

Activator protein-1 (AP-1): a bridge between life and death in lung epithelial (A549) cells under hypoxia

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Abstract Activator protein-1 (AP-1) transcription factor plays a central role in hypoxia to modulate the expression of genes that decides the fate of the cell. The aim of the present study was to explore the role of AP-1 subunits in lung epithelial (A549) cells under hypoxia. Cell cycle studies by flow cytometry indicated that cell viability was unaffected by the initial hypoxia exposure (0.5% O₂ at 37 °C) for 6 and 12 h. However, both transient cell cycle arrest and cell death was detected at 24 and 48 h. Flow cytometry and spectrofluorometry data confirmed the increase in ROS levels. Elevated ROS and calcium levels activated the stress-related MAPK signaling cascade. ERK and JNK were activated in early hypoxic exposure (within 6 h), whereas p38 were activated in 48 h of hypoxia. These subtypes further stimulated the subunits of AP-1 at different times of hypoxia exposure to orchestrate different genes responsible for cell proliferation (6 and 12 h) and apoptosis (24 and 48 h). Our results clearly depict the role of AP-1 heterodimer, i.e., p-c-jun/c-fos, p-c-jun/fosB, junD/c-fos, and junD/fosB in cell proliferation/survival by regulating the expression of Bcl-2 and cyclins (D1 and B1) at 6 h and 12 h of hypoxia, whereas junB/Fra-1 heterodimer have important role in apoptosis by regulating the expression of p53, Bax, and cyclin-dependent kinase inhibitors (p16, p21, p27) at 24 h and 48 h of hypoxia.

Also, the cell survival signaling pathway NO-AKT interrupted at 24 h and 48 h of hypoxia indicating cell death. In conclusion, hypoxia for different time points activated different subunits of AP-1 that combined to form different heterodimers. These dimers regulated the expression of genes responsible for cell proliferation and apoptosis. Since, AP-1 plays a role in the decisive phenomenon of the cell to choose between proliferation and apoptosis; thus, its subunits or dimers could be a good therapeutic target for many diseases.

Keywords Hypoxia · Activator protein-1 (AP-1) · Mitogen-activated protein kinase (MAPK) · Apoptosis · Cell proliferation

Abbreviations

p-c-jun	Phosphorylated-c-jun
MAPK	Mitogen-activated protein kinase
JNK	Janus N-terminal kinase
ERK	Extracellular signal-regulated kinases
AKT	Protein kinase B
ROS	Reactive oxygen species
NO	Nitric oxide

Introduction

Oxygen is a vital element required to sustain life on earth. Insufficient oxygen causes pathophysiological changes to the basic structural and functional unit of life, i.e., the cell. A normal cell can grow, proliferate, differentiate, and dies after completing its life span. Whereas, insufficient or low oxygen concentration known as ‘Hypoxia’ may halt or interrupt this normal functioning of the cell. Hypoxia may either be caused due to natural consequences of

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environment, e.g., high altitude (acute mountain sickness) or is a common feature of many diseases such as chronic obstructive pulmonary edema (COPD) [1], acute respiratory distress syndrome, myocardial infarction (low cardiac output or perfusion) [2, 3], anemia (reduced oxyhemoglobin content), tumors or cancers [4, 5], Asperger's syndrome [6], Parkinson's disease (death of vital nerve cells) [7], Alzheimer's (neurodegenerative) disease [8, 9], atherosclerosis (proliferation of intimal-smooth-muscle cells) [10], and sickle cell disease [11]. These diseases are caused either due to excessive proliferation or excessive cell death under hypoxia. Thus, hypoxia is considered to be a solemn problem worldwide as it can lead to death of the individuals.

The phenomenon of cell proliferation and apoptosis are interrelated and depend upon the cell cycle progression that is controlled by cyclins and cyclin-dependent kinases (CDKs). Cyclins such as D, E, A, and B are the most required proteins that are mandatory to progress the cell cycle through G1-S-G2 and M phases of the cell cycle, respectively. These cyclins get activated through CDKs whose activities are in turn constrained by CDK inhibitors (CKIs) such as p15, p16, p18, p19, p21, p27, and p57. These CKIs that are regulated through p53, inhibit the phosphorylation of cyclins at G1, G1-S, and S-G2 stage [12–16]. The Bax and Bcl-2 (Bcl family genes) are also related to cell survival and apoptosis. The mechanism of holding cells in quiescent stage is important as it gives time to repair or make cell temporarily inactive in the stressed condition. But if the stress prolongs, then the phenomena of programmed cell death, i.e., apoptosis, gets activated and prepares the cell for death.

The adequate quantity of reactive oxygen and nitrogen species (RONS) are essential requirement of a body to initiate various physiological processes, whereas imbalance in these RONS generates harmful effects in the normal functioning of the cell and results in oxidative stress [17, 18]. ROS is known to activate a number of stress-mediated signaling cascades; one among these is MAPK under hypoxia stress [19, 20]. MAPK includes ERK, JNK, and p38 as subtypes which regulate downstream transcription factors to control cell proliferation and apoptosis. MAPK transmits the signals to downstream 'immediate early response genes' like c-jun and c-fos gene (subunits of AP-1 transcription factor) to carry out stress-adaptive responses [21–24]. The expression of immediate early genes depends upon the type and duration of the hypoxic stress. The products of early immediate genes, i.e., fos and jun family homo/heterodimerize to form an active AP-1 transcription factor. AP-1 is a redox-sensitive transcription factor which belongs to leucine-zipper family proteins that play roles in cell proliferation, apoptosis, inflammation, cell differentiation, and angiogenesis. The jun family

members includes c-jun, junB and junD, fos family members—c-fos, fosB and Fra-1. The cellular function that decides the fate of the cell highly depends upon the homo/heterodimers formed by the combination of different subunits of AP-1 transcription factor [17, 25–28].

Therefore, the aim of this study, was to investigate the compositions of active heterodimers and their functional role in cell proliferation and apoptosis at 6, 12, 24, and 48 h of hypoxia exposure in lung epithelial cells. A stress activated MAPK signalling was also explored to connect the pathway for the conversion of signals at gene levels through its concerning transcription factor.

Materials and methods

Materials

All chemicals and culture reagents (Dulbecco's Modified Eagle's High Medium—DMEM, Fetal Bovine Serum—FBS) were purchased from SIGMA-Aldrich. Assay kits, ELISA kits, and antibodies were purchased from Invitrogen, SIGMA-Aldrich, and Santa Cruz Biotechnology, respectively.

Lung epithelial cell (A549) culture

Lung epithelial cells (A549) (purchased from National Centre for Cell Science, Pune, India) were routinely grown in DMEM supplemented with 10% FBS, 100 mg/L ampicillin, and 100 mg/L streptomycin at 37 °C in a humidified atmosphere of 5% CO₂/95% air. For the experiments, cells were seeded in 96- and 6-well plates, T-25, and T-175 flasks. 70–80% confluent cells were exposed to hypoxia (0.5% O₂) for 6, 12, 24, and 48 h. Samples were collected at these time points of hypoxia and normoxia groups, respectively.

