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Nucleus Pulposus Cells Express HIF-1α Under Normoxic Culture Conditions: A Metabolic Adaptation to the Intervertebral Disc Microenvironment

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Abstract Nucleus pulposus (NP) cells of the intervertebral disc reside in an environment that has a limited vascular supply and generate energy through anaerobic glycolysis. The goal of the present study was to examine the expression and regulation of HIF-1α, a transcription factor that regulates oxidative metabolism in nucleus pulposus cells. Nucleus pulposus cells were isolated from rat, human, and sheep disc and maintained at either 21% or 2% oxygen for various time periods. Cells were also treated with desferrioxamine (Dfx), a compound that mimics the effects of hypoxia (Hx). Expression and function of HIF-1α were assessed by immunofluorescence microscopy, Western blot analysis, gel shift assays, and luciferase reporter assays. In normoxia (Nx), rat, sheep, and human nucleus pulposus cells consistently expressed the HIF-1α subunit. Unlike other skeletal cells, when maintained under low oxygen tension, the nucleus pulposus cells exhibited a minimal induction in HIF-1α protein levels. Electromobility shift assays confirmed the functional binding of normoxic HIF-1α protein to its putative DNA binding motif. A dual luciferase reporter assay showed increased HIF-1α transcriptional activity under hypoxia compared to normoxic level, although this induction was small when compared to HeLa and other cell types. These results indicate that normoxic stabilization of HIF-1α is a metabolic adaptation of nucleus pulposus cells to a unique oxygen-limited microenvironment. The study confirmed that HIF-1α can be used as a phenotypic marker of nucleus pulposus cells. J. Cell. Biochem. 98: 152–159, 2006.

Key words: intervertebral disc; nucleus pulposus cells; HIF-1α; hypoxia; microenvironment; normoxic stabilization; metabolic adaptation

In eukaryotes, a continual supply of oxygen is required to drive oxidative phosphorylation, the major energy generating system of the cell. The actual oxygen requirements are tissue specific. In some tissues, a momentary decrease in the oxygen supply is sufficient to cause irreversible damage. In those tissues where energy generation is not dependent on oxidative metabolism the oxygen needs are more modest. The oxygen requirement of connective tissues is not known with certainty. Of these tissues, almost nothing is known of the tissues of the intervertebral disc. However, there is reason to believe that as the cells of the intervertebral disc reside in an environment that has a limited vascular supply, there is downregulation of oxidative metabolism [Bartels et al., 1998; Urban, 2002; Urban et al., 2004]. Our previous work has shown that in this specialized microenvironment, nucleus pulposus (NP) cells express HIF-1α, a key transcription factor that regulates anaerobic metabolism [Rajpurohit et al., 2002].

HIF-1α is a member of the basic helix-loop-helix (bHLH)-PER-ARNT-SIM (PAS) family of proteins and composed of a constitutively expressed β subunit and an α subunit that is

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rapidly degraded under normoxic conditions, but stable under hypoxic conditions [Wang et al., 1995]. Transactivation of HIF-1 target genes involves dimerization of the two subunits and binding to an enhancer element the hypoxia-response element (HRE) in target genes [Semenza, 2002]. The bHLH and PAS motifs are required for dimerization while the downstream basic region affords specific binding to the HRE DNA sequence 5′-RCGTG-3′ [Semenza, 2002]. HIF-1α promotes transcription of key glycolytic enzymes that allows a cell to switch from aerobic to anaerobic metabolism [Firth et al., 1995; Semenza et al., 1996; Riddle et al., 2000; Obach et al., 2004].

The goal of the present study is to examine the expression and regulation of HIF-1α in nucleus pulposus cells. Surprisingly, it was found that unlike other mammalian cells, this protein is not significantly induced under low oxygen tension, although a modest increase in transcriptional activity was observed. This finding lends strength to the view that cells of the nucleus pulposus are functionally adapted to an avascular hypoxic environment.

