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**RESEARCH ARTICLE**

# Detection of Hypoxia inducible factor-1 By DNA Binding Studies

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**ABSTRACT**

Stabilization of HIF-1α, its translocation into the nucleus and dimerization with its HIF-1β subunit to form HIF-1 complex is an index of hypoxia. Until recently, most studies involving HIF1 were carried out in animal cell line models. In this study, we show the translocation of HIF-1 in freshly isolated human lymphocytes treated with hypoxia mimicking conditions. In order to check for expression of HIF-1 and its nuclear translocation, we used conditions of hypoxia and hypoxia mimicking conditions. *in vivo* hypoxia conditions were analyzed in rat models by exposing them to oxygen partial pressure relevant to an altitude of 7500 m ($pO_2$=59 mm Hg), whereas hypoxia mimicking conditions were established by using certain agents known to stabilize HIF-1. Agents like $CoCl_2$ and DFO were used in this context in freshly isolated lymphocytes. The translocation of HIF-1 is shown using electrophoretic mobility shift assay of nuclear and cytoplasmic extracts.



## INTRODUCTION

Hypoxia occurs in many cases such as exposure to high altitude, disease processes such as atherosclerosis, ischemia, stroke, myocardial infarction and also, cancer. Recent evidences have suggested similarities of hypoxia with physiological events occurring in dementia caused in Alzheimer's and age related dementia (Sun, *et al.*, 2006). Based on severity, hypoxia can be broadly categorized into two types: acute and chronic. Acute hypoxia occurs when cells are exposed to very low oxygen concentration and chronic hypoxia occurs when cells are exposed to low oxygen concentrations for a long duration. Hypoxia prevailing at high altitude locations is the root cause of many maladies, such as AMS, HACE and HAPE, that develop in unacclimatized persons shortly after ascent. HACE had been considered to be the end stage of AMS (Ward and West, 1995), eventually leading to death. Thus the present study deals with detection of HIF-1 *in vitro* and *in vivo* conditions, Not only hypoxia, but also chelating agents like cobalt chloride, desferrioxamine, growth factors like insulin, etc, have been observed to stabilize HIF-1α (Semenza, 2000). Structurally, HIF-1 is a dimer composed of a β subunit, which is relatively stable and acts as a constitutive factor (Wood *et al.*, 1996), and the α subunit, which is degraded rapidly and is therefore the limiting factor in the reaction chain (Huang *et al.*, 1998). During hypoxia, the α-subunit becomes stabilized, leading to stabilization of the dimers, its binding to DNA, and induction of DNA translation into mRNA. In this paper, we demonstrate for the first time HIF-1 nuclear translocation in freshly isolated lymphocytes treated with hypoxia mimicking conditions.

## MATERIALS AND METHODS

### Materials

Freshly collected blood samples, Histopaque 1077, Phosphate buffer saline, Buffer A (10 mM HEPES pH 7.9; 10mM KCI; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF; protease inhibitor cocktail), Buffer C (20mM HEPES pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF; protease inhibitor cocktail), Nonidet P-40, 5 X binding buffer (12 mM HEPES pH 7.5; 70 mM KCl; 1 mM EDTA; 1 mM DTT; 12 % Glycerol), annealing buffer (67 mM Tris-Cl pH 7.6, 13 mM MgCl2, 6.7 mM DTT, 1.3 mM spermidine, 1.3 mM EDTA), radioactive [$\gamma$-$^{32}$P] ATP, DNA oligonucleotide probes containing HIF-1 dimer binding site(wild type-W18 and mutant M16), herring sperm DNA(non-specific), Cobalt Chloride, desferrioxamine, etc. The following custom-made DNA probes were obtained from Genetix Biotech Asia Pvt. Ltd. Probe-W18 (wild type), forward strand 5'- GCCCTACGTGCTGTCTCA-3' corresponding to nt 3454-3471 of the published EPO gene sequence and corresponding reverse strand. Probe-M18 (mutant) forward strand 5'-CCTAAAAGCTGTCTCA-3' and its corresponding reverse strand (nt 7-9 mutation underlined, do not bind HIF-1α) (Tian *et al.*, 1997).

