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Attenuation of NFkB Activation Augments Alveolar Transport Proteins Expression and Activity under Hypoxia

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Abstract: ***Epithelial sodium channels (ENaCs) controls vectorial Na^+ across epithelia thus playing a central role in all aspects of fluid clearance in the body. The aim of this study was to investigate in vitro time dependant model of hypoxia induced reduction in vectorial Na^+ and fluid transport across the alveolar epithelium. AEC exposed to 3% O_2, for different time periods (1 h, 3h, 6h, 12h, 24h and 48 h) showed increased oxidative stress (↑ROS,↑MDA) and NF-κB up-regulation. Hypoxia induced a time-dependent decrease in Na^+/K^+-ATPase activity and α_1 Na^+/K^+-ATPase protein expression reaching to negligible levels after 6h of exposure. Intriguingly, hypoxia also decreased copy number of ENaC α-subunit in AEC beyond detectable levels after 12h. Curcumin reinstate survival signalling by blunting NFkB signalling and reinforced the levels of alveolar antioxidants along with significant ($p<0.001$) increase in ENaC and Na^+/K^+-ATPase expression in lungs of rats under hypoxia. Therefore, we propose that, curcumin prophylaxis abate NFkB aberrant signalling induced down regulation of Na^+/K^+-ATPase and ENaC under hypoxia.***

Keywords: NF-κB; Na^+/K^+-ATPase; ENaC; alveolar epithelium; curcumin; hypoxia

1. Introduction

Active transport of Na^+ across alveolar epithelial cells play an important role in the regulation of the volume of alveolar lining fluid and in the reabsorption of edema fluid (Sartori et al. 2010) in pathological conditions like high altitude pulmonary edema (HAPE). Alveolar fluid reabsorption is chiefly attributed to active sodium ion (Na^+) influx via apically located epithelial sodium channel (ENaC) and is subsequently "pumped" out of the cells by the basolaterally located sodium-potassium adenosine triphosphatase (Na^+/K^+-ATPase) (Monika et al. 2011). One of the reasons speculated for alveolar hypoxia induced pulmonary edema is mainly by inhibiting proteins involved in this alveolar fluid transport (Mairbäurl et al. 1997; Verghese et al. 1999; Wodopia et al. 2000). Numerous studies have revealed that internalisation of transporters also serve to contribute to the decreased sodium channel activity in hypoxia exposed alveolar epithelial cells (Dada et al., 2003, Planes et al., 1995; Sartori et al., 2002). Earlier studies by Stelzner et al. (1988) proved that glucocorticoids placement in adrenalectamized rats reversed the augmented protein leak, suggesting important physiological role of both exogenous and endogenous glucocorticoids on vascular permeability under hypoxia support this notion. Following these studies, Lazrak et al. (2000) further proved that glucocorticoids might help in synthesizing the new ion transporters in alveolar epithelial cells. To add these findings Dagenias et al (2001) reported an increase in mRNA expressions of α ENaC on dexmethasone treatment in primary alveolar type II (ATII) cells isolated from adult rat. All these reports clearly reveal that, drugs like dexamethasone and salbutamol (β-adrenergic agonists) are known to blunt the hypoxic inhibition of alveolar Na^+ transport and fluid reabsorption by increasing expression and activity of Na^+ transporters in alveolar epithelial cells and therefore prevents the fluid efflux in the lungs. (Maggirioni 2006) *in vitro* and *in vivo* (Mairbäurl et al. 2003; Urner et al. 2012).Further Nifedipine (Ca^{2+} channel blocker) is nowadays recommended as prophylactic drug of choice against HAPE if progressive high altitude acclimatisation is not possible (Maggirini 2006). Nifedipine effectively inhibits NF-κB activation there by contributing to decreased inflammation followed by enhanced endothelial function in coronary circulation (Takase et al 2005) leading to reduction in transvascular leakage in lungs (Sarada et al., 2012). In addition to these, drugs like acetazolamide and phosphodiesterease inhibitor (sildenafil and tadalafil) promote pulmonary vasodilation (Swenson et al., 2006; Maggiornirni 2006) and reduce inflammation (Roumeguere et al., 2010; Sawtzley et al., 2005). It is well known that in individuals with a history of HAPE pharmacological prophylaxis is prudent. But these drugs have various side effects like nausea, headache, psychiatric or psychological problems immune suppression etc**.** (Mokra et al. 2000; Stuart 2005; Downs et al. 2012; Rassler 2012; Bassfordet al. 2012). Currently researchers are focusing attention on phytochemical drugs to replace these existing drugs.