MATERIALS AND METHODS

Cell Isolation and Culture

Male Wistar rats (200–250 g) were euthanized with CO_2. The spinal columns, rib cartilage, and long bones were removed under aseptic conditions. Lumbar intervertebral discs were separated from the spinal column, while bone and cartilage pieces were collected from calvaria and ribs, respectively. Human disc tissue (minimally degenerative) was obtained from patients undergoing cervical spinal surgeries as per a protocol approved by Institutional Review Board of the Thomas Jefferson University. The gel-like nucleus pulposus was separated from the annulus fibrosus using a dissecting microscope and both the tissues were treated with 0.1% collagenase and 10 U/ml hyaluronidase for 4–6 h separately. The partially digested tissue was maintained as an explant in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS) supplemented with antibiotics in a humidified atmosphere containing 5% CO_2 at 37°C. Nucleus pulposus and annulus fibrosus cells migrated out of the explant after 1 week. When confluent, the cells were lifted using trypsin (0.25%) EDTA (1 mM) solution and sub-cultured in 10 cm dishes. Rat chondrocytes (R-CD) and calvarial osteoblasts were isolated and cultured as reported previously [Masquelier et al., 1990; Lin and Oberbauer, 1999].

Cells were cultured in a hypoxia work station (Invivo$_2$, Ruskinn, UK) with a mixture of 2% O_2, 5% CO_2, 93% N_2 for 24–72 h. To induce chemical hypoxia some cells were treated with 130 μM desferrioxamine (Dfx) for 24 h.

Immunofluorescence Microscopy

Cells were plated in flat bottom 96-well plates and maintained in normoxia (Nx) or hypoxia (Hx) for 24 h. After incubation, cells were fixed with 4% paraformaldehyde on ice for 20 min and washed with phosphate buffered saline (PBS). Cells were permeabilized with 0.5% Triton-X 100 in PBS for 15 min and then incubated in blocking solution (PBS containing 10% FBS) for 45 min. Cells were then incubated with anti-HIF-1α antibody (ABR, Golden, CO) in blocking solution at a dilution of 1:200 at 4°C overnight. As a negative control, cells were reacted with mouse isotype IgG under similar conditions. After thoroughly washing the cells with PBS, the experimental and control cells were incubated with Alexa Fluor-488 conjugated anti-mouse secondary antibody (Molecular Probes, St. Louis, MO), at a dilution of 1:50 for 1 h at room temperature. After washing, some cells were also incubated with 5 μM propidium iodide for 20 min at room temperature. Cells were imaged using a laser scanning confocal microscope (Olympus Fluoview, Japan).

Nuclear Extracts and Western Blotting

Nuclear extracts were prepared from sub-confluent cells according to the method of Dignam et al. [1983] using the CellLytic NuCLEAR extraction kit (Sigma-Aldrich, St. Louis, MO). After desired culture period in hypoxia or normoxia cells were immediately placed on ice to prevent oxidative degradation of HIF-1α. Cells were then placed in hypotonic buffer (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT) and incubated on ice for 15 min. Igeapal CA-630 was added to a final concentration of 0.6% and the mixture was vortexed vigorously for 10 s. Nuclei were recovered by centrifugation at 3,300*g* for 30 s at 4°C and extracted by gentle shaking in buffer containing 20 mM HEPES pH 7.9, 0.42 M NaCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 0.5 mM DTT for 30 min at 4°C. The extract

was then centrifuged for 15 min at 25,000*g*, and the supernatant was snap frozen at −70°C. All buffers contained a protease inhibitor cocktail: 2 mM 4-(2-aminoethyl) benzenesulfonylfluoride, 1.4 pM *trans*-epoxysuccinyl-L-leucylamido [4-guanidinobutane], 130 pM bestatin, 1 μM leupeptin, and 0.3 pM aprotinin; and phosphatase inhibitors 5 mM NaF and 200 μM Na_3VO_4.

Nuclear extracts were resolved on 10% SDS–polyacrylamide gels. Proteins were transferred by electroblotting to nitrocellulose membranes (Bio-Rad, CA). The membranes were blocked with 5% non-fat dry milk in TBST (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween 20) and incubated overnight at 4°C in 3% non-fat dry milk in TBST with the anti-HIF-1α antibody; 1:500 (R&D Systems, CA and Novus Biologicals, CA). Immunolabeling was detected using the enhanced chemiluminescence reagent (ECL, Amersham Biosciences).