Intracellular reactive oxygen species quantification

Generation of ROS was assessed by spectrofluorometer and through flow cytometer using 2',7'-dichlorofluorescein-diacetate (DCFH-DA) as a probe as described earlier by LeBel et al. [29]. Briefly, ROS in cells cause oxidation of DCFH, yielding the fluorescent product 2, 7-DCF. Cells were incubated with DCFH-DA (10 μM) for 30 min in incubator. Thereafter, the medium was removed, and fluorescence was measured using a spectrofluorometer (excitation, 485 nm; emission, 530 nm). The result was expressed in RFU/minute. For FACS analysis, after incubation with DCFH-DA, cells were trypsinized after removing media and assessed immediately through FACS.

Data were normalized to values obtained from normoxia cells.

Intracellular calcium quantification

Intracellular calcium was estimated using Fluo-4 NW calcium assay kit (Invitrogen). The green fluorescent signal was quantified using multimode plate reader (FLUOstar Omega) at excitation and emission wavelength of 490 nm and 520 nm, respectively. Quantitative fluorescence data were represented as relative fluorescence units (RFU)/min.

Nitric oxide assessment

Nitrite is a stable end product of NO, and its concentration was determined by the Griess reagent (Sigma-Aldrich). In brief, 100 μ l of supernatant medium was incubated with 100 μ l of Griess reagent (40 mg/ml in distilled water) (Sigma, USA) for 20 min in dark and absorbance was measured at 540 nm. Quantitative data were given in μ M/ml of media supernatant.

Cell cycle analysis

Cells were cultured at 37 °C in a 5% CO₂, 95% air atmosphere, and exposed to 0.5% O₂ for 6, 12, 24, and 48 h. Cells were harvested, washed with PBS, and centrifuged at 1000 rpm/min for 10 min, after which the supernatant was carefully aspirated. Five milliliters of cold 75% ethanol was added in a drop-wise manner to the centrifuge tubes which were then incubated at –20 °C overnight. Cells were washed once with PBS and once with stain buffer, after which they were centrifuged for 10 min at 1500 r/min, and the supernatant was aspirated. Cells were resuspended in 0.5 mL of PI/RNase staining buffer, incubated for 15 min and analyzed on a flow cytometer (BD Biosciences) immediately thereafter. Data were analyzed using Flow Jo software. Results are expressed as the percentage of cells in G1, S, and G2 phase in each group.

Apoptosis analysis

Cells were cultured at 37 °C in a 5% CO₂, 95% air atmosphere and exposed to 0.5% O₂ for 6, 12, 24, and 48 h. Cell apoptosis analysis was performed using an annexinV-FITC & PI apoptosis detection kit (Sigma-Aldrich). Briefly, cells were harvested and washed with PBS. After centrifuging, cells were resuspended in 300 μ l 1 \times binding buffer provided in the kit and incubated with 5 μ l annexinV-FITC and 5 μ l PI for 5 min at room temperature in the dark. Cells were analyzed on a flow cytometer. The results are expressed as the percentage of apoptotic cells in each group.

Cell lysate preparation

Following hypoxia exposure, A549 cells were harvested on ice and thoroughly washed with cold PBS. Nuclear and cytosolic proteins were extracted using protein extraction kit (Sigma-Aldrich). Protein estimation was performed using Bradford's method [30].

ELISA and Western blotting

Trans AM AP-1 Family Transcription factor Assay kit, Active Motif, Catalog no 44296 was used to evaluate the levels of AP-1 subunits.

For western blotting, thirty microgram protein sample was mixed with 6X Laemelli buffer (0.25 M Tris-HCl pH 6.8, 10% SDS, 0.5% Bromo Phenol Blue, 0.5 M di-thiothreitol and 50% glycerol) and boiled for 10–15 min. These samples were resolved on a 10% SDS-PAGE, the resolved proteins were blotted on nitrocellulose membrane and blocked in 3% BSA solution for 1 h. Blocking solution and antibody dilutions were prepared in Tris-buffered saline with 0.1% Tween-20. Blot was incubated with primary antibodies such as p-ERK, ERK, p-JNK, JNK, p-p38, p38, p-c-jun, c-jun, junB, junD, c-fos, fosB, Fra-1, cyclin D1, cyclin B1, p16, p21, p27, p53, Bax, and Bcl-2 (dilution ranging from 1:500 to 1:1000) overnight at 4 °C and then with HRP-labeled secondary antibody (1:20,000, Santa Cruz) for 2 h, at room temperature. Enhanced chemiluminescence detection kit (SIGMA-Aldrich) was used to develop the blots and capture on X-ray film (Fuji Films). β -actin was used as the loading control (Santa Cruz) (1:1000). Densitometry was done using image J software (NIH).

Statistical analysis

Values are expressed as the mean \pm SD of at least three independent experiments. For experiments, one-way ANOVA was used. Differences were considered significant when $p < 0.05$.

Results

Hypoxia exposure-induced reactive oxygen species (ROS) generation and calcium level in A549 cells

Lung epithelial (A549) cells were exposed to hypoxia (0.5% O₂) for 6, 12, 24, and 48 h and ROS generation was assessed by spectrofluorometer and flow cytometry. The results showed that the increase in duration of hypoxia exposure led to significant elevation in intracellular ROS generation with 1.2, 2.3, 2.9, and 4 folds, respectively, as

compared to normoxia cells (Fig. 1a, b). Intracellular calcium level significantly increased by 1, 1.2, 2.1, and 2 fold in 6, 12, 24, and 48 h, respectively (Fig. 1c).

Hypoxia led to decrease in nitric oxide (NO) and AKT level

NO, a vasodilator, also an important signaling molecule, decreased one-fold at each time point of hypoxia exposure in A549 cells (Fig. 2a). The ratio between p-AKT/AKT decreased significantly at 24 and 48 h by 0.3 and 0.7 folds, respectively, under hypoxia exposure compared to normoxia cells (Fig. 2b).

Hypoxia exposure at 24 and 48 h arrested the cell cycle

The different phases of cell cycle (G1, S, and G2) were determined by flow cytometry. Hypoxia exposure for 6 h decreased 4.7% of A549 cell population in G1 phase,

whereas increased 2% in S and 13% cells in G2 phase compared to normoxia cells. At 12 h of hypoxic exposure, 19% of cells were increased in G1/G0 phase, whereas 6% and 2.5% decrease in S and G2/M phase, respectively. At 24 and 48 h, cell percentage increased up to 14.7% in G1/G0 phase (blocking of cells in G0 stage), 8–9% in S, and 2–4% in G2 phase. The blocked cell population in G1/G0 phase at 24 and 48 h are important because these cells have to decide whether they have to wait in G0/G1 phase to re-enter into the cell cycle or they have to follow apoptosis, a programmed cell death pathway (Fig. 3).