Curcumin is an active component of turmeric (*Curcumin longa* Linn), used as a spice and recommended in Ayurvedic medicine for centuries on the Indian subcontinent. Turmeric contains curcumin along with other chemical constituents known as the "curcuminoids" (Srinivasan 1952). The antioxidant and the anti-inflammatory effect of curcumin are well established via the suppression of NF-κB. NF-κB is a nuclear transcription factor required for the expression of genes involved in cell proliferation, cell invasion, metastasis, angiogenesis, and resistance to chemotherapy. Several studies have shown that NFkB is activated under hypoxia which in turn elicits the inflammatory responses (Loercher et al. 2004; Sarada et al. 2008; Culver et al. 2010; Himadri et al. 2010; Melvin et al. 2011) leading to increased transvascular leakage. However, to our knowledge no report is available addressing the prophylactic effect of curcumin on the alveolar epithelial ion channels. Therefore, the objectives in this study were to determine 1) time dependent effect of hypoxia on alveolar fluid transport proteins 2) the

molecular mechanisms responsible for decrease in alveolar ion transporters and/or channels, and 3) the modulatory effect of curcumin on expression of alveolar fluid transport proteins in A549 cells and in lungs of rats exposed to hypoxia

2. Materials and Methods

2.1 Reagents

Reagents for cell culture media like Dulbecco's modified Eagle's medium F-12 (DMEM F-12), trypsin- EDTA, penicillin, streptomycin, foetal bovine serum (FBS) and curcumin powder were purchased from Sigma Life Technologies (St. Louis, MO USA).

2.2. Invitro and Invivo studies

The A549 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM F-12) supplemented with 10% fetal bovine serum, 100 units of penicillin, and 50µg/ml streptomycin and maintained at 37° C, 5% CO_2, 21% O_2.Experiments were carried out on male Sprague Dawley rats weighing 150-200 gm body weight (BW). Rats were maintained at 25±1˚C with day and night cycles of 12 h each and given food and water ad libitum. The institute ethics committee approved all the experimental protocols for this study and followed the guidelines of Universities of Federation for Animal Welfare (UFAW) for animal research.

2.3. Preparation of curcumin:

The drug curcumin was diluted in a vehicle (DMSO-0.5%). A549 cells were treated with varying concentration of curcumin (2.5, 5, 10, 20, 50 and 100µM) prior to hypoxia exposure. Similarly, freshly prepared curcumin (50mg/kg BW) was administered orally to rats 1h prior to hypoxic exposure.

2.4. In-vitro studies.

Cells were plated at a density of $0.5x10^5$ cells per well in 12 well plates and let adhered over 12 h under normal conditions and/or cultured under hypoxic conditions (3% O_2– 5% CO_2– 92% N_2. Hypoxic conditions were achieved in a humidified variable aerobic incubator (Galaxy 170R, New Brunswick Scientific, CT, USA) for different durations *viz.* 1h, 3h, 6h, 12h and 48h. The time dependent studies of hypoxia exposure revealed that, 6h at 3% of hypoxia showed approximately 50 % reduction in cell viability and significant ($p<0.001$) reduction in alveolar transport proteins expression as wellcompared to control cells. Hence, rest of the experiments were carried out till 6h of hypoxia exposure. Further cells were pre-incubated with 2.5µM, 5µM, 10µM, 25µM, 50µM and 100µM curcumin for different time durations viz. 30, 60, 90 and 180 minutes prior to hypoxia exposure and then exposed to hypoxia (6h).10 µM curcumin dose was found to be suitable dose. Here after curcumin in vitro dose refers to this dose.

2.5. *In-vivo* studies: Exposure to hypobaric hypoxia

The rats were exposed to simulated altitude of 7620m in a hypobaric chamber (Decibel Instruments, India) for 6h. The reason for exposing the rats to hypobaric hypoxia for 6 h is based on our previous studies (Sarada et al., 2008). Curcumin was administered at (50mg/Kg BW) standradised based on our previous studies (Sarada et al., 2014).

2.6. Analysis of Cell Viability:

The cell viability was assayed by the method of Repetto et al. (2008). To further strengthen our results cell viability was also quantitatively assessed by lactate dehydrogenase (LDH) assay using Randox kit as per manufacture's instruction.

2.7. Determination of Oxidative Stress Parameters:

Reactive oxygen species (ROS) generated in control and hypoxia exposed cells was estimated flowcytomertically by the method of Le Bel et al. (1990) using fluorescent probe 2',7'-dichlorfluorescein-diacetate (*DCFH-DA)* with aid of BD FACS Aria (BD bioscience San Jose, California, USA) and analysed using BD FACSD v6 software. The lipid peroxidation occurred in line with ROS production in cells was estimated by measuring malondialdehyde (MDA) level in normoxia and hypoxia exposed cells as mentioned earlier (Okhawa et al. 1979). The reduced glutathione (GSH) levels in control and hypoxia exposed cells were determined as per Kum-Talt and Tan (1974) method. The anti oxidant enzymes such as glutathione peroxidase (GPx) and superoxide dismutase (SOD) were determined using RANSEL and RANSOD (Randox Laboratories, Antrim, United Kingdom) kits respectively as per manufacture's instructions.