### Isolation and induction of lymphocytes from peripheral blood

Freshly drawn 5 ml peripheral blood (used within 4 hrs of collection) treated with an anticoagulant preferably EDTA was layered over Histopaque 1077 (Ficoll, Sigma-Aldrich) and lymphocytes were purified as mentioned by the manufacturer. Isolated lymphocytes were suspended in 100 µ1 of PBS and treated with substances stabilizing hypoxia inducible factor-1 such as, $CoCl_2$ and desferrioxamine. In the following experiment, a 100 mM $CoCl_2$ stock was made in 50 mM HEPES (pH 5.5) and an aliquot of this stock was added to the lymphocyte suspension to get a final concentration of 1 mM. Then the sample was incubated at 37°C for 15-20 min. After incubation 500 µl of RPMI 1640 medium (without Fetal Calf serum) was added to this and the sample was further incubated for 2 h. In case of samples to be treated with desferrioxamine, a 100 mM stock of desferrioxamine is made in sterile MQ water and added to the lymphocyte sample to get a final concentration of 100 µM and the sample was processed in same fashion as lymphocytes treated with $CoCl_2$.

### Animals

Adult male Sprague-Dawley rats (weights: ~175 g) were maintained at Defence institute of Physiological and Allied Sciences, DRDO,

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Delhi. Animals were maintained at a constant temperature (22 $^{o}$C ± 2 $^{o}$C) with 12 h light-dark cycle. Animals had access to food and water *ad libitum* and all efforts were made to minimize animal suffering. The experimental protocols followed were in accordance with the guidelines approved by the DIPAS ethical committee (DRDO, Delhi) in compliance with the European Communities Council directive.

**Exposure to hypoxia**

The animals were exposed to simulated hypobaric hypoxia of 7500 m (25,000 ft), $pO_2$= 59 mm Hg) in an animal decompression chamber (Decibel instruments, Delhi, India) at 22 $^{o}$C ± 2 $^{o}$C coupled to mercury barometer (Bhatia *et al.*, 1966). The animals were kept in this chamber operated under 12 h light–dark cycles. Ambient $CO_2$ in the chamber was constantly monitored and maintained at < 0.03% by adjustments in overall chamber ventilation, and humidity was maintained at < 40%. Animals (n=6 / group) were exposed to either normoxia in room air, or continuous simulated hypobaric hypoxia corresponding to oxygen partial pressure at 7500 m altitude for a period of 5 days with a break of 30 min each day for replacing food and water. After exposure to the above conditions, animals were sacrificed by cervical dislocation; brains were dissected on ice to retrieve hippocampus and cortex. The samples were stored at -80 $^{o}$C until further use.

**Preparation of Nuclear extracts from Lymphocytes and Rat brains**

After incubation, the samples were centrifuged at 5000 X g for 5 min at room temperature. Pellet was washed with 1 ml PBS and centrifuged again. PBS was removed and the pellet was suspended in 400 µ1 cold buffer by gentle pipeting with a yellow tip. Cells were then allowed to swell on ice for 15 min, and 25 µ1 of a 10 % solution of NP-40 was added. The homogenate was vigorously vortexed for 10 sec and centrifuged for 5 min at 10,000 X g. The supernatant containing cytoplasm and RNA was transferred into a fresh tube. The contents were mixed and immediately stored at - 20 °C. The nuclear pellet was washed with cold buffer A and resuspended in 50 µ1 ice-cold buffer C. The sample is then vigorously rocked at 4 $^{o}$C for 30 min on a shaking platform and then centrifuged for 5 min at 10,000 X g at 4 °C. The supernatant (~ 55 µ1) was transferred into a fresh eppendorf tube and stored at -80 °C. In case of rat tissues 100 mg rat brain samples were homogenized on ice with a tissue tearer in 500 µl of buffer A and processed further in a method similar to that of extraction of lymphocyte nuclear extracts.

