (7620 m) for different time points at $25 \pm 1^\circ\text{C}$ and the vascular leakage was determined using a fluorescent probe sodium fluorescein. The increase in fluorescein content in the lungs of rats began at 3 hrs of hypoxic exposure and reached the peak at 6 hrs of exposure. The results suggest that hypoxia increased the pulmonary vascular permeability. Values are mean $\pm$ SD ($n = 12$). Significant difference between groups were determined by ANOVA followed by Student-Newman-Keuls test. ^a $P < 0.001$ compared with normoxia group (0 hrs). ^b $P < 0.05$ compared with hypoxia group (3 hrs and 12 hrs). ^c $P < 0.01$ compared with hypoxia group (3 hrs and 6 hrs).

hypoxic exposure resulted in marginal decrease in MDA levels.

Because the maximum increase in pulmonary vascular leakage was observed during 6 hrs of hypoxic exposure, all other parameters were determined by exposing the animals for 6 hrs only.

Antioxidant Levels. A marginal but nonsignificant increase in GSH levels was observed in the lungs of hypoxia exposed rats related to control animals. However, exposure to hypoxia resulted in to an appreciable fall in GPx levels while the SOD remained higher as compared with control animals (Table 2).

NFκB Levels. There was a nearly 13-fold increase in NFκB levels (nuclear fraction) in the lungs of hypoxia-exposed animals as compared with that of control animals. However, the cytoplasmic levels of NFκB remained more or less similar (Fig. 3). To further confirm whether increased translocation of NFκB also results in increased DNA-binding activity, gel shift assays were performed using a highly specific biotinylated-oligonucleotide probe. The results revealed a significant increase in NFκB DNA-binding activity in the lungs of rats exposed to hypoxia over control animals (Fig. 4).

Table 1. Effect of Hypobaric Hypoxia on ROS and MDA Levels in Lungs of Rats Exposed to Different Hours of Hypoxic Exposure (7620 m) at $25 \pm 1^\circ\text{C}$ ^a

Parameters	Hours of Hypoxic Exposure					
	0	1.5	3	6	12	24
ROS (nmol/min/mg protein)	73 $\pm$ 15	95 $\pm$ 5*	113 $\pm$ 11*	189 $\pm$ 2*,***	229 $\pm$ 14*	271 $\pm$ 14*
MDA (nmol/g tissue)	11 $\pm$ 2	19 $\pm$ 3*,**	23 $\pm$ 9*	58 $\pm$ 4*,***	48 $\pm$ 1*	46 $\pm$ 1*

^a Values are mean $\pm$ SD ($n = 12$). Significant difference between groups were determined by analysis of variance followed by Student-Newman-Keuls test.

* $P < 0.001$ compared with normoxia group (0 hrs); ** $P < 0.05$ compared with hypoxia group (3 hrs); *** $P < 0.01$ compared with all other hypoxia groups (1.5, 3, 12, and 24 hrs).

Table 2. Effect of Hypobaric Hypoxia on GPx, GSH, and SOD Levels in Lungs of Rats Exposed to Simulated Hypobaric Hypoxia (7620 m) for 6 hrs at $25 \pm 1^\circ\text{C}^a$

Parameters	Hours of Hypoxic Exposure	
	0	6
GPx U/mg	0.12275 ± 0.02	$0.0802 \pm 0.015^*$
GSH $\mu\text{mol/gm}$	4.624 ± 1.67	6.365 ± 1.08
SOD U/mg	0.2788 ± 0.024	$0.37206 \pm 0.062^*$

^a Values are mean $\pm$ SD ($n = 12$).

* $P < 0.05$ compared with normoxia group (0 hrs).

Proinflammatory and Cell Adhesion Molecules. To confirm whether the enhanced levels of NFkB lead to upregulation of inflammatory molecules, which are known to be regulated by it, we measured the expressions of IL-1, IL-6, and TNF- α levels in the lungs of animals during hypoxic exposure by immunoblotting. In control animals, IL-1 was not detectable; however, its level was increased appreciably upon exposure to hypobaric hypoxia (Fig. 5a). A significant increase in IL-6 and TNF- α levels also were noticed in the lungs of hypoxic rats. The fold increase in IL-6 and TNF- α was 7 and 4.5 times, respectively, relative to control levels (Fig. 5b, c).

We also determined the expression profiles of cell adhesion molecules such as ICAM-I, VCAM-I and selectins (P-selectin) in the lungs of control and hypoxic rats. ICAM-I was virtually undetectable in the lung of control rats; however, upon exposure to hypoxia, a significant increase in

its level was observed (Fig. 6a). At the same time, VCAM-1 levels increased by about 3-fold in the lungs of rats exposed to hypoxia over control animals (Fig. 6b). Similarly, exposure of rats to hypoxia also resulted in to a significant increase in lung P-selectin levels although in control animals P-selectin was not detectable (Fig. 6c).