2.8. Preparation of Cell Membrane:

Rat lung homogenates and A549 cells lysate was taken and from which the Cell membrane was separated as described earlier (Wodopia et al. 2000). The protein concentration in cell membrane and whole cell lysates were measured by Lowrey et al. (1951).

2.9. Determination of Na^+ K^+ ATPase activity.

The ATPase activity of the basolateral membranes were measured using the method of Fritz and Hamrick (1966) as modified by Desaiah and Ho (1979) and end point phosphate analysis was done by the method of Fiske and Subbarow (1921). The enzyme activity was expressed as µMPi/mg protein/h in A549/lung membrane preparation.

2.10. Western Blot analysis

Cells in 6 well or 24 well plates were washed twice and scraped off the filters in ice-cold PBS, then centrifuged at 1500 rpm for 10 min at 4 °C. The pellet was resuspended in 300 µl of ice-cold lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.6), 1% Triton X-100, 0.1% SDS, and protease inhibitors and kept on ice for 1h. The cell lysates were then centrifuged (8,000 rpm, 15 m) at 4 °C. Both cytosolic and nuclear fractions were separated and stored at -80°C for further analysis. The proteins were resolved

through 8%, 10% and 12% acrylamide gels, electro blotted (Bio Rad, USA), to nitrocellulose paper (Millipore, USA). For α_1- Na^+/K^+-ATPase and α-ENaC (Santa Cruz Biotechnology, Santa Cruz, USA) analysis, the cell membrane fractions were used. Nuclear fraction and cytoplasmic extracts were used for transcription factor NF-κB (mouse monoclonal anti-p65) and TNF α expression analysis respectively (Santa Cruz Biotechnology) by western blot**.** The membranes were washed and probed with their respective secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, USA). Finally, the membranes were developed using kit Chemiluminescence substrate (Sigma MO, USA) and bands were visualized on X-ray film (Fuji Films, India). Densitometric analysis was carried out by using Gel Doc System (Alpha Innotech CA, USA)

2.11. Electrophoretic mobility shift assay (EMSA)

The EMSA for NF-κB carried out using a commercial kit as per manufactures instruction (Pierce,USA).

2.12 mRNA expression studies (RT-PCR)

The total cellular RNA was extracted from 1 g weight of lung tissue using Trizol reagent following the manufacturer's recommended protocol. The concentration of RNA was measured by absorbance at 260 nm. Reverse transcription was performed with Invitrogen c DNA synthesis kit as per manufactures instruction (Invitrogen, Singapore). The PCR products were separated by electrophoresis loading onto a 1.2% agarose gel. After ethidium bromide staining, all DNA bands were photographed using Gel Doc System (Alpha Innotech CA, USA).

αENaC	5'TCCCAGGATTGGATCTTCGAG3' 5'ACCACAGAGAGCACGGACGA3'	209bp	NM031548
α_1Na+/K+ ATPase	5'AACCTACTACTAGCCCACTGC3' 5'CTTTGGTGCTTTCCTACCAGG3'	129bp	NM 012504
β-Actin	5'GAGGATATCGCTGCGCTGG-3' 5'ATCTTTTCACGGTTGGCCT3'	350bp	Gen bank V01217

2.13 Statistical Analysis

Statistical analysis was performed using SPSS for windows (15.0) software (SPSS Inc., Chicago, IL). Comparison between experimental groups and curcumin treated groups were made by using two way ANOVA with student Newman-Kluls test for comparison between groups. Differences were considered statistically significant for $P<0.05$. Results are expressed as mean ± SD.

3. Results

3.1. Hypoxia decreases cell viability in A549 cells by disrupting oxidant to antioxidant balance

Hypoxia induced time dependent decrease in cell viability (Fig. 1 a) with increased intracellular ROS production (Fig. 1c) under hypoxia. A gradual and significant increase in LDH (Fig. 1b) levels were observed in cells exposed to different time points tested ($P<0.05$) as compared to control.

3.2. Hypoxia blunts alveolar ion transport proteins expressions and activity

Hypoxia inhibited the activity and expression of the Na^+/K^+-ATPase in A549 cells in a time-dependent manner. The inhibition increased with time of exposure (as early as 1h) reached a maximum at 6 h (~ 6% of control; $P<0.001$) and virtually no activity was detected upon further hypoxic exposures (Fig. 2a and 2b). We have correlated this activity with expression pattern of α- ENaC proteins at apical surface of the cell membrane of A549 cells (Fig. 2c).

3.2.1. Hypoxia induced alveolar transport inhibition is mediated by NF-κB:

Western blot analysis revealed a rapid and robust increase in NF-κB following 1h of hypoxia treatment (Fig 2e) which remained more or less constant as the hypoxic durations increased. These levels of induction were comparable to those observed with TNF-α (Fig 2d).