**Labeling the oligonucleotide probe**

**A. Preparing probes for Phosphorylation reaction**

W18 and M16 probes were obtained as single stranded oligonucleotides from Genetix Biotech Asia Pvt. Ltd.

W18 forward- 5'-GCCCTACGTGCTGTCTCA- 3'
W18 reverse-5'-TGAGACAGCACGTAGGGC-3'
M16 forward-5'-CCTAAAAGCTGTCTCA-3'
M16 reverse-5'-TGAGACAGCTTTTAGG-3'

The probes were first annealed to synthesize double stranded probes. Both forward and reverse strands of each probe were taken and approximately 5 µg of W18 and M16 probes, containing 400 pmol of available 5' ends were first annealed in annealing buffer. Annealing was carried out in a thermal cycler (Techgene, USA) adjusted to carry out the following temperature changes in 2 cycles. After annealing, 100 pmol of the annealed dsDNA probe was used for labeling 5'-$PO_4^{-3}$ with [γ-$^{32}$P] ATP.

| Temperature ($^{o}$C) | Duration (min) |
|---|---|
| 88 | 2 |
| 65 | 10 |
| 37 | 10 |
| 21-23 (RT) | 5 |

**B. Phosphorylation Reaction**

**The following reaction was assembled in a sterile microcentrifuge tube**

| | | |
|---|---|---|
| a. | Consensus labeled Oligonucleotide (22 pmoles/µl) | 2.0 µl |
| b. | T4 Polynucleotide Kinase 10X Buffer | 2.5 µl |
| c. | [γ-$^{32}$P]ATP (3,000Ci/mmol at 10mCi/ml) | 20.0 µl |
| d. | T4 Polynucleotide Kinase (5–10u/µl) | 1.0 µl |
| e. | Total volume | 25.5 µl |

The reaction mix was incubated at 37 °C for 15 minutes. 2 µl of cold ATP (10 pmoles) was added to the reaction mixture and the reaction was further incubated for 30 min. after incubation, the reaction was stopped by heating at 65 $^{o}$C. The labeled oligonucleotide was further purified to remove unincorporated [γ-$^{32}$P] ATP using sephadex G-25 gel filtration column.

**C. Purification of labeled oligonucleotide probe.**

2 ml of sephadex G-25 matrix was packed into a 5 ml column. After washing extensively with autoclaved MΩ water, void volume was calculated for each column, which was approximately 1 ml. [γ-$^{32}$P] ATP labeled W18 probe was loaded onto the column. 100 µl of autoclaved MΩ was let to pass through the column for up to 4 times and discarded. From the 5$^{th}$ 100 µl loaded, up to 12 fractions of 100 µl each were collected. The same procedure was followed to purify labeled M16 probe in a separate 2 ml sephadex G-25 column. After purification 5 µl each of fractions 3,5,7 and 9 were loaded onto a 20% native polyacrylamide gel and the probes were electrophoresced at 30 mA. After electrophoresis, the gel was dried at 80 $^{o}$C on a gel drier (comp name) connected to vacuum for 1 hr. The dried gel was exposed overnight to a Kodak X-ray film (XK-5 grade, USA) and then subjected to autoradiography. An aliquot of 5 µl was removed for determination of percent incorporation from the fractions showing maximum labeling as observed from the developed autoradiogram.

**D. Determination of Percent Incorporation**

1µl of the labeled oligonucleotide was spotted onto Whatman ® DE81 filter paper strips. The filter papers were dried into individual vials, and counted in Beckman's liquid scintillation counter. Under the labeling conditions described above, typically 50% or more of the radioactivity is incorporated in the 5′ end-labeling reaction. Ideally, the specific activity of the labeled oligonucleotide will be 1,00,000–2, 00,000 cpm per µl.