Electrophoretic Mobility Shift Assays

Electromobility shift assays were carried out as previously described with minor modifications [Stokes and Perry, 1995; Stokes et al., 2001]. Briefly, the binding reaction was perfomed in a solution containing 12.5 mM HEPES, pH 7.9, 50–100 mM NaCl, 5% glycerol, 2 mg/ml BSA, 1–2 μg poly-dIdC, 0.1 mM EDTA, 0.1 mM DTT, 0.5–1 ng of ^{32}P-end-labeled double-stranded oligonucleotide VEGF probe (top strand sequence 5′acagtgcatacgtgggctccaac3′) or 4–5 ng end-labeled PCR-generated probe (95 bp) (SantaCruz Biotechnology) and 10–15 μg of nuclear protein. Following incubation for 30 min at room temperature, extracts were loaded onto 5% acrylamide-0.5× tris-borate-EDTA gels electrophoresed at 130 V for 2–3 h. To demonstrate the specificity of binding, nuclear extracts were also incubated with a probe containing a mutation in HRE. To perform supershift experiments, 1–2 μl (1 mg/ml) of anti-HIF-1α antibody (Novus, CA) was added to the binding reaction for 25 min at room temperature, prior to the addition of labeled DNA probe. After addition of labeled DNA-probe, the reactants were incubated together for an additional 20 min at room temperature. Gels were vacuum dried and exposed to X-ray film at −70°C.

Reporter Plasmids

Reporter plasmids p2.1 and p2.4 harbor a 68-base pair sequence from ENO1 promoter (nt −416 to −347) flanked by *Kpn*I and *Xho*I restriction sites inserted upstream of an SV40 promoter and *Photinus pyralis* (firefly) luciferase coding sequences are described previously [Semenza et al., 1996] (kindly provided by Dr. Gregg Semenza). In p2.4, 3-bp substitutions (CGT–AAA) were introduced that disrupt HIF-1 binding sites at the 5′ and 3′ ends of the 68-bp sequence. As an internal transfection control, vector pRL-TK (Promega) containing *Renilla reniformis* (sea pansy) luciferase genes under the control of the herpes simplex virus thymidine kinase (HSV-TK) promoter was used. The amount of transfected plasmid, the pre-transfection period after seeding, and the post-transfection period before harvesting were optimized for rat nucleus pulposus cells using pSV β-galactosidase plasmid (Promega). For HeLa cells, we utilized the manufacturer's optimized protocol.

Transfections and Dual Luciferase Assay

Nucleus pulposus and HeLa cells were transferred to 24-well plates at a density of 5×10^4 cells/well on the day before transfection. LipofectAMINE 2000 (Invitrogen, CA) was used as a transfection reagent. For each transfection, 500 ng of reporter gene plasmid (p2.1 or p2.4) and 500 ng of control plasmid pTK-RL were premixed with the transfection reagent. Forty-eight hours after the transfection, some cells were exposed to hypoxia or maintained in normoxia overnight. After treatment, the cells were harvested and a Dual-Luciferase™ reporter assay system (Promega) was used for sequential measurement of firefly and Renilla luciferase activities with specific substrates that is, beetle luciferin and coelenterazine, respectively. Quantification of luciferase activities and calculation of relative ratios were carried out using a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA); at least three independent transfections were performed in triplicate.

Statistical Analysis

Data were analyzed by *t*-test; the obtained *P*-values are indicated in the text and figures. Quantitative data is representative of three experiments (n = 3).

RESULTS

Nucleus Pulposus Cells Show Stable Expression of HIF-1α Under Normoxic Conditions

Figure 1 shows expression of HIF-1α protein by both rat and human nucleus pulposus cells. HIF-1α protein expression by nucleus pulposus cells was compared with expression by annulus fibrosus cells, chondrocytes and osteoblasts cultured in both hypoxia and normoxia. Both rat and human nucleus pulposus cells showed high HIF-1α staining in normoxia compared with annulus fibrosus cells. Similarly, normoxic expression of HIF-1α by rat chondrocytes and osteoblasts was less than HIF-1α expression by rat nucleus pulposus cells. As might be expected, the HIF-1α protein is present in all the cells cultured at 2% oxygen. Unlike other skeletal cells, there is little change in HIF-1α staining in nucleus pulposus cells when maintained under normoxic or hypoxic conditions.

Nucleus Pulposus Cells Show Nuclear Localization of HIF-1α Protein in Normoxia with Little Hypoxic Induction

Figure 2A shows the time dependence of HIF-1α expression by nucleus pulposus cells under hypoxic and normoxic conditions. There is an immediate and robust expression of HIF-1α under normoxic conditions. The level of expression did not change over 48-h period. A comparable level of expression is seen in cells maintained at 2% oxygen till 48 h. When the cells were fractionated, HIF-1α protein was localized to the nuclear fraction (Fig. 2B). This localization was evident in cells maintained in