3.3. Curcumin rescued AEC cells and rat lung tissue by blunting NF-κB thereby restoring Na^+/K^+-ATPase and ENaC under hypoxic stress.

A concentration of 10 μM of curcumin was found to be ideal among the concentrations tested as it has given more than 60 % cell viability in A549 cells under hypoxia with 60 minutes preconditioning with curcumin (Fig. 3a). The hypoxic induced depletion in antioxidant levels (GSH, GPx and SOD) were successfully restored by curcumin treatment as compared to hypoxic cells (Table 1).

Curcumin treatment prior to hypoxia exposure in A549 cells attenuated NF-κB activation (Fig 3b and c) in A549 cells. We further estimated whether translocation of NF-κB into nucleus has triggered an inflammatory milieu under hypoxia? Upon further investigation it was found that TNF α levels in hypoxia exposed cells and lung tissues were elevated by significantly curcumin treatment compared to control (Fig 3 d).

3.5.3. Curcumin restored the hypoxia impaired alveolar ion transport proteins both *in-vitro*and *in-vivo*

Curcumin treated hypoxia exposed A549 cells and male SD rats were able to maintain the Na^+/K^+-ATPase activity significantly at higher levels ($p<0.001$) as compared to control (hypoxia, Fig 4 a and b). Similar trend was observed in α ENaC and α_1Na^+/K^+-ATPase protein expressions when subjected to western blot analysis (Fig.4 c and d). We further examined the gene expression(Fig 4 e and f) pattern of the alveolar transport proteins in lungs of rats exposed to hypoxia.

4. Discussion

High altitude exposure leads to altered activity of reactive oxygen species, which in turn leads to oxidative damage. ROS are formed during stress conditions in the human body. When an extremely low availability of oxygen occurs, such as during ischemia or exposure to very low oxygen pressure, reactive oxygen species can exceed the capability of the defense mechanism, causing oxidative damage to lipids, proteins and to DNA (Karakucuk 2011). We observed hypoxia increased cell death which was confirmed by neutral inclusion and LDH assays (Fig 1 and 2). The impact of hypoxia on initiation of cell death *versus* cell survival is matter of debate still as researchers have noted that A549 cells are relatively tolerant to hypoxia (Clerici and Plane`s 2009, Vivona et al 2000, Wodopia et al 2001). However, in our current study we observed a decreased cell viability under hypoxia which could be possibility due to (a) the cytotoxic effect of hypoxia is dependent on the concentration of oxygen and time duration of exposure (Singh et al 2013), for instance Santore et al (2002) report that there is no increase in cell death at 15% at 24h in A549 whereas, Dada et al (2003) noted that there were no differences in cell viability or in ATP levels in A549 cells exposed to 1.5% O2 for 1h as compared with cells incubated at 21% O_2 (b) cancerous cells have stabilised HIF-1α; activates HIF-1, thereby protects cells under hypoxia however cancerous cells upon further exposure to moderate or severe hypoxia causes further inhibition of hydroxylases, modulate inflammation via key posttranslational modifications in the IL-1β pathway (Scholz et al. 2013). This stimulated inflammatory mediators could be possible reason for the rise in cell death observed in current study. We observed rise in NF-kB levels consecutively an increased TNF-α levels (Fig 4 b) in A549 cells which suggest possible role played by the inflammatory mediators.

Curcumin treatment protected A549 cells under hypoxia against GSH depletion (Table 1) suggesting that curcumin either scavenges the oxidant species responsible for GSH consumption or increases the biosynthesis of GSH. This phenomena could be achieved by peculiar chemical structure of curcumin i.e. curcumin have two o-methoxy phenolic OH groups attached to the β-diketone moiety having methylene CH_2 group. It is believed that the H abstractions from these groups are responsible for the remarkable antioxidant activity of curcumin. The free radical scavenging activity of curcumin can arise by the resonance stabilization of its radicals from two phenolic OH groups (mainly) or from the CH2 group of the β -diketone moiety (Mathews et al., 2012). Therefore, it seems likely that curcumin may be involved in scavenging of free radicals to protect the cells against the fall in GSH levels (Biswas et al., 2005). Increased in GSH levels under curcumin supplementation could have facilitated maintenance of GPx and SOD levels upon hypoxia exposure (Tale 2) leading to increased cellular tolerance to stress. Curcumin confers cellular antioxidant activity by maintaining antioxidant/oxidant balance.

TNF-α reduce alveolar epithelial sodium and chloride and the associated fluid transport and can hence contribute to pulmonary edema accumulation (Eisenhut 2006). TNF-α was found to reduce epithelial sodium channel (ENaC) expression in alveolar type II cells (Dagenais et al., 2004). This indicates that, the oxidative stress–induced NF-κB regulates key ion channels in alveolar epithelium under hypoxia.