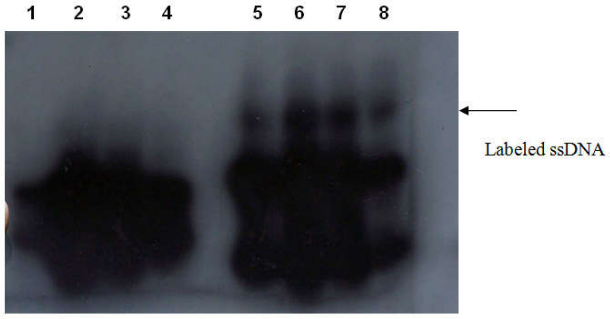


**Fig 1. Autoradiogram of W18 and M16 oligonucleotide probes after 5'-end labeling with [γ–$^{32}$P] ATP**

1µg of synthetic W18 and M16 dsDNA probes were labelled at 5'-$PO^{3-}_4$ end with [γ–$^{32}$P] ATP using the enzyme T4 polynucleotide kinase (Genei, Bangalore). The labeled probe was then purified to remove unincorporated [γ–$^{32}$P] ATP using Sephadex G-25 column. The purified probe was loaded onto 20% native polyacrylamide gel and electrophoresced. The gel was then subjected to autoradiography.

Lanes 1-4= fractions 3,5,7,9 of labelled W18 probe; Lanes 5-8= fractions 3,5,7,9 of labelled M16 probe.

**Electrophoretic Mobility Shift Assay (EMSA)**

After the preparation of nuclear extracts and successful labeling of W18 and M16 probes, EMSA was carried out. Binding reaction was assembled using 5-10 µg nuclear extract and 5 X binding buffer. The reaction mixture was incubated on ice or at 4 $^{o}$C for 30 min and 4 µl of [□-$^{32}$P] ATP labeled W18 or M16 probe (approximately 1 X $10^{6}$ cpm or 0.2 pmol) was added to this and the mixture was incubated for 45 min to 1 h on ice or 4 $^{o}$C. Parallel reactions with only labeled probes without nuclear extracts were kept as controls. After incubation, samples were subjected to 6 % nondenaturing PAGE (pre run at 160 V for 15 min) and electrophoresis was performed at 180 V in 0.5 X TBE at 4 $^{o}$C. After the completion of electrophoresis, the gel was placed on a sheet of Whatman 3mm filter paper and covered with plastic wrap and dried at 80 $^{o}$C on a gel dryer connected to vaccum for 30 min . The dried gel was then exposed to Kodak (XK-5) X-ray film overnight at –70 $^{o}$C and autoradiographed.

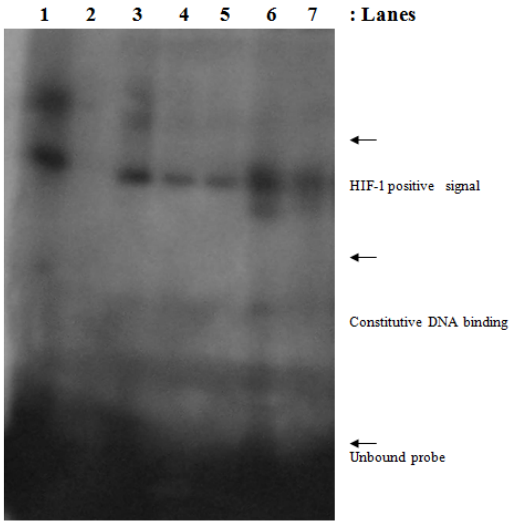


**Fig. 2. A representative autoradiogram of EMSA carried out with nuclear extracts from human lymphocytes exposed to hypoxia mimicking conditions**

Lymphocytes exposed to 1 mM $CoCl_2$ and 100 µM desferrioxamine were lysed to extract nuclear proteins. 10 µg of nuclear extracts were incubated with 1 pmole of 5'-[γ-$^{32}$P] labeled W-18 probe containing the wild type HRE element and M-16 probe containing mutated HRE sequence, in a binding reaction. The DNA-protein complex was then electrophoresced by 6 % native PAGE at 300 V. The figure shows image of the autoradiogram developed from the electrophoresced PAGE. Lane 1, 3 = treated with W18 probe (1= 100 µM Def; 3 = 1 mM $CoCl_2$); Lane 2 = only probe; Lane 4, 5 = treated with M16 probe (4= 100 µM Def; 5 = 1 mM $CoCl_2$); Lane 6, 7 = treated with W18/M16 (Control lymphocyte samples).