NFkB Blockade by Curcumin. To understand the involvement of NFkB in the development of pulmonary vascular leakage, the animals were administered curcumin (50 mg/kg BW) orally 1 hr before hypoxic exposure. Interestingly, a significant reduction in water content (45.6%) and transvascular leakage (60%) was noted in curcumin + hypoxia group when compared with the hypoxic group (Fig. 7a, b). However, the normoxia group administered with curcumin did not show any significant difference in vascular leakage compared with control. Similarly, as expected, curcumin administration markedly inhibited hypoxia-induced NFkB levels by about four times (Fig. 8a, b) compared with the hypoxic group.

Discussion

The present study was undertaken to determine the association of oxidative stress and activation of NFkB in causing vascular leakage during exposure to hypobaric hypoxia. An animal model (rat) was used, where rats were exposed to simulated hypobaric hypoxia for different durations, that is, 3 hrs, 6 hrs, 12 hrs, and 24 hrs at 7620 m at $25 \pm 1^\circ\text{C}$. The results showed that exposure of animals to hypoxia for 6 hrs led to increased vascular leakage as evidenced by increased water content and fluorescein

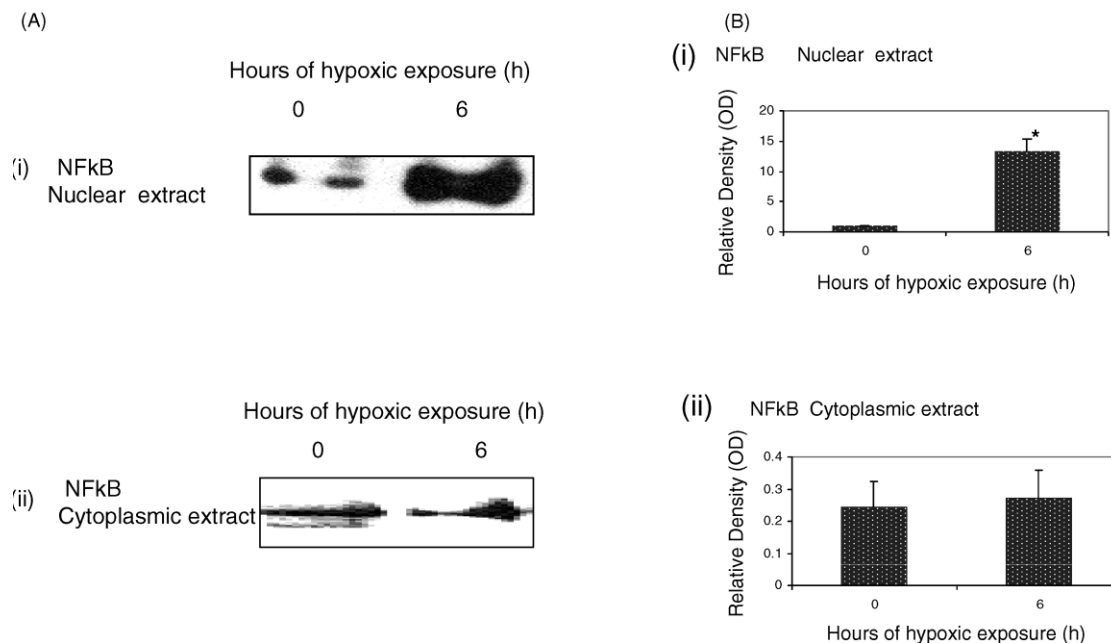


Figure 3. Expression of NFkB protein in lungs of rats exposed to hypobaric hypoxia (7620 m) for 6 hrs at $25 \pm 1^\circ\text{C}$ (A) shows Western blot analysis and (B) represents densitometry analysis in (i) nuclear and (ii) cytoplasmic fractions. Hypoxic exposure resulted in a 13-fold increase in NFkB levels in the lungs of rats compared with control, whereas no significant NFkB expression was observed in lung cytoplasmic extracts. Values are mean $\pm$ SD ($n = 12$). * $P < 0.05$ compared with normoxia group (0 hrs).