AEC tolerate hypoxia by regulating ATP consumption. This adaptation occurs through down regulation of proteins involved in processes that consume ATP, such as Na+/K+-ATPase which accounts for 30% of cellular ATP consumption (Celrici and Planes 2009; Comellas et al., 2006). In order to maintain ambient level of cellular ATP the system promotes endocytosis of Na^+/K^+-ATPase from the basal membrane in event of inadequate oxygen supply. This might be the reason for reduction in copy number of Na^+/K^+-ATPase and activity in AEC exposed to hypoxia. Hummler et al (1996) demonstrated αENaC-deficient mice died within few minutes of birth due to defective neonatal lung liquid clearance. Hence it is clear that the maintenance of both ion transport channels is of equal importance for alveolar fluid clearance.

Our results showed an interesting phenomenon that, αENaC expression in A549 cells was sustained up to 12 h of hypoxia exposure, whereas, basolateral α_1 Na^+/K^+-ATPase expression and activity dropped below detectable levels within 6h of hypoxia exposure. In general the Na^+ enters through ENaC, in to the alveoli, then the basolaterally located Na^+/K^+ ATPase mediates the removal of Na^+ that entered the cells across the alveolar interface. Therefore, inhibiting the Na^+/K^+ pump without reducing the apical Na^+ entry would cause the alveolar epithelial cells to swell and lyse. This could be the reason in the present study that, the ENaC expressions at apical region of the A549 cells were functional up to 12h of hypoxic exposure indicating that Na^+ are getting accumulated up to this time of exposure. But $\alpha_1 Na^+/K^+$-ATPase stopped functioning from 6h of hypoxic exposure. Our results clearly indicate that $\alpha_1 Na^+/K^+$-ATPase activity stopped before ENaC becomes non-functional. In our previous study we showed the maximum transvacular leakage in lungs of rats exposed to hypoxia was at 6h (Sarada et al., 2008). Therefore, these results point out to hypothesize that the activity of α_1 Na^+/K^+-ATPase is the critical factor in pulmonary edema formation. Moreover, recent study showed possible role of NF-κB and ROS lead to decrease transport protein expression levels during hypoxia and inflammation (Matthay et al. 2002). Specifically, NF-κB up-regulation is an inflammatory stimulus that can disrupt cell polarity, thereby decreasing the number of transport proteins.

At the molecular levelcurcumin down-regulate NF-κB, maintain oxidant/antioxidant balance hence preventing inflammation and rescuing the expression of ion channels involved in alveolar fluid clearance. Thus explaining the vital role played by NF-κB in ENaC and Na^+/K^+-ATPase endocytosis in hypoxia. The RT-PCR data revealed the decrease in copy numbers of alveolar transport proteins is in turn due to the regulated gene expression. The data presented in this study pursue us to believe that this regulated gene expression is mediated by the up regulated NFkB levels. But how NFkB regulates the $\alpha_1 Na^+/K^+$-ATPase expression at gene level warrants further investigation? Here, we present for the first time in-vivo and

in-vitro evidence that, curcumin plays a protective role against hypoxia induced impairment of cation transport in A549 cells and in lungs of rats. Hypoxia causes inhibition of fluid clearance before the fluid accumulation in the lungs. Perhaps this would result into accumulation of fluid in to the lungs leading to pulmonary edema. These results further explain that, both the channels work independent to each other but together contribute in causing pulmonary edema.

5. Conclusion

The current study revealed that hypoxia inhibited Na^+/K^+-ATPase function, decreased the copy number of transporter proteins in the cell membrane. This process is mediated by the increased generation of ROS followed by increased lipid peroxidation. Hence the increased oxidative stress further upregulated the transcription factor NF-κB, thereby switching on to an intracellular pro- inflammatory signalling pathway, triggering decrease in copy number of α_1 - Na^+/K^+-ATPase and α- ENaC proteins. Curcumin being a powerful antioxidant and anti-inflammatory molecule is capable of providing a reduced oxidative stress environment in cells as well as in rat lungs thereby capping the activation of NF-κB and inflammation induced by hypoxia. Thus curcumin is a potent molecule that can facilitate alveolar fluid clearance during hypoxia and improve the lung's ability to clear the accumulated fluid. As such, a better understanding of the mechanisms that increase Na^+/K^+-ATPase function, may lead to the development of better therapeutic approaches to tackle the fluid accumulation in lungs. If this is also the case in in-vivo humans, the natural phytochemical molecule curcumin, is potential enough to fill this lacuna as a prophylactic drug that can up regulate the alveolar fluid clearance under hypoxic conditions.