## RESULTS

### Labeled oligonucleotide W18 and M16 probes

The oligonucleotide probes containing HRE element were labeled and purified using sephadex G-25 gel filtration columns. In this method, fractions 3 to 11 were observed to have optimal labeling (*Fig* 3.1) and hence were used further for EMSA. We could detect up to less than 0.1 pmol of labeled probe bound to HIF-1.

### EMSA with lymphocytes incubated in HIF-1α stabilizing conditions

In order to detect HIF-1α, electrophoretic mobility shift assays were carried out. The principle behind the assay is that HIF-1α once stabilized dimerizes with its HIF-1β subunit in the nucleus and the HIF-1 complex thus formed binds specific DNA sequences known as Hypoxia Responsive Elements. A synthetic oligonucleotide probe is custom synthesized (W18) which binds active HIF-1α in nuclear extracts and when the extracts are separated on 5% native acrylamide gels, the DNA-protein complex mobility is retarded, while unbound radiolabeled DNA probe W18 will run faster along with tracking dye. The retarded radioactive bands seen in autoradiogram indicate the induction of HIF-1 in the cell extract. To confirm the specificity of probe and HIF-1 interactions, a mutated probe M16 was used as a negative control (*Fig* 3.2). Also, to confirm translocation of HIF-1α subunit into the nucleus, EMSA was carried out in a similar fashion with cytosolic extracts. As observed in *Fig* 3.3, HIF-1 was observed to be absent in the cytoplasm both in control and hypoxia mimicking conditions indicating its rapid degradation in normoxia and swift translocation into the nucleus in stabilizing conditions.

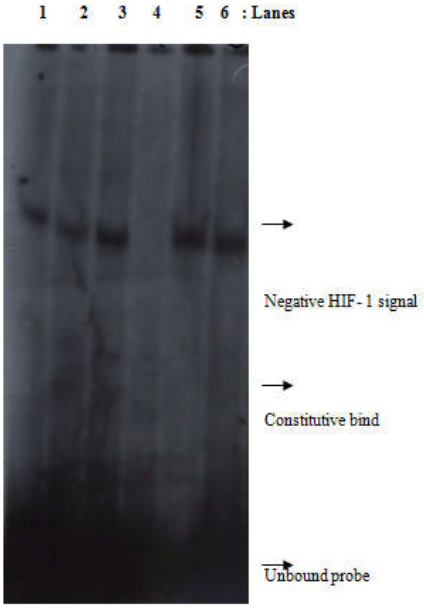


**Fig. 3. A representative autoradiogram of EMSA carried out with cytosolic extracts from human lymphocytes exposed to hypoxia mimicking conditions**

Lymphocytes exposed to 1 mM $CoCl_2$ and 100 µM desferrioxamine were lysed to extract nuclear proteins. 10 µg of nuclear extracts were incubated with 1 pmole of 5'-[γ-$^{32}$P] labeled W18 probe containing the wild type HRE element and M16 probe containing mutated HRE sequence, in a binding reaction. The DNA-protein complex was then electrophoresced on 6 % native PAGE at 300 V. The figure shows image of the autoradiogram developed from the electrophoresced PAGE. Lane 1 = control cytosolic extract with W18 probe; Lane 2, 3 = treated with W18 probe (2= 100 µM Def; 3 = 1 mM $CoCl_2$); Lane 4= only probe; lane 5, 6 = treated with M16 probe (5= 100 µM Def; 6 = 1 mM $CoCl_2$).