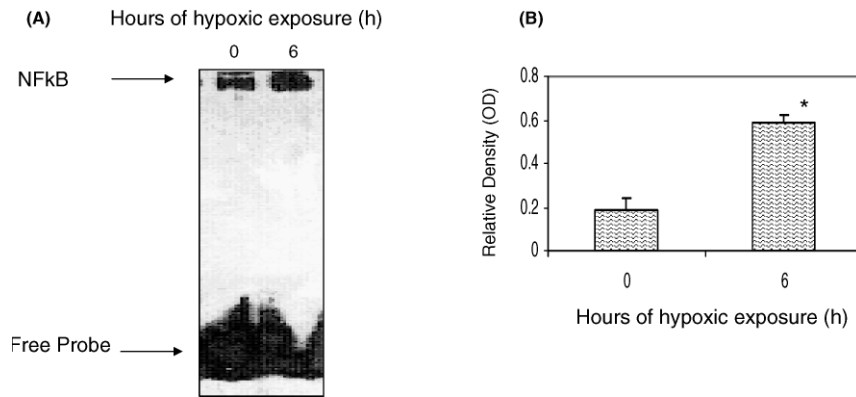


Figure 4. Expression of NFκB-DNA binding in the lungs of rats exposed to hypobaric hypoxia (7620 m) for 6 hrs at $25 \pm 1^\circ\text{C}$. (A) shows Western blot analysis and (B) represents densitometry analysis. Nuclear extracts were prepared and analyzed for NFκB binding to DNA by EMSA. The arrow indicates the position of NFκB and free probe. Hypoxic exposure for 6 hrs resulted in increased DNA binding activity of NFκB significantly over control. Values are mean $\pm$ SD ($n = 12$). * $P < 0.05$ compared with normoxia group (0 hrs).

leakage in lungs as compared with control animals. Further, there was a significant increase in ROS generation and lipid peroxidation, decreased antioxidative enzymes, enhanced expression of NFκB, and higher levels of proinflammatory cytokines and cell adhesion molecules in the lungs of rats exposed to hypoxia compared with normoxic animals. It was also observed that the curcumin administration 1 hr prior to hypoxic exposure significantly ($P < 0.01$) reduced the transvascular leakage in the lungs of rats by down-regulating the activation of NFκB compared with the hypoxia-exposed rats. In this limited study, we speculated

the possible role of oxidative stress and NFκB in causing the vascular leakage. Further, using the NFκB blocker curcumin, we showed the involvement of NFκB for the first time in the cause of pulmonary edema. These findings provide clues for developing new therapeutic drugs against HAPE. The schematic representation of hypoxia-induced transvascular leakage in the present study will be sequentially discussed in the ensuing paragraphs (Fig. 9).

HAPE is associated with an increase in leakage of both fluid and proteins in the alveolar space, with enhanced arterial pressure followed by pulmonary vasoconstriction. It

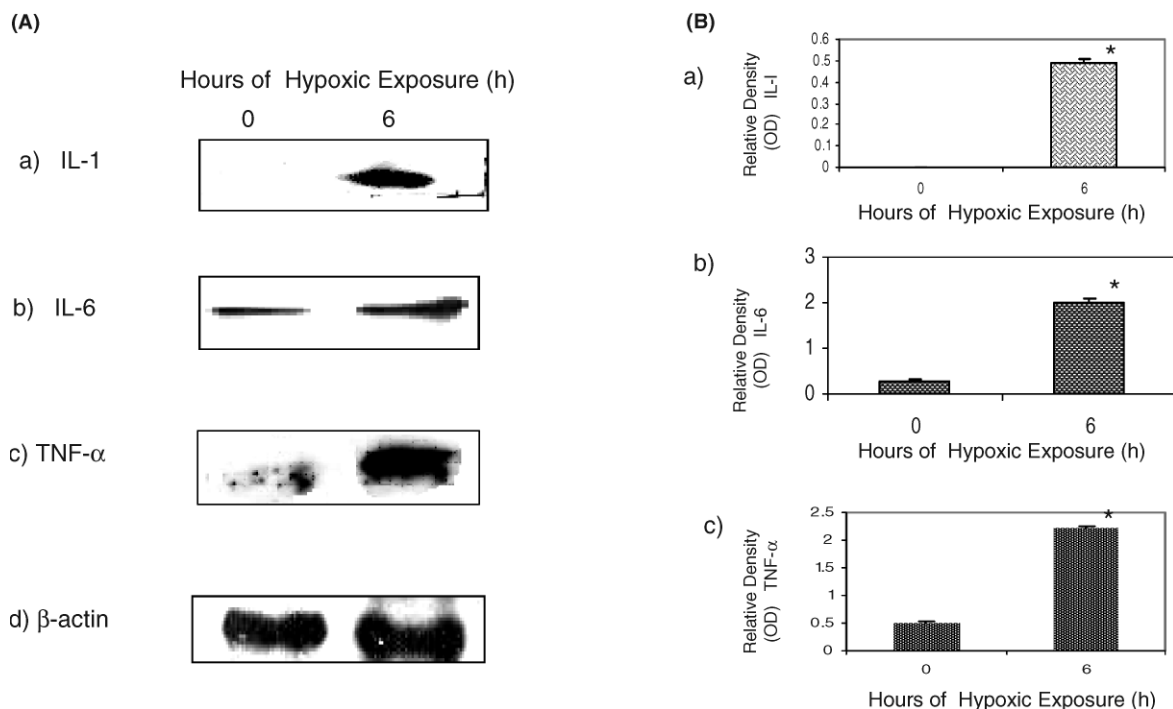


Figure 5. Expression of proinflammatory cytokines (a) IL-1, (b) IL-6, and (c) TNF-α in the lungs of rats exposed to hypobaric hypoxia (7620 m) for 6 hrs at $25 \pm 1^\circ\text{C}$. (A) shows Western blot analysis and (B) represents densitometry analysis. The hypoxic exposure resulted in enhanced expression of proinflammatory cytokines in lung homogenates of rats compared with control. Values are mean $\pm$ SD ($n = 12$). * $P < 0.05$ compared with normoxia group (0 hrs).