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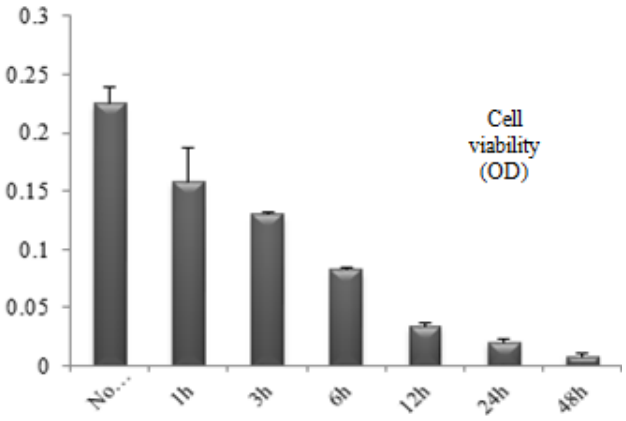


Figure 1(a)

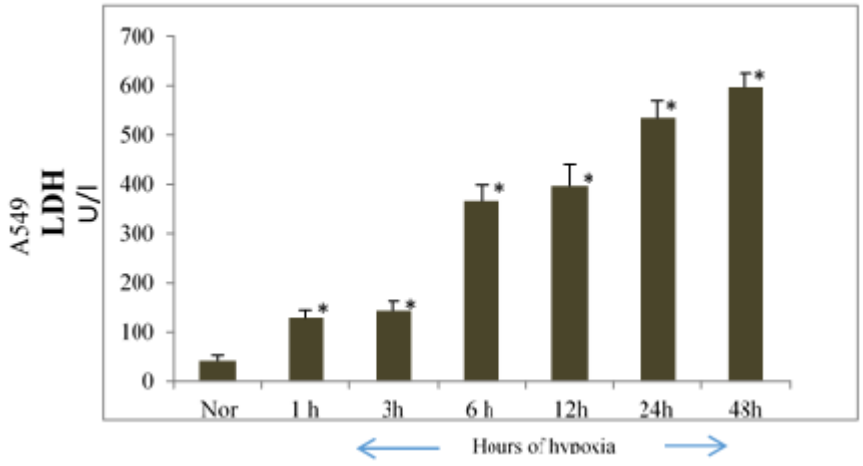


Figure 1b

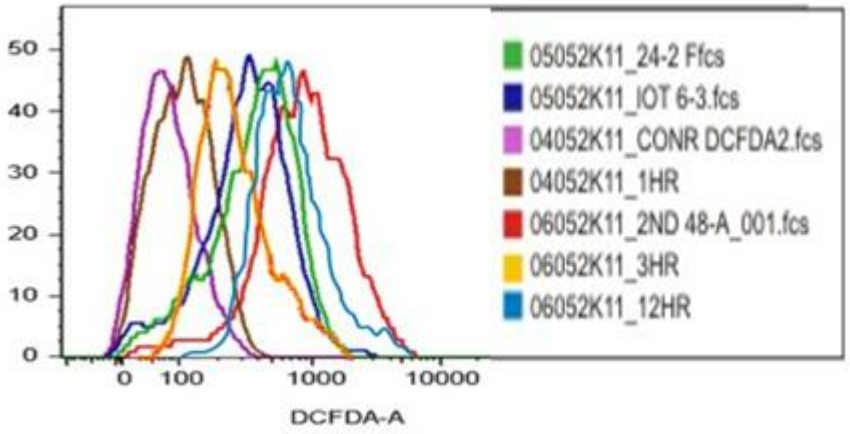


Figure 1c

Figure1. **Hypoxia induced cell death in A549 cells.** A549 cells were incubated with 3% oxygen for different durations. (**a)** Cell viability in hypoxia exposed A549 cells were evaluated by monitoring the neutral red uptake. **(b)** LDH levels in culture medium after hypoxic challenge. **(c)**effect of hypoxia on reactive oxygen species (ROS) generatio; the DCF fluorescence intensity was measured by flow cytometry.*n* = 4-6 experiments. Bar graph shows means ± S.D. * $p < 0.05$ *vs.* control, # $p < 0.01$ *vs.* 6 h hypoxia, (Nor: normoxia).