Hypoxia for 24 and 48 h led to apoptosis in A549 cells

Initial hypoxia exposure (6 h) of A549 cells, led to 1.4 and 0.6% of cells in early and late apoptosis. At 12 h, the percentage of cells in early and late were 7% and 2.9%, respectively. At 24 h the percentage of cells in early and late apoptosis increased to 12.5% and 3.3 furthermore, at

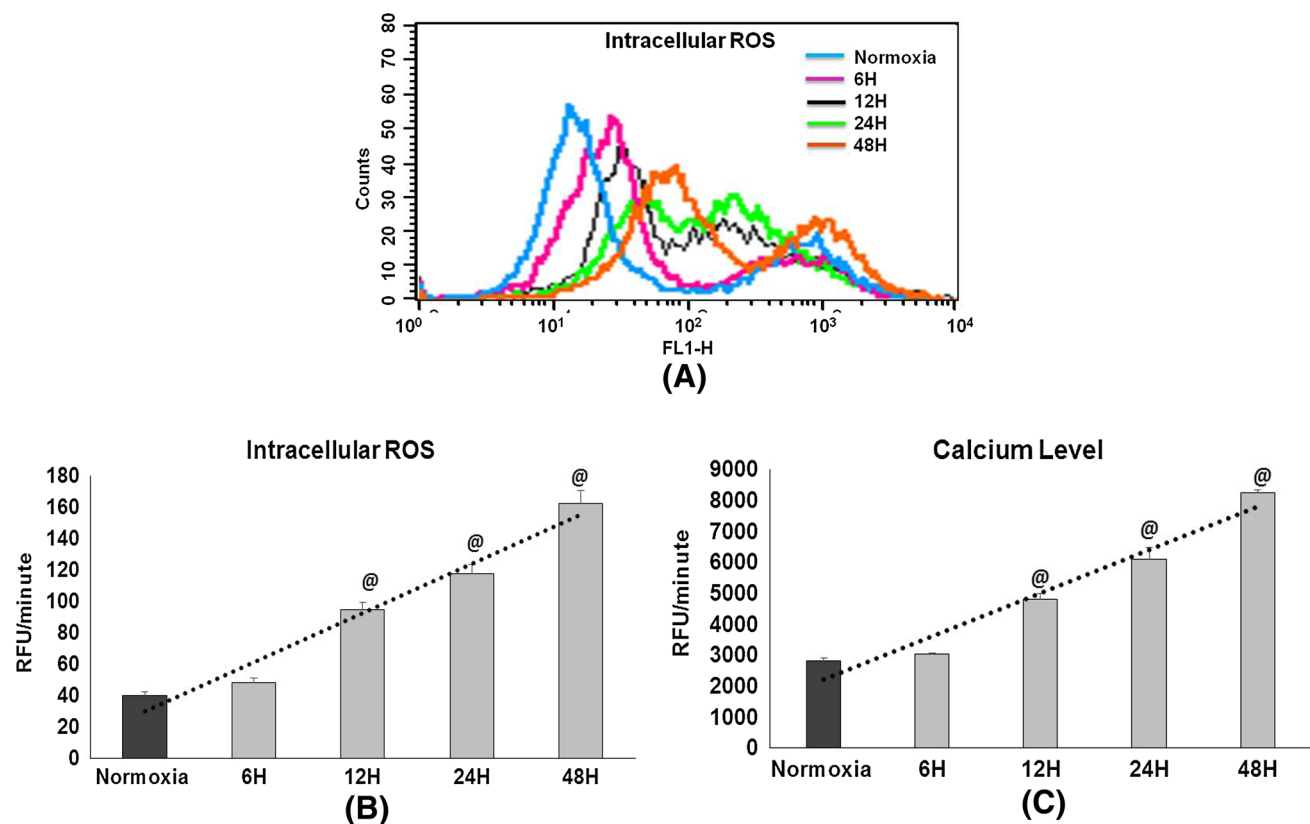


Fig. 1 Hypoxia exposure-induced reactive oxygen species (ROS) generation and calcium level in A549 cells. A549 cells were exposed to hypoxia (0.5% O₂) for 6, 12, 24, and 48 h and intracellular ROS generation was estimated by flow cytometry and spectrofluorometer. **a** Flow cytometry results depicted the increased ROS generation at each time point. **b** Spectrofluorometry result confirmed that intracellular ROS level increased with increase in duration of hypoxia

exposure. **c** Calcium level was evaluated by spectrofluorometry, increase in the duration of hypoxia exposure led to the elevation in intracellular calcium level. All experiments were carried out on at least three separate cell cultures. Data are given as mean \pm SD @ statistically significant difference (i.e., $p < 0.05$) compared with normoxia control values

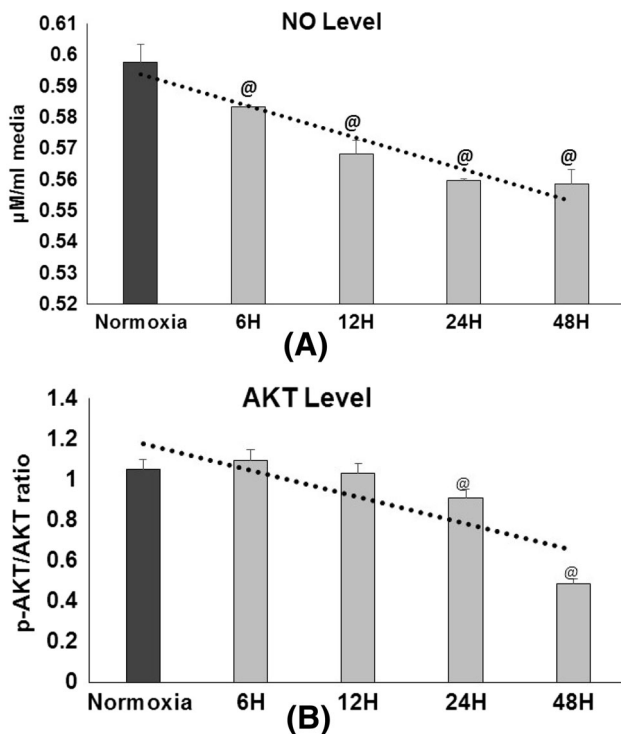


Fig. 2 Hypoxia led to decrease in nitric oxide (NO) and AKT level. A549 cells were exposed to hypoxia (0.5% O₂) for 6, 12, 24, and 48 h. **a** NO level was decreased with increasing hypoxia exposure time. **b** The levels of phospho-AKT and AKT were evaluated by ELISA. Phospho-AKT/AKT ratio decreased with the increasing duration of hypoxia exposure. All experiments were carried out on at least three separate cell cultures. Data are given as mean \pm SD @ statistically significant difference (i.e., $p < 0.05$) compared with normoxia control values

48 h annexin V/PI-stained cell percentage increased to 36.6% and 12.4%, respectively. The results clearly showed that blockage of large number of cells within G1 phase (cell cycle) at 24 and 48 h of hypoxia exposure forced them to enter in apoptosis (Fig. 4).

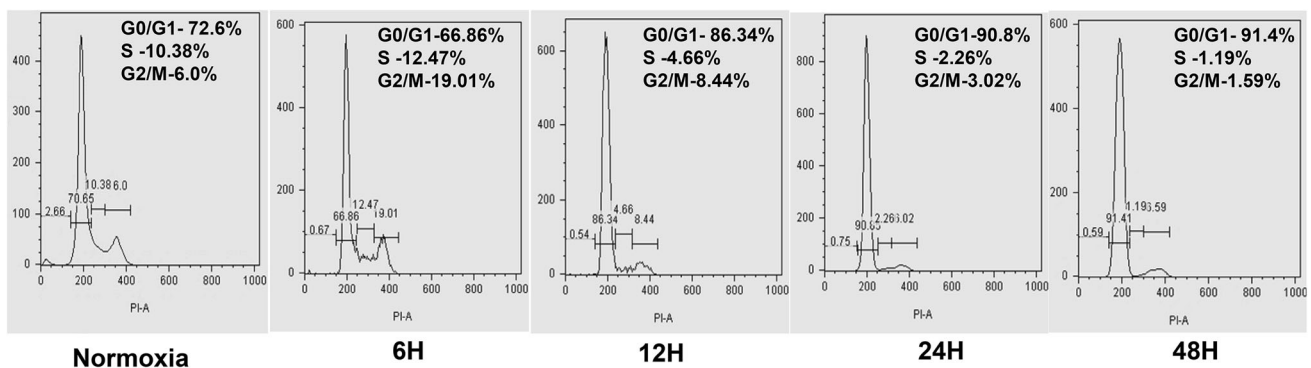


Fig. 3 A549 cells were harvested after hypoxia exposure for 6, 12, 24, and 48 h and cell cycle analysis was performed by flow cytometry. Initial hypoxia exposure (6 and 12 h) increased the cell percentage in S and G2 phase. Further increase in hypoxia exposure