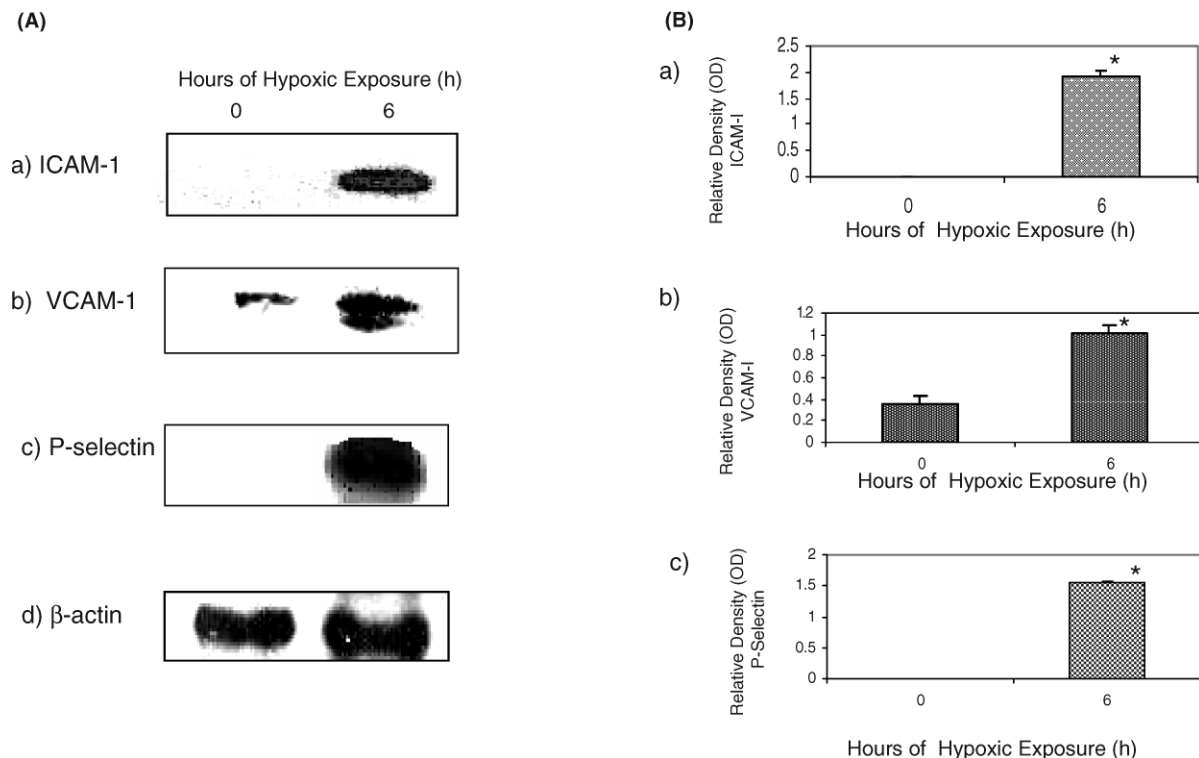


Figure 6. Expression of cell adhesion molecules (a) ICAM-I, (b) VCAM-I, and (c) P-selectin in the lungs of rats exposed to hypobaric hypoxia (7620 m) for 6 hrs at $25 \pm 1^\circ\text{C}$. (A) shows Western blot analysis and (B) represents densitometry analysis. The results suggested that hypoxia induced the upregulation of cell adhesion molecules. Values are mean $\pm$ SD ($n = 12$). * $P < 0.05$ compared with normoxia group (0 hrs).

is well known that HAPE occurs on ascent to altitudes above 2450 m (22) while its incidence decreases with lowering altitudes. In our studies, we have used rat as an animal model because of the following reasons: (1) the physiologic mechanisms of its acclimatization to HA are very much similar to that of humans (17); (2) smaller animals have higher capillary density and shorter diffusion distance compared with larger species (37); and (3) hypoxia can lead to pulmonary edema in some species (especially in the rat) as occurs in the development of HAPE in humans,

and hypoxia may also exaggerate the effects of a prior inflammatory insult into the lung (12, 25). Therefore, in the present study, we exposed the rats to a simulated altitude of 7620 m at $25 \pm 1^\circ\text{C}$ for different durations, that is, 3 hrs, 6 hrs, 12 hrs, and 24 hrs in order to find out the optimum time for appearance of vascular leakage in the lungs. We found that maximum lung wet/dry weight ratio was obtained in rats exposed to 6 hrs to 12 hrs duration. Further exposure resulted into a marginal decrease in wet/dry weight ratio. This could be attributed to considerable water loss caused