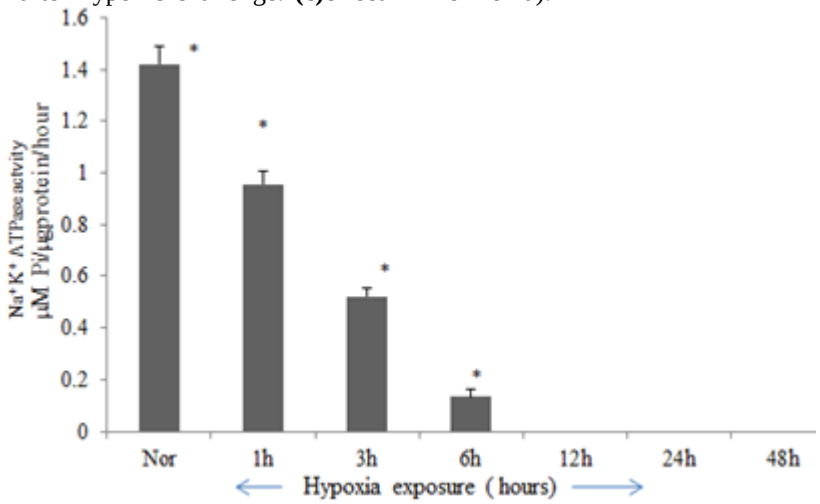


Figure 2

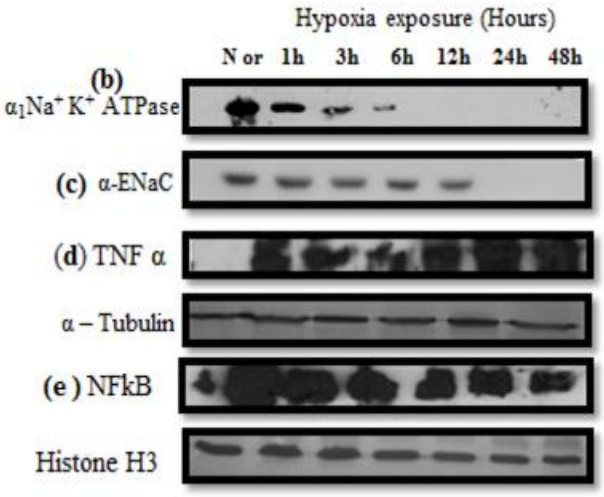


Fig 2**. Hypoxia decreases Na $^+$ K$^+$ -ATPase activity and α1 protein abundance at the plasma membrane.(a)**Hypoxia decreased theactivity of Na $^+$ K $^+$ ATPase time dependently. * $p < 0.05$ *vs.* control (0h). **(b)** Na $^+$ K $^+$ ATPase expression in cell membrane preparations also decreased under hypoxia.**(c)** Hypoxia significantly down regulated the αENaC expression at every time point tested. Western blot analysis for**(e)** NF-κB and **(d)**TNF α expression levels in A549 cells .

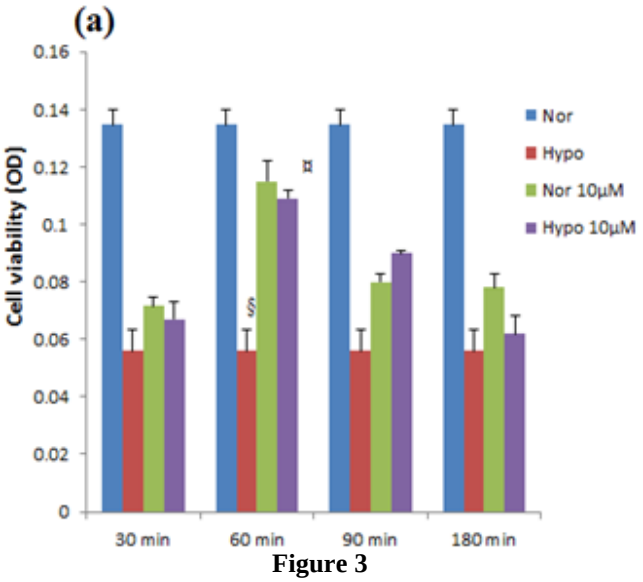


Figure 3

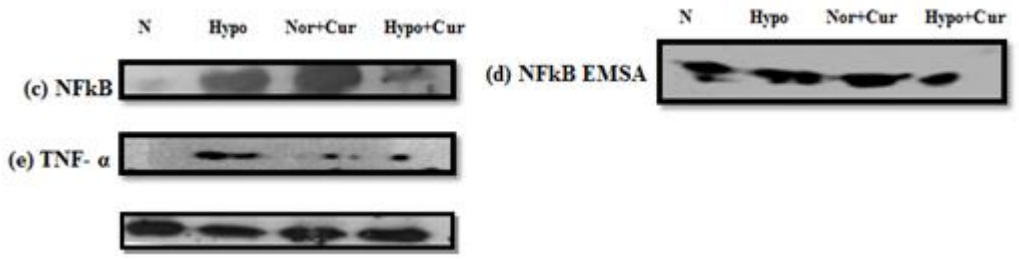
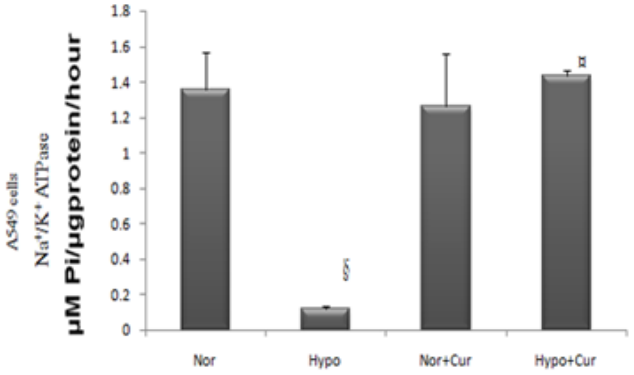
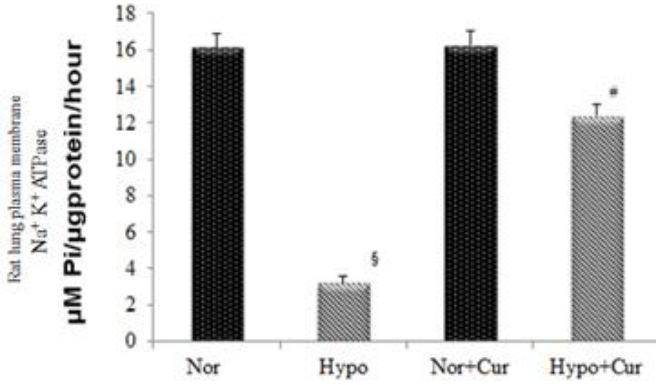