Hypoxia led to the activation of mitogen-activated protein kinases (MAPK) signaling pathway

MAPK signaling pathway is known to activate AP-1 transcription factor as an early response to environmental stress. Among the different subtypes of MAPK (ERK, JNK, and p38), p-ERK expression was increased significantly by 1 fold at 12, 24, and 48 h. Expression of p-JNK was increased with 0.8, 1 folds in 6 and 12 h; however, it decreased significantly by 1.50 folds at 24 and 48 h, respectively. No change was observed in p-p38 expression at 6 and 12 h, its expression increased significantly by one-fold at 24 and 48 h of hypoxia exposure (Fig. 5a–c).

Expression of different subunits of AP-1 Transcription Factor

p-c-jun: c-jun is a subunit of AP-1 transcription factor that can form heterodimer and homodimer with fos and jun family members. The ratio of p-c-jun to c-jun was not changed in 6 and 12 h compared to normoxia. However, this ratio decreased by 0.7 folds at 24 and 48 h of hypoxia exposure compared to normoxia cell (Fig. 6a).

junB: junB belongs to jun family. Its expression was not changed at 6 h; however, it increased significantly by 2.6 folds each at 12, 24, and 48 h, respectively, of hypoxia exposure (Fig. 6b).

junD: The other important subunit of AP-1 transcription factor is junD, which belongs to jun family and its expression was significantly decreased by 2.1 and 1.1 folds 24 and 48 h, respectively, in hypoxia-exposed A549 cells (Fig. 6c).

c-fos: c-fos belongs to fos family. Its expression was not changed much in 6 and 12 h; however, it decreased by 2.2 and 1.9 folds at 24 and 48 h, respectively (Fig. 6d).

for 24 and 48 h increased the percentage of cells in G0/G1 phase of cell cycle. All experiments were carried out on at least three separate cell cultures

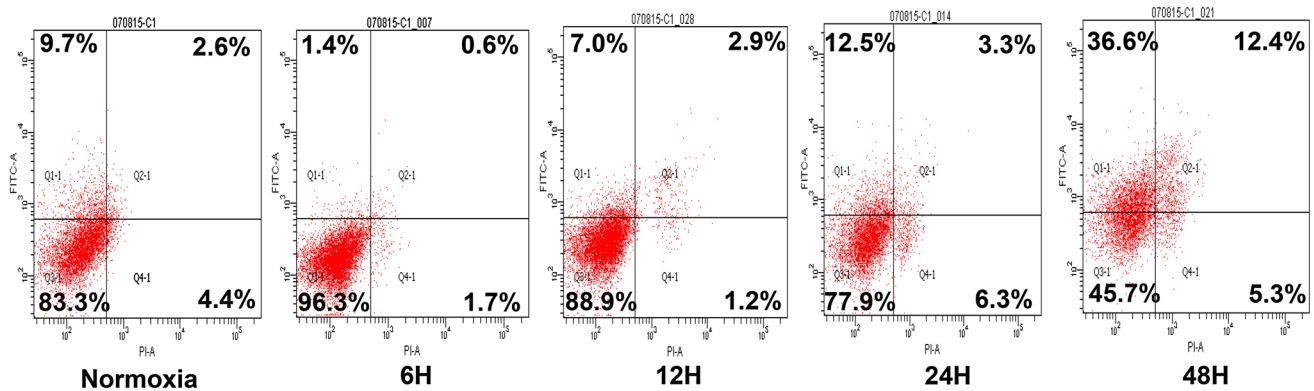


Fig. 4 A549 cells were harvested after hypoxia exposure for 6, 12, 24, and 48 h and annexinV/PI staining was performed by flow cytometry. Results indicate decreased apoptosis (early and late) in A549 cells due to hypoxic stress for 6 and 12 h, whereas at 24 and

48 h results showed the increased percentage of apoptotic cells compared to normoxia cells. All experiments were carried out on at least three separate cell cultures

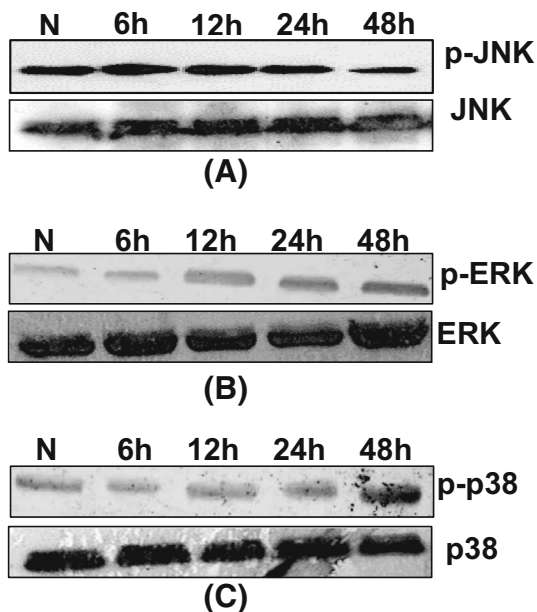


Fig. 5 Typical western immunoblot of subtypes of MAPKs, JNK (a), ERK (b), and p38 (c) in the cell lysate of A549 cells exposed to hypoxia for 6, 12, 24, and 48 h

fosB: fosB is also a member of fos family and its expression was significantly decreased with 1.1 folds in 48 h (Fig. 6e).

Fra-1: Fos family also includes Fra-1. Its expression was not changed at 6 and 12 h of hypoxia; however, its expression was significantly increased with 1.9 folds at 24 and 48 h in hypoxia-exposed cells (Fig. 6f).

Hypoxia exposure led to change in expression of genes responsible for proliferation

Our FACS data confirmed the initial proliferative response (6 and 12 h) in A549 cells therefore, we evaluated the

expression of genes, responsible for cell proliferation like cyclin D1 and cyclin B1. The expression of cyclin D1, cyclin B1 was not much changed at 6 and 12 h of hypoxia exposure; however, it decreased significantly by 0.7 folds at 24 and 48 h (Fig. 7a, b).

The expression of an anti-apoptotic Bcl-2 gene increased at 6 and 12 h and decreased at 24 and 48 h of hypoxia compared to normoxia (Fig. 7c).

Hypoxia led to increased expression of genes responsible for apoptosis

Since, the flow cytometry results also showed the increase in cell death at 24 and 48 h of hypoxia. Therefore, the expression of cyclin-dependent kinases inhibitors (CDK inhibitors) like p16, p27, and p21 was evaluated. The expression of p16, p27, and p21 proteins increased significantly up to 0.9 folds at 24 and 48 h. p53 and Bax (induce apoptosis through intrinsic pathway) are also the downstream targets of AP-1 that regulate cell growth, our results revealed that the expression of these genes increased significantly by 1 and 1.2 fold at 24 and 48 h of hypoxia exposure in A549 cells (Fig. 8a–e).