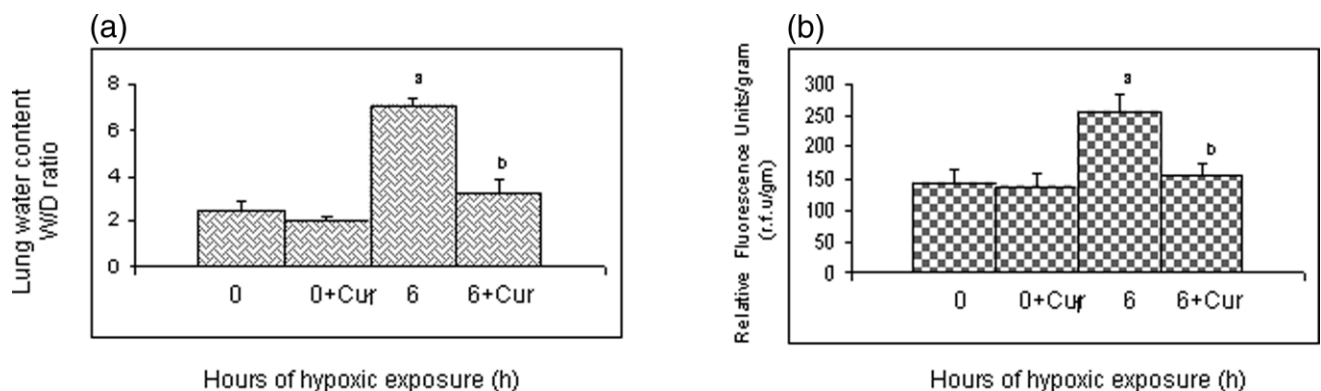


Figure 7. Determination of pulmonary edema in rats exposed to 6 hrs of hypobaric hypoxia and prevention by curcumin (a) depicts the lung water content and (b) represents transvascular leakage. The rats were exposed to simulated hypobaric hypoxia (7620 m) for 6 hrs at $25 \pm 1^\circ\text{C}$. Curcumin (50 mg/kg BW) was administered orally to rats 1 hr prior to exposure (6 hrs) to hypobaric hypoxia. Values are mean $\pm$ SD ($n = 12$). Significant difference between groups were determined by ANOVA followed by Student-Newman-Keuls test. ^a $P < 0.001$ compared with normoxia group (0 hrs). ^b $P < 0.01$ compared with hypoxia group (6 hrs). Cur = curcumin.

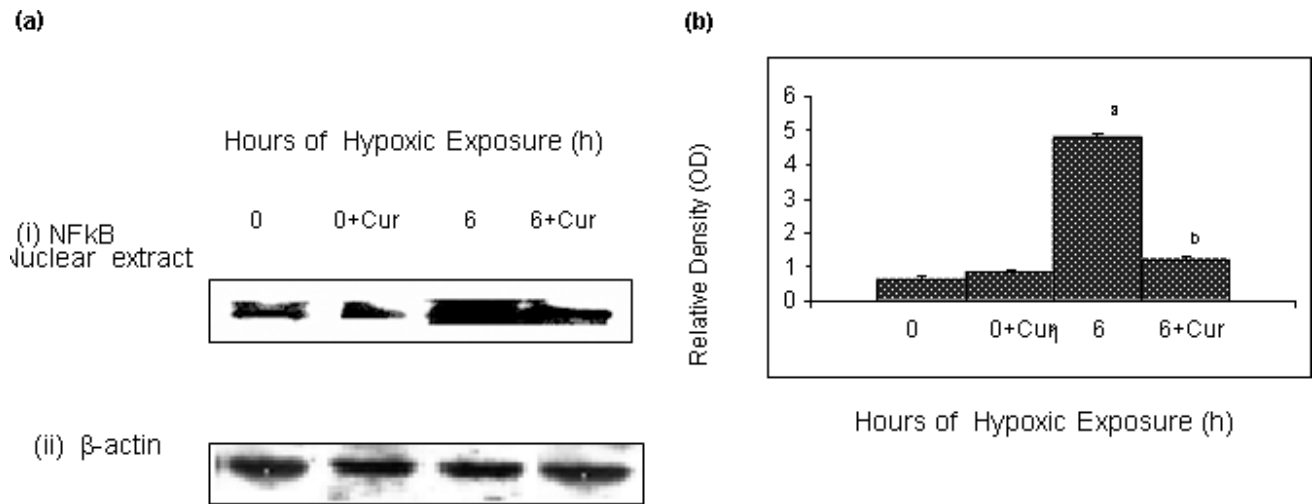


Figure 8. Effect of curcumin on NFκB protein expression in the lung nuclear fraction of rats exposed to hypoxia (7620 m) for 6 hrs at 25 ± 1°C. (a) shows Western blot analysis and (b) represents densitometry analysis. Curcumin (50 mg/kg BW) was administered orally to rats 1 hr prior to exposure (6 hrs) to hypobaric hypoxia. The results revealed that prior administration of curcumin to the rats exposed to hypoxia significantly downregulated the NFκB activity compared with hypoxia-exposed rats. Values are mean ± SD ($n = 12$). Significant differences between groups were determined by ANOVA followed by Student-Newman-Keuls test. ^a $P < 0.001$ compared with normoxia group (0 hrs). ^b $P < 0.01$ compared with hypoxia group (6 hrs). Cur = curcumin.