Figure 3 **Protective effect of curcumin on A549 cells under hypoxia**. **(a) Prior treatment of** A549 cells with 10 µM curcumin for different time points and exposed to hypoxia for 6h, The 60 min incubation with curcumin prior to hypoxia showed maximum cell viability. **(b)** expression of NFkB protein in A49 cells. **(c)** NFkB sequence specific DNA binding (EMSA) in A549 cells**.** (d) Pro inflammatory molecule TNFα protein **expression in** A549 cells.Nor-Normoxia, Hypo-Hypoxia, Cur- curcumin.



(a)



(b)

Figure 4 **Figure 4 Effect of curcumin on the expression of sodium pump activity and its protein expression** (a) α_1Na^+/K^+ ATPase pump activity in (a) A549 cells and (b) rat lung tissue (c) αENaC protein expression (d) α_1Na^+/K^+ ATPase protein expression in rat lung tissues and A549 cells exposed to hypobaric hypoxia for 6 hours. mRNA expressions levels of (e) α-ENaC and (f) α_1Na^+/K^+ ATPase in rat lung tissue. Administration of curcumin 1 h prior to hypoxia exposure have significantly up regulated the expression of these molecules and its activity as compared to control (hypoxia). §= Normoxiavs Hypoxia, ¤= Hypoxia vs Hypoxia + cur., $p<0.001$. Nor- Normoxia, Hypo-Hypoxia, Cur- curcumin.

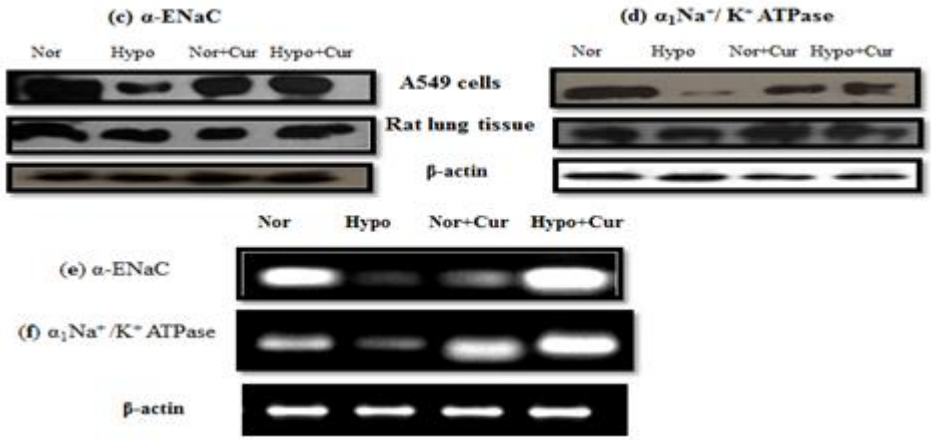


Table 1

Parameter	*Normoxia*	*Hypoxia*	*Normoxia + Curcumin*	*Hypoxia+ Curcumin*
GSH nmol/gm protein	263.27±10.8	139.66±9.5[a]	285.59±11.9	250.24±8.03[b]
GPx U/mg protein	12.96±0.9	5.62±1.02[a]	13.6±1.3	12.28±1.3[b]
SOD U/mg protein	0.247±0.06	0.106±0.08[a]	0.276±0.05	0.194±0.02[b]

Table 1: Effect of Curcumin on antioxidant status (GSH, GPx, and SOD) in A549 cells exposed to hypoxia 3%. Hypoxia significantly reduced the antioxidant levels in cells as compared to control. Whereas, curcumin treatment maintained the antioxidant levels more or less similar to that of control, indicating the potent antioxidant activity of curcumin under hypoxic conditions. Cells from at least 4-6 independent experiments were used for each time point; values are mean ± SD. [a]$p<0.05$ compared with Normoxia (0h) group. [b]$p<0.001$ compared with hypoxia (6h).Nor-Normoxia, Hypo-Hypoxia, Cur- Curcumin.