Discussion

The study investigated the hypoxia-induced molecular response in human lung epithelial type II cells, which is an alveolocapillary barrier that maintains alveolar integrity. It secretes surfactant to lower the surface tension, clear edema, and is involved in repair of injured alveolar epithelium. During ascent to high altitude or in pathological disorders, the decrease in oxygen tension leads to the injury of the epithelial cells. The injured epithelial cells impair fluid clearance and results in flooding of alveolar

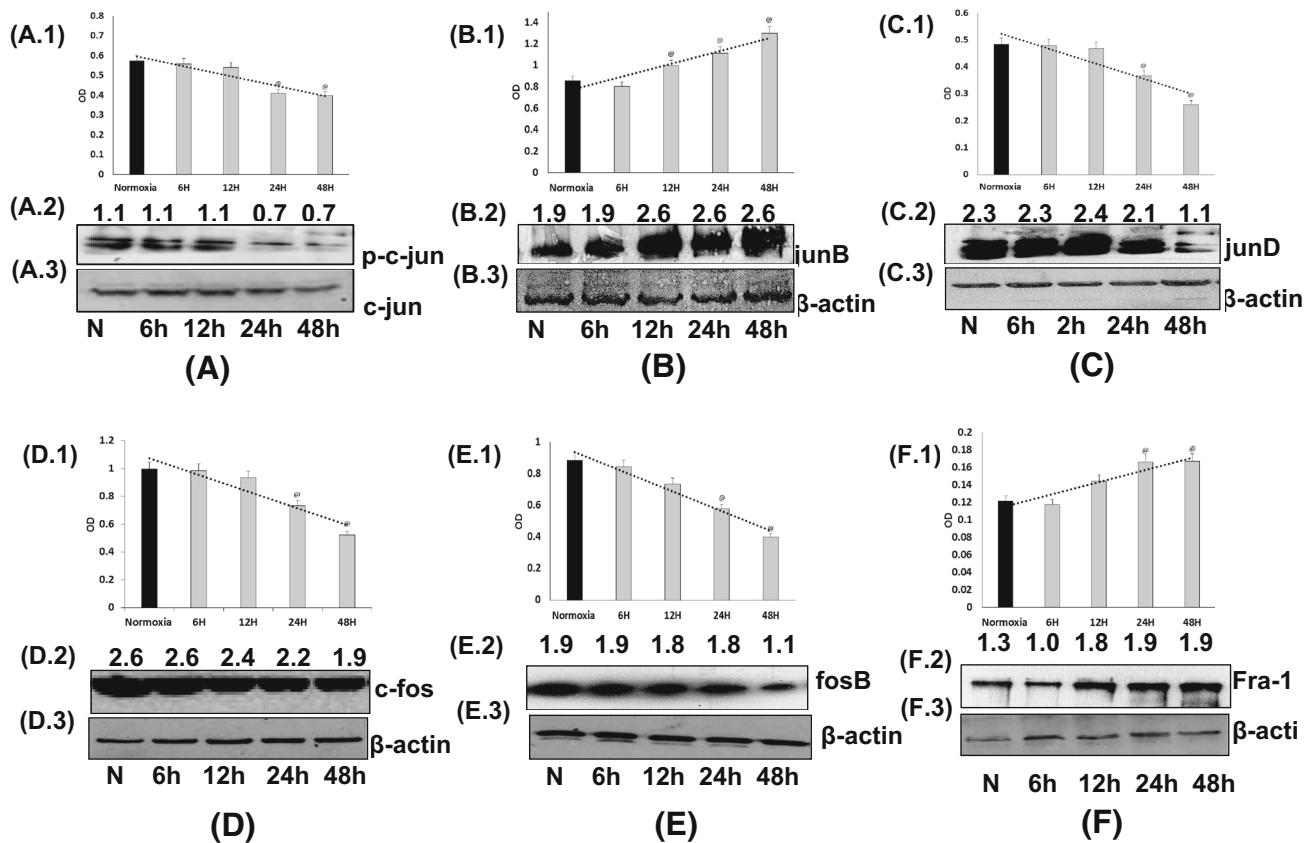


Fig. 6 The expression levels of different subunits of AP-1, p-c-jun (a), junB (b), junD (c), c-fos (d), fosB (e), and Fra-1 (f) estimated through ELISA (1) and confirmed by western blot (3) in A549 cells under hypoxia exposure for 6, 12, 24, and 48 h. Densitometry values

normalized to beta actin for each blot and is given above each blot (2). Data are given as mean \pm SD @ statistically significant difference (i.e., $p < 0.05$) compared with normoxia control values

spaces, which leads to the development of pulmonary edema. Therefore, the aim of this study was to trail the mechanism that could define the path followed after the initial stimuli to the downstream molecules including transcription factor and its regulated genes using A549 cells as a model system.

In present study, A549 cells were exposed to hypoxia at different time durations 6, 12, 24, and 48 h. The cell cycle analysis through flow cytometry was carried out and results depicted that, 6 and 12 h of hypoxia exposure increased the percentage of cells in S-G2 phase indicating that the cell cycle is progressing; however, at 24 and 48 h, the percentage of cells in G1 phase increased. The higher number of cell population in G1 phase indicated the interruption in movement of cells from G1 to S phase (blocking of cell cycle in G_0 –G1 phase) and leading cells towards apoptosis. Normally, every cell undergoes proliferation, differentiation, and apoptosis during its lifespan, whereas any type of stress may alter normal functioning of the cell by activating certain downstream signaling pathways, promoting cell survival, and cellular suicidal programs [31]. Both the processes of cell survival and cell death are well regulated

at biochemical and molecular levels by signaling pathways that finally target the transcription factor for the activation of genes. Depending upon the level and duration of stress, transcription factors activate the responsive genes resulting in ‘loss of function’ and ‘gain in function.’ The expression level of these genes decides either to adopt survival strategies or typically undergo apoptosis. To trace the changes at biochemical levels, we evaluated ROS, calcium level, and NO. Our results indicated that the hypoxia exposure led to increase in ROS generation and calcium levels as compared to normoxia cells. This result is in agreement with other reports that hypoxia or low oxygen concentration leads to the overshoot of ROS and calcium levels in the cell [6, 32]. Another important molecule that plays a role in cell survival is nitric oxide, which protects the cells by activating NO-AKT signaling pathway [33]. We observed that NO was decreased at each time point; however, its downstream target i.e., AKT levels were the same as normoxia at 6 and 12 h, and its level decreased at 24 and 48 h. This maintained level of AKT (6 and 12 h) helps cells to survive or proliferate under hypoxia. Since the levels of ROS and calcium increased under hypoxia,