by severe hyperventilation during exposure to hypoxia. To confirm further, we measured the transvascular leakage (common cause of edema) by direct method using a fluorescent probe, sodium fluorescein. Sodium fluorescein is a fluorescence tracer that can be measured easily in lungs to exactly quantify dye extravasation and therefore vascular permeability. We found that the fluid accumulation, as revealed by increase in fluorescein content in the lungs of rats, began at 3 hrs of hypoxic exposure and reached the peak at 6 hrs of exposure. In this regard our results are in consistence with the earlier studies (9).

Because exposure of animals to hypobaric hypoxia for 6 hrs resulted in a maximum fluid leakage in lungs, we studied the role of oxidative stress, that is, ROS in the development of HAPE. The increased MDA levels ($P < 0.001$) in lung tissue indicated increased free-radical production. Hypoxia is known to increase plasma and tissue MDA levels at high altitudes and even in *in vitro* conditions (36, 47), and our results are in accordance with earlier studies (33). To cope with the oxidative stress, a marginal but insignificant increase in GSH levels was noticed in the the lungs of rats exposed to hypoxia as compared with control animals. Further, there was a significant increase in SOD levels in the lungs of rats exposed to hypoxia as compared with control animals. It is speculated that the increased SOD levels will attenuate the superoxide radicals (O_2^-), which are produced during exposure to hypoxia. Recently, it has been shown that ROS are involved and may even play a causative role in the AMS, HAPE, and HACE (4, 8). However, the mechanism through which ROS contribute to the pathophysiology of organ injury to the lung remains incompletely understood. It is not yet completely known whether pulmonary edema is an inflammatory disease or not. Studies on human subjects

showed elevated RBC counts and serum proteins in bronchioalveolar lavage (BAL) fluid of HAPE subjects within a day of ascent to 4559 m. However, there was no significant difference in proinflammatory mediators, that is, IL-1, IL-6, TNF- α , IL-8, thromboxane, PEG2, and leukotriene B4 in the BAL fluid of HAPE subjects (40). The main drawback of this study is that the levels of inflammatory cytokines were measured in subjects at the beginning and at end of the study, but no afford was made to measure these cytokines before the onset of HAPE. Therefore, it is possible that these inflammatory cytokines would have disappeared after causing the inflammation. This would possibly explain the fact that production of inflammatory mediators might precede the onset of pulmonary edema. Interestingly, in the present study, we found that NFκB levels are elevated at 6 hrs of hypoxic exposure (starting of increased vascular leakage). Because NFκB directly regulates proinflammatory cytokine production, we speculate that production of inflammatory cytokines also precedes the vascular leakage, and once their action is over, they disappear. However, studies on BAL fluid of mountaineers with advanced HAPE at Mount McKinley (38) and also hospitalized HAPE patients in Japan (26) showed elevated levels of cytokines and increased granulocytes. This shows that in advanced cases of HAPE, inflammation may occur and contribute to enhanced pulmonary capillary permeability. Earlier, Ono *et al.* (35) showed that the rats primed with endotoxin developed pulmonary edema. Further, the BAL fluid of endotoxin-primed and hypoxia-exposed rats contained a greater number of white blood cells and a higher concentration of proteins than that of endotoxin-primed normoxic rats. This indicates the involvement of inflammatory components in the occurrence of pulmonary edema. The present study is focused mainly on the role of oxidative