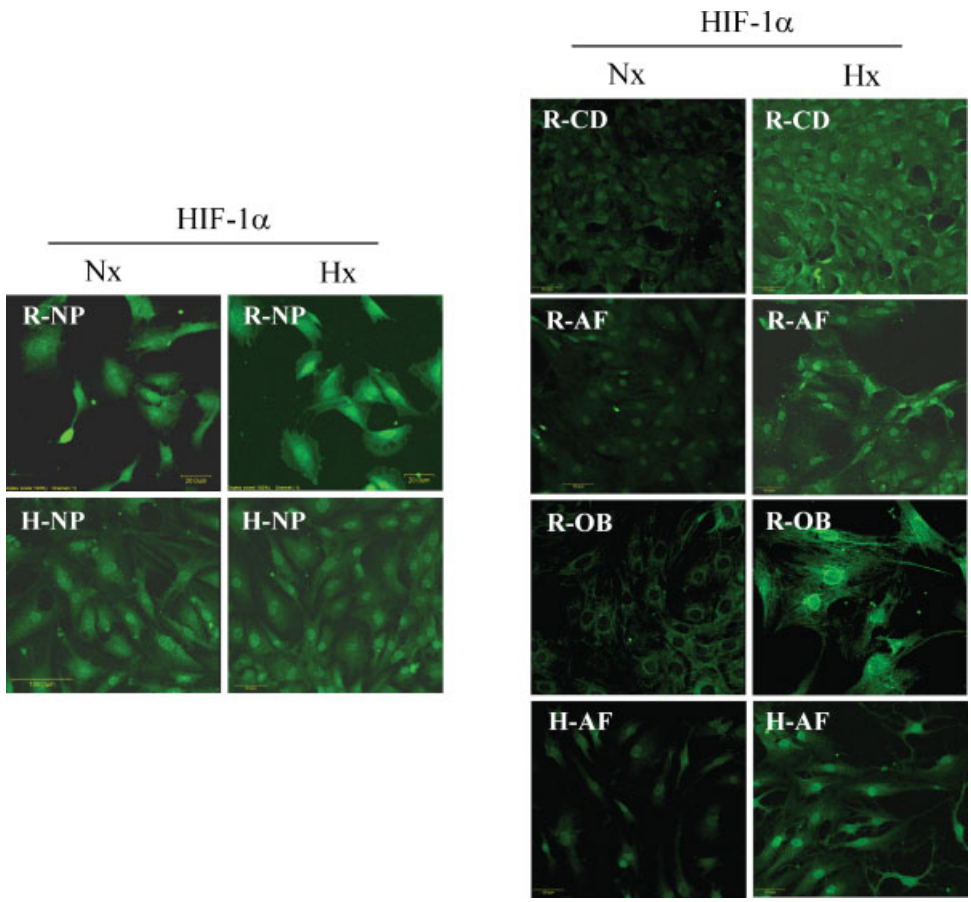

Fig. 1. Expression of HIF-1α protein by nucleus pulposus (NP) and other skeletal cells under hypoxic and normoxic conditions. Nucleus pulposus cells were grown in normoxia (Nx) and hypoxia (Hx) and treated with an antibody to HIF-1α. The cell nuclei were stained with propidium iodide. HIF-1α is expressed by nucleus pulposus cells in normoxia. In contrast, rat chondrocytes (R-CD), rat and human annulus fibrosus (R-AF and H-AF) cells and rat osteoblasts (R-OB) express minimal level of this protein in normoxia. When the cells are cultured in 2% O_2, the latter cells (R-CD, R-AF, H-AF, and R-OB) upregulate expression of HIF-1α. In contrast, in hypoxia, there is little induction in HIF-1α levels in nucleus pulposus cells. Isotype and secondary antibody controls were negative for staining (not shown). Mag. ×20.

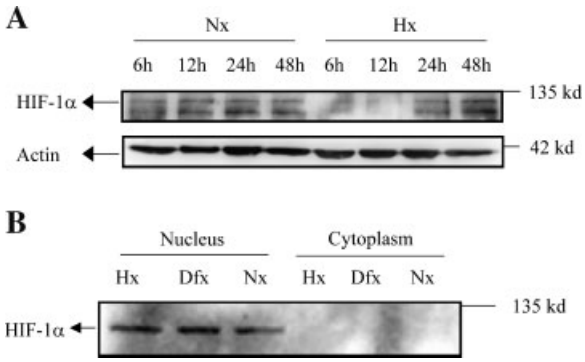

Fig. 2. Expression of HIF-1α protein by nucleus pulposus cells. **A**: Rat nucleus pulposus cells were cultured at 21% (Nx) and 2% (Hx) oxygen for various time periods and total cell protein was isolated. 40 μg of total cell protein was resolved by SDS–PAGE and blots were probed with an antibody to HIF-1α. Note, by 24 h there is similar levels of HIF-1α expression (shown by an arrow) by cells maintained under normoxic and hypoxic conditions. Moreover, in hypoxia there appears to be no time-dependent increase in HIF-1α expression. **B**: Rat nucleus pulposus cells were cultured under normoxic (Nx) and hypoxic (Hx) conditions; some cells were treated with desferrioxamine (Dfx), a hypoxia mimic for 24 h. Nuclear and cytoplasmic proteins were separated and analyzed by Western blot. Note that in both Nx and Hx, HIF-1α is localized to the nuclear fraction.