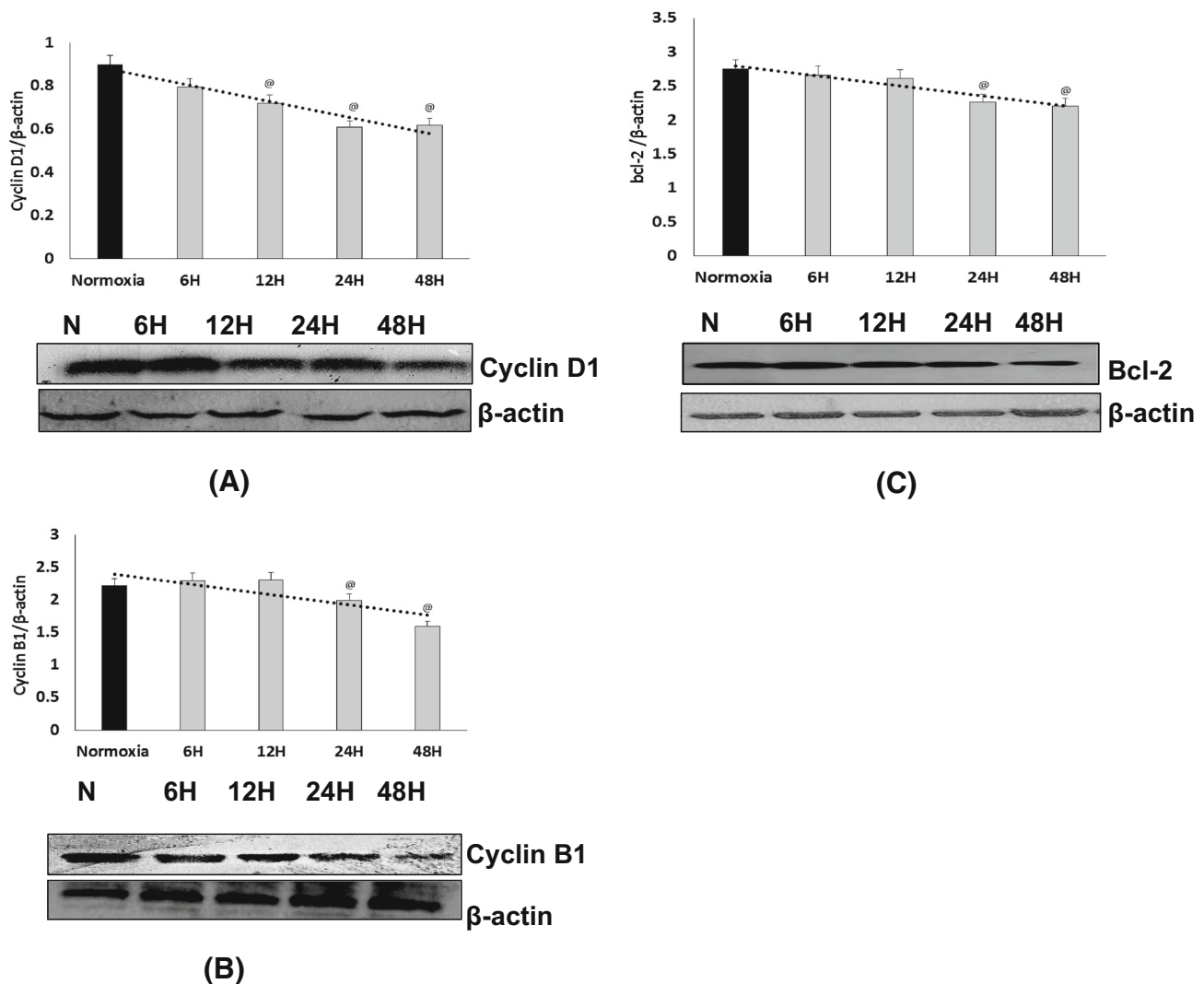


Fig. 7 Representative western blot of cyclin D1 (a), cyclin B1 (b), and bcl-2 (c) genes in A549 cells exposed to hypoxia for 6, 12, 24, and 48 h. Graph shows densitometry values normalized to beta actin. Each point is the mean \pm SD of three experiments @ $p < 0.05$

these molecules might be acting as a signaling molecule to initiate the signaling cascade, we therefore explored the signaling pathway in cells exposed to hypoxia for different times.

Yong Son et al. and other researchers have reported that MAPK signal transduction pathway is an important cascade to be activated under stressed conditions (increased ROS, cytokines, or growth factors). These MAPKs are the midway connectors that connect the upcoming extracellular signals to its concerning transcription factor for the conversion of signals to cellular response. Therefore, we checked the expression levels of MAPKs and our western blotting results confirmed the phosphorylation of extracellular receptor kinases (ERK), c-jun N-terminal kinases (JNK), and P38 at different times. These results demonstrated that the increased ROS and calcium levels might have activated MAPK signaling pathway under hypoxia. The expression

levels of JNK were not changed at 6 and 12 h, however, it decreased at 24 and 48 h, p38 expression increased in 24 and 48 h, and p-ERK expression remains elevated at each time point indicating that ERK and JNK, as an early response (within 6 h) pathway, whereas p38 as late response (24 and 48 h) pathway to hypoxia stress. These findings are line with other studies that MAPK plays the major role in signal transduction from cell surface to nucleus under hypoxia [6, 19, 20, 32]. Since the downstream targets for ERK and JNK are fos and jun family members, respectively, [34] therefore, in the next experiment, we checked their expression levels under hypoxia. The jun and fos family combines to form an active AP-1 transcription factor that regulates the genes responsible for cell proliferation and apoptosis. According to our results, AP-1 subunits show a variable expression pattern during the hypoxia exposure for 6, 12, 24, and 48 h. The changed expression levels of different