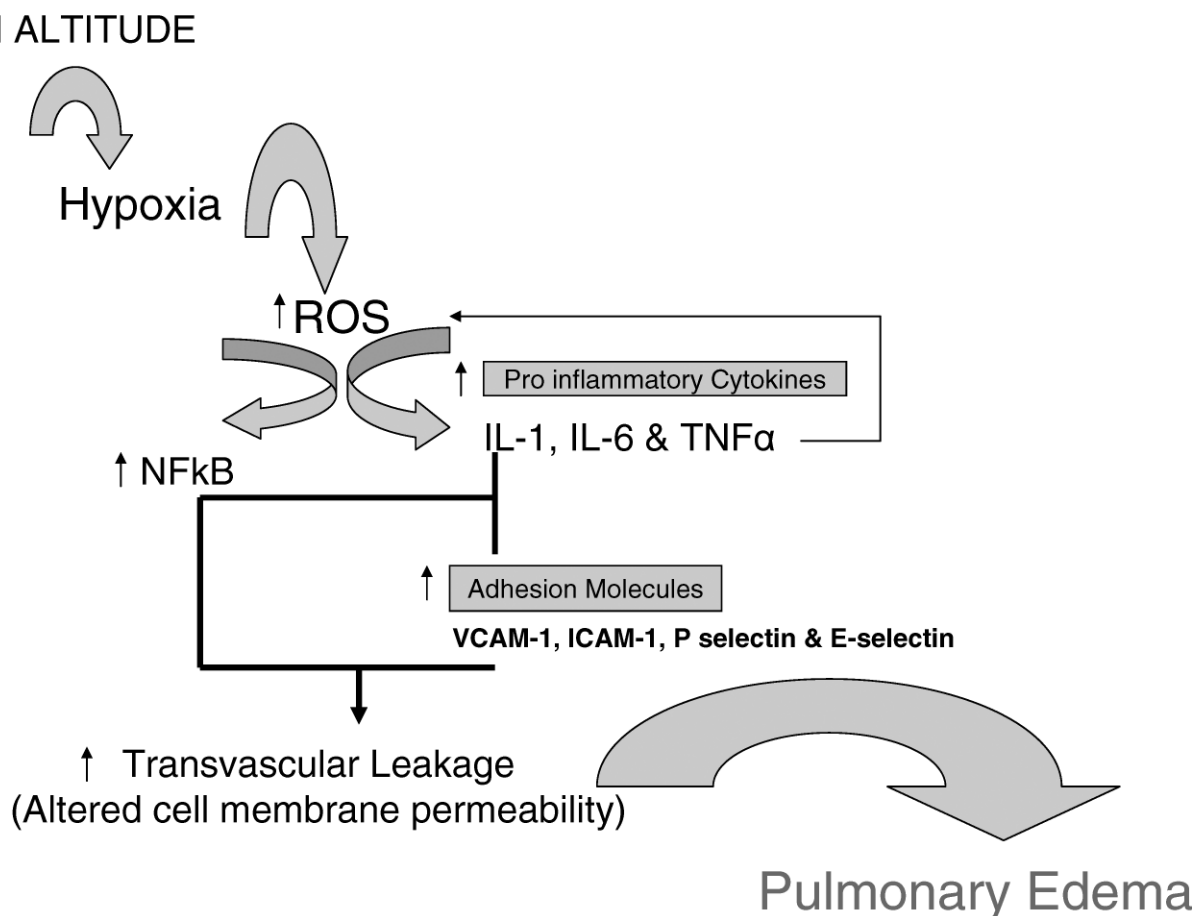


Figure 9. Schematic representation of possible mechanism of high-altitude-induced inflammation causing transvascular leakage. Under hypoxic conditions, increased production of ROS (reactive oxygen species) overwhelms the oxidative stress. The increased oxidative stress in turn upregulates the NFkB, which plays a pivotal role in the regulation of many genes involved in the inflammatory response. The activated NFkB further upregulates the proinflammatory cytokines (IL-1, IL-6, and TNF- α) and cell adhesion molecules (ICAM-1, VCAM-1, and P-selectin), leading to increased transvascular leakage in the lungs of rats.

stress (hypoxia) driven–inflammation mediated by NFkB in causing vascular leakage in the lungs.

It is reported recently that oxidant stress increases the vascular endothelial permeability and expression of redox-sensitive transcription factor NFkB (29). In the present study too, we found an appreciable (nearly 13-fold) increase in NFkB levels in the nuclear extract of the lungs of animals exposed to hypoxia over control animals. NFkB is normally found in its inactive form in the cytosol as the heterodimer p50/P65 unbound to its inhibitory unit I κ B α . Upon appropriate cell stimulation, I κ B α and I κ B β are rapidly phosphorylated on specific amino-terminal serine, signaling for ubiquitination and degradation by the 26 S proteasome. This results in the exposure of a nuclear localization sequence and DNA-binding domains, allowing the NFkB to enter the nucleus and stimulate the transcription of target genes. The DNA-binding studies in the present study have shown an increased NFkB-binding (nearly 3-fold) activity in the lungs of hypoxia-exposed rats compared with control. NFkB and I κ B α are present in the nucleus in low concentrations even in nonactivated cells, and the signaling molecules of NFkB shuttle between the cytoplasm and the

nucleus. This could be the reason for the presence of NFkB in the cytoplasmic fraction of the lung in the present study. To the best of our knowledge, we report for the first time the involvement of NFkB in the development of pulmonary vascular leakage.

Earlier it was reported that altered NFkB can mediate an inflammatory cytokine response and also induce gene transcription of adhesion molecules, that is, ICAM-1, VCAM-1, etc. (21). Therefore, we sought to determine whether increased NFkB levels lead to enhanced inflammatory cytokines. Interestingly, we found a significant increase in IL-1 and IL-6 levels in the lungs of rats exposed to hypoxia over control animals. Further, there was a significant increase in TNF- α levels during hypoxia exposure. TNF- α is a polypeptide that influences endothelial cell function by promoting neutrophil adherence to vascular endothelium (20). TNF- α has been shown to inhibit the release of endothelium-derived relaxing factor (EDRF) in isolated carotid artery rings, and therefore causes endothelial dysfunction besides increasing reactive oxygen species levels (15). Deruelle *et al.* (18) showed that the increased level of TNF- α is responsible for development of right

ventricular hypertrophy (RVH) in rats exposed to 10% oxygen for 3 weeks.