normoxia as well as hypoxia. Nuclear localization was also evident in nucleus pulposus cells treated with desferrioxamine, an agent that causes chemical hypoxia. To confirm that HIF-1α expression by nucleus pulposus cells was evident in normoxia and localized to the nucleus, cells from a number of species were examined (Fig. 3). Thus, nuclear extracts of nucleus pulposus cells isolated from rat, sheep, and human intervertebral discs was assessed by Western blot analysis. Figure 3 shows that nucleus pulposus cells evidenced a high basal level of HIF-1α expression in normoxia. In contrast, annulus fibrosus cells, chondrocytes and osteoblasts cultured at 21% O_2 showed very little or no HIF-1α protein. There was a significant induction of this protein when these control skeletal cells were cultured in a low-oxygen environment or treated with Dfx.

Normoxic HIF-1α Binds to HRE and is Transcriptionally Active

To assess whether the HIF-1α retains its DNA binding and transcriptional activity, the rat and human nuclear extracts were examined by the electromobility shift assay. Figure 4A shows that there was a small increase in binding to probe derived from human ceruloplasmin gene promoter when extracts of hypoxic cells were used. Likewise, higher level of binding was evident in hypoxia when the HRE probe (from VEGF promoter) was incubated with nuclear extracts from rat and human nucleus pulposus cells (Fig. 4B). Incubation with mutant probe abolished the binding of HIF-1α protein to the HRE resulting in significant reduction in binding signal (Fig. 4A). Likewise, when nuclear extracts from nucleus pulposus cells were incubated with anti-HIF-1α antibody prior to incubation with labeled HRE probe, a super shift (gel retardation of oligo-HIF-1α complex) was observed (data not shown).

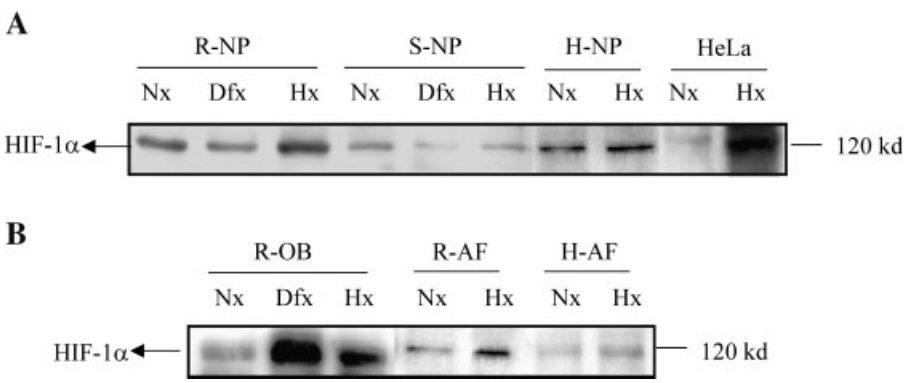

Fig. 3. Expression of HIF-1α by nucleus pulposus and other skeletal cells. **A**: Western blot analysis of nuclear fraction of rat (R), sheep (S), and human (H) nucleus pulposus (NP) cells maintained under normoxic (Nx) and hypoxic (Hx) conditions. Rat and sheep nucleus pulposus cells were also treated with desferioxamine (Dfx) for 24 h. Note, all of these cells express HIF-1α under normoxic condition. In hypoxia, there is a little increase in HIF-1α expression by rat nucleus pulposus cells. No increase is evident in cells from the sheep or human nucleus pulposus. Moreover, Dfx does not increase HIF-1α expression by all species examined. In contrast, control HeLa cells show strong induction of HIF-1α protein in hypoxia. **B**: Expression of nuclear HIF-1α protein by osteoblast (OB), rat annulus fibrosus (R-AF), and human annulus fibrosus (H-AF) cells. In all these cells, there is induction of HIF-1α in hypoxia, or after treatment with Dfx.