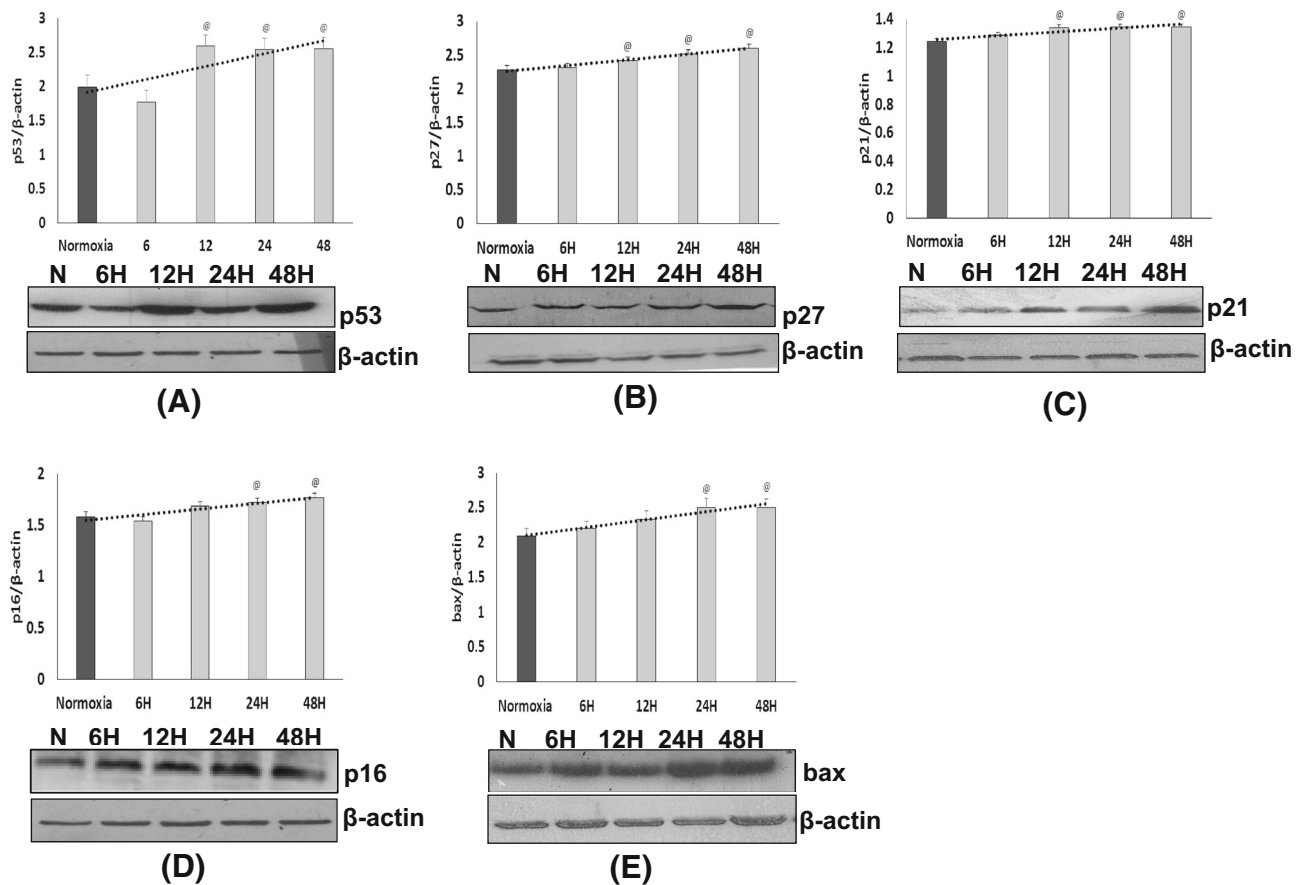


Fig. 8 Figure showing the western blot of p53 (a), p27 (b), p21 (c), p16 (d), and bax (e) genes in A549 cells exposed to hypoxia for 6, 12, 24, and 48 h. Graph shows densitometry values normalized to beta actin. Each point is the mean \pm SD of three experiments @ $p < 0.05$

subunits at different time points also affect the normal cell functioning. The subunits p-c-jun, junD, c-fos, and fosB were expressed at 6 and 12 h leading to the possibility of strong bond formation among these subunits. These subunits combine to form functional homo/heterodimer that are required for ‘to grow’ or ‘to die’ phenomenon of the cell. The possible and strong heterodimers formed at 6 and 12 h were p-c-jun/c-fos, p-c-jun/fosB, junD/c-fos, and junD/fosB as shown in Fig. 9. The impact of each dimer of AP-1 is still unclear, some researchers have concluded that p-c-jun and c-fos form the active c-jun/c-fos heterodimer playing essential role in cell proliferation. So, there is a strong possibility that these heterodimers may have positive control over the cell, causing cell survival and proliferation at 6 and 12 h of hypoxia. Wolfram Jochum [35] also proposed that the same subunit of AP-1 is able to perform cell proliferation and apoptotic functions depending upon the type and duration of stress. At longer duration of hypoxia, i.e., 24 and 48 h, the expression levels of junB and Fra-1 subunits increased; however, the expression of p-c-jun, junD, c-fos, and fosB decreased therefore there was a possibility that the aforementioned heterodimer was replaced by strong junB/Fra-1

heterodimer (shown in Fig. 9). The change in heterodimer compositions at longer duration of hypoxia activated the genes responsible for blockage of cells in G_0 phase and directed them to follow the programmed cell death (apoptosis). Our results are in accordance with previous reports [36] stating that JunB has a negative effect over cell proliferation.

Having confirmed the formations of different heterodimers at different times of hypoxia exposure, we sought the role of these subunits in the regulation of proliferative and apoptotic genes. For the progression of cell cycle, cyclins like D, A, E, and B form the complex with cyclin-dependent kinases 2, 4, and 6. Cyclin-dependent kinase inhibitors (CKIs) (p16, p21, p27) inhibit the formation of the complex between cyclins and the cyclin-dependent kinases at G1 to S and G2 to M phase, respectively. These CKIs are the downstreams of a tumor suppressor gene p53, which is regulated by AP-1 transcription factor [37]. In our study, the expression of genes responsible for cell proliferation such as cyclin D1 and cyclin B1 was decreased at 24 and 48 h. The levels of Bcl-2, an anti-apoptotic gene also decreased at 24 and 48 h of hypoxia; this gene has

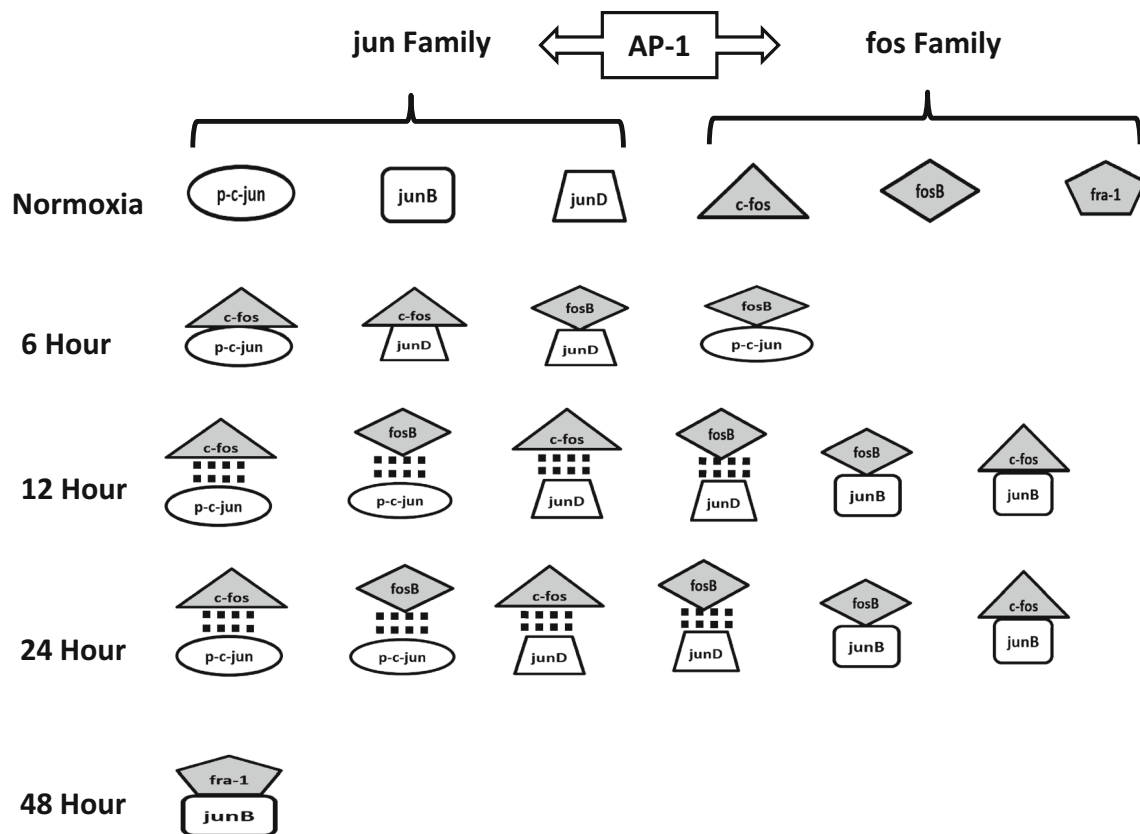


Fig. 9 Schematic representation of the AP-1 subunits and their possible combination of active heterodimers at 6, 12, 24, and 48 h of hypoxia. At different times of hypoxia the expression of AP-1 subunits was changed. At 6 h p-c-jun/c-fos, p-c-jun/fosB, junD/c-fos,