The cytokines such as TNF- α and IL-1 cause the appearance of ICAM-I and VCAM-I and the selectins such as P-selectin and E-selectin in the capillary endothelial cells and permit lymphocytes and monocytes to adhere and move into inflamed tissues. In the present study, there was a concomitant upregulation of cell adhesion molecules such as ICAM-I, VCAM-I, and P-selectin besides proinflammatory cytokines in the lungs of rats exposed to the hypoxia over control animals. It has been shown earlier that O₂ deprivation (a common cause of hypoxia) leads to upregulation of genes involved in inflammation. Carol and Brain (10) reported that the cell adhesion molecules like ICAM-I and VCAM-I genes contain NFκB site on the promoter region and are known to be regulated by NFκB. We therefore hypothesize that the observed increase in these proinflammatory and cell adhesion molecules could be attributed to enhanced NFκB levels in the lungs of hypoxic rats.

Recent reports revealed that curcumin, a yellow pigment and a major component of turmeric (*Curcumin longa* L.), has the ability to downregulate the NFκB activation, which has been linked to a number of inflammatory diseases (1, 14, 45). Therefore, NFκB blockade studies were carried out to know whether this strategy would inhibit hypoxia-induced fluid accumulation in the lungs. It was evident from our present data that curcumin administration significantly attenuated vascular leakage in the lungs of rats exposed to hypobaric hypoxia and maintained their values similar to that of normoxic animals. Further, curcumin significantly inhibited hypoxia-induced NFκB expression and maintained its levels similar to that of control levels. Curcumin from *C. longa* has been demonstrated as an anti-inflammatory agent in *in vivo* animal models (2). Curcumin is reported to scavenge free radicals, inhibit lipid peroxidation, protect the SH group of GSH, and activate glutathione-S-transferase as well as inhibit nitrite radical-induced oxidation of hemoglobin and prostaglandin biosynthesis (32, 43). However, recent studies revealed that dexamethasone—a corticosteroid recommended as prophylactic drug to prevent AMS and HACE—has been shown to inhibit NFκB (11) and leads to a reduction in systemic pulmonary artery pressure in HAPE subjects (30, 39). Further, nifedipine is nowadays recommended as prophylactic drug of choice against HAPE if progressive high-altitude acclimatization is not possible (31). Nifedipine is effective in inhibiting NFκB activation and thereby contributing to decreased inflammation followed by increased endothelial function in the coronary circulation (41). However, in the present study, the ability of curcumin to prevent hypoxia-induced vascular permeability is not linked to its antioxidant activity, as administration of antioxidants such as vitamin C or vitamin E have little effect on hypoxia-induced vascular leakage in lungs (data not shown). Further in the present study, there is no linear relationship between ROS generation and vascular leakage. ROS continued to

accumulate even after 24 hrs while maximum lung water content was observed between 6 hrs to 12 hrs. However, increased NFκB activity was directly associated with pulmonary edema. This shows that ROS are not solely responsible for occurrence of vascular leakage although they might be contributing to occurrence of vascular leakage, probably through upregulation of NFκB. This also possibly explains the inability of antioxidant vitamins to prevent hypoxia-induced pulmonary vascular leakage. Therefore, the observed reduction in fluid efflux in our study might be caused by the anti-inflammatory activity of curcumin. We therefore hypothesize that exposure of animals to hypoxia activates NFκB, which in turn results in upregulation of inflammatory mediators, finally leading to pulmonary vascular leakage. Further research, including a generation of overexpression/knockout studies of NFκB and microarray analysis of rat lungs, will help us to unravel the exact molecular mechanism involved in hypoxia-induced transvascular leakage.

Conclusion

Taken together, our results revealed that exposure of rats to hypoxia increased the oxidative stress in the animals as evidenced by enhanced ROS, increased MDA levels, and marginal elevation in endogenous antioxidants such as SOD and GSH levels in the lungs of rats. This increased oxidative stress might have activated NFκB and its translocation into the nucleus, leading to upregulation of the proinflammatory cytokines (IL-1, IL-6, and TNF- α) and cell adhesion molecules (ICAM-1, VCAM-I, and P-selectin). This in turn might be responsible for causing vascular leakage in rats exposed to hypoxia. Administration of curcumin significantly inhibited hypoxia-induced vascular leakage and NFκB levels in lungs. These findings, therefore, suggest that oxidative stress-driven increases in lung NFκB content can contribute to the formation of pulmonary edema (seen in this model). Because NFκB plays a significant role in inflammation, the present study opens a new area for developing better therapeutic strategies for the prevention of HAPE.

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