### Exposure to high altitude simulating conditions induces hypoxia

Rats exposed to high altitude simulating conditions for a period of 5 days were sacrificed and their brain nuclear extracts were used to carry out EMSA. Autoradiogram of EMSA thus carried out indicated stabilization and nuclear translocation of HIF-1α (*Fig* 3.4).

## DISCUSSION

Hypoxia resulting from high altitude exposure or mimicking conditions is known to cause significant changes in cellular gene expression (Ward and West, 1995; Guo *et al.*, 2006). HIF-1 is one of the most important transcription factors that induces up-regulation of specific proteins including VEGF, EPO, heme oxygenase1, inducible

nitric oxide synthase, aldolase, enolase, LDH, etc (Semenza, 2002). In this work, we used HIF-1 stabilisation as marker of hypoxic induction. Freshly isolated adult human lymphocytes were isolated and exposed

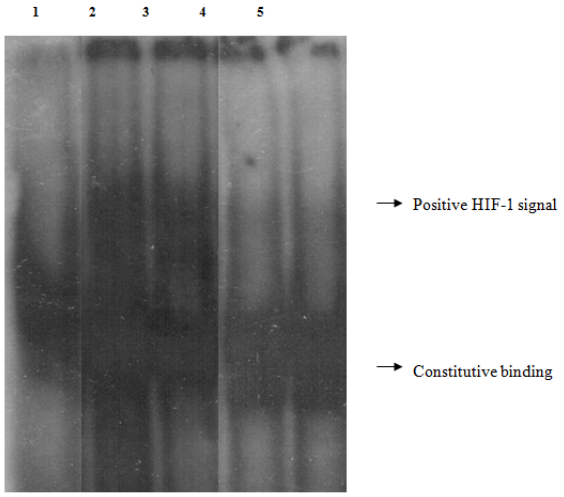


**Fig. 4. A representative autoradiogram of EMSA Carried out with brain nuclear extracts from rats exposed to hypobaric hypoxia**

Rats exposed to high altitude simulating conditions at an oxygen partial pressure corresponding to 7500 m were sacrificed after 5 days. Brain samples were extracted and homogenized to extract nuclear extracts. 10 µg of the extracts were used in a binding reaction. 10 µg of nuclear extracts were incubated with 1 pmole of 5'-[γ-$^{32}$P] labeled W18 probe containing the wild type HRE element and M16 probe containing mutated HRE sequence, in a binding reaction. The DNA-protein complex was then electrophoresced on 6 % native PAGE at 300 V. The figure shows image of the autoradiogram developed from the electrophoresced PAGE. Lane 1= control nuclear extracts with W18; Lane 2, 3 = hypoxia nuclear extracts with W18; Lane 4, 5 = hypoxia nuclear extracts with M16 (hypoxia).

to hypoxia mimicking conditions using $CoCl_2$ or desferrioxamine (Guo, *et al.*, 2006). After exposure nuclear and cytosolic extracts were prepared from the lymphocytes and EMSA was carried out using an 18 bp oligonucleotide probe and containing wild type HRE element, and a 16 bp oligonucleotide probe containing mutated HRE element (Miyazaki *et a.l*, 2002). *Fig* 2.1 indicates stabilization of HIF-1α and its nuclear translocation and dimerisation with HIF-1β, thus confirming induction of HIF-1α stabilizing conditions. We further proceeded towards standardizing conditions of hypoxia using hypobaric decompression chamber. Rat brain nuclear extracts on examination by EMSA showed translocation of HIF-1 complex. This finding is concurrent with the evidence that hypoxia stabilizesHIF1-α, its translocation into the nucleus and dimerization with HIF1-β subunit. This dimerization results in its DNA binding activity and activation of downstream gene expression. HIF-1 is known to bind hypoxia response element in more than fifty genes (Semenza, 2001). This activity regulates adaptation to high altitude and also modulates gene expression in hypoxia like conditions like cancer, Carbon monoxide exposure, pulmonary and cerebral ischemia, stroke, etc (Kumar and Klein, 2004). Hence the presence of HRE in such genes is a reliable indicator in analysis of conditions that influence their expression.

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