and junD/fosB heterodimers were formed. At 12 and 24 h, the bonding among aforementioned subunits became weak (.....) and new active heterodimers were formed (fosB/junB and c-fos/junB). At 48 h, the strong bond was formed between junB and Fra-1 subunits

sites for AP-1 in its promoter region. This clearly demonstrated that the active heterodimers among p-c-jun/c-fos, p-c-jun/fosB, junD/c-fos, and junD/fosB were involved in cell proliferation by regulating cyclin D1, cyclin B1, and Bcl-2 genes under initial hypoxia exposure (6 h). In addition, at 24 and 48 h junB/Fra-1, heterodimer formed had negative impact over cell cycle progression and led to apoptosis by changing the expression of cyclins and CKIs through p53. We observed increased expression of p53 at longer duration of hypoxia, this might lead to G1 cell cycle arrest through transactivation of p21, p27, and p16, CKIs. The changes in the expression of cyclins and CKIs at 48 h were linked to the strong heterodimer formed between junB and Fra-1. Another apoptotic gene, i.e., bax, regulated by AP-1 was increased in its expression at 24 and 48 h of hypoxia exposure. From these results, we have concluded that, at different times of hypoxia different heterodimers of AP-1 were formed, which could be responsible for the change in the pattern of gene expression that led to switch the cell proliferation pathway to a programmed cell death. Figure 10 concluded our study, which showed a correlation

among signaling molecules, AP-1 transcription factor, and its regulated genes. Previously, reports suggested that AP-1 plays an important role in various human diseases. Its subunits, i.e., c-jun, junB, junD, c-fos, fosB, and Fra-1 have a major contribution in various types of human cancers such as lung cancer [38], colorectal cancer [39], breast cancer [40], and skin cancer [41]. Due to the diverse possibilities of formation of heterodimers and their involvement in a variety of roles, made AP-1 a vital transcription factor to target for research purpose.

Conclusion

In conclusion, subunits of AP-1 express in a time-dependent manner and act on specific target genes that may contribute to the differential effects, i.e., cell proliferation and apoptosis. The expression of AP-1 subunits increased or decreased depend upon the duration and level of hypoxia exposure. The composition of AP-1 heterodimers decide the fate of lung cells to choose between life and death. To the

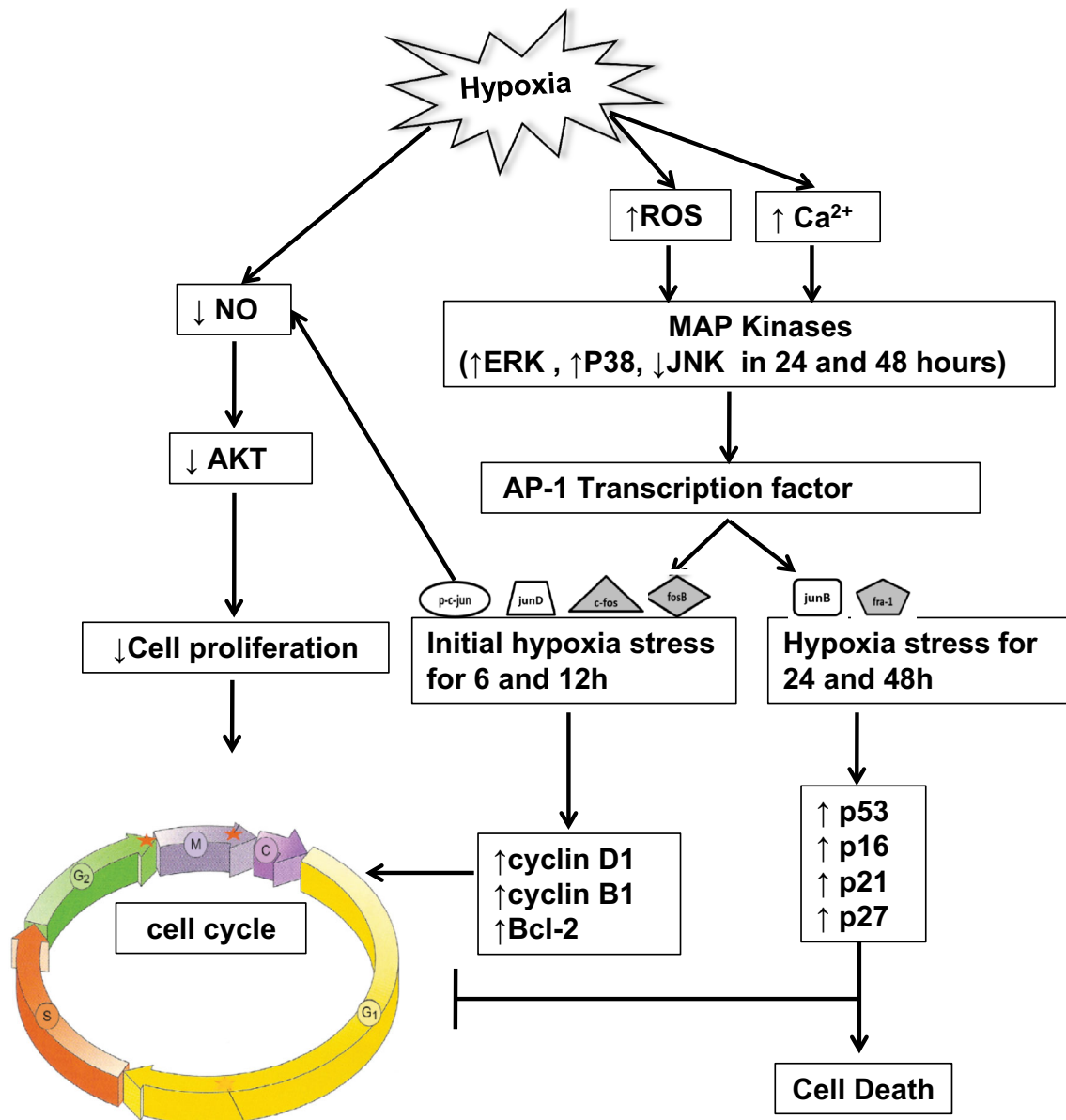


Fig. 10 Schematic representation of proposed signaling pathway. mitogen-activated protein kinases (ERK, JNK, and p38) activated AP-1 transcription factor via ROS and calcium. ERK and JNK were activated during initial hypoxia exposure and p38 was expressed at longer duration of hypoxia exposure. These MAPKs activated

different AP-1 subunits (c-jun, junD, junB, c-fos, fosB, and Fra-1) at different time durations of hypoxia. The heterodimers of AP-1 subunits are involved in the expression of several genes that regulate cell proliferation (bcl-2, cyclin D, and cyclin B) and apoptosis (bax, p16, p21, p27, and p53)

best of our knowledge we, for the first time reported that the heterodimer formed among p-c-jun, junD, c-fos, and fosB during the initial hypoxic stress (6–12 h) increases the cell proliferation, whereas junB/Fra-1 heterodimer at longer exposure (24 and 48 h) increased the programmed cell death i.e., apoptosis. So, AP-1 plays a dual role in deciding the fate of the cell in hypoxic condition. Therefore, this transcription factor or its specific subunit can be a major therapeutic target in hypoxic conditions.

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Compliance with ethical standards

Conflict of interest The author(s) declare that they have no conflict of interest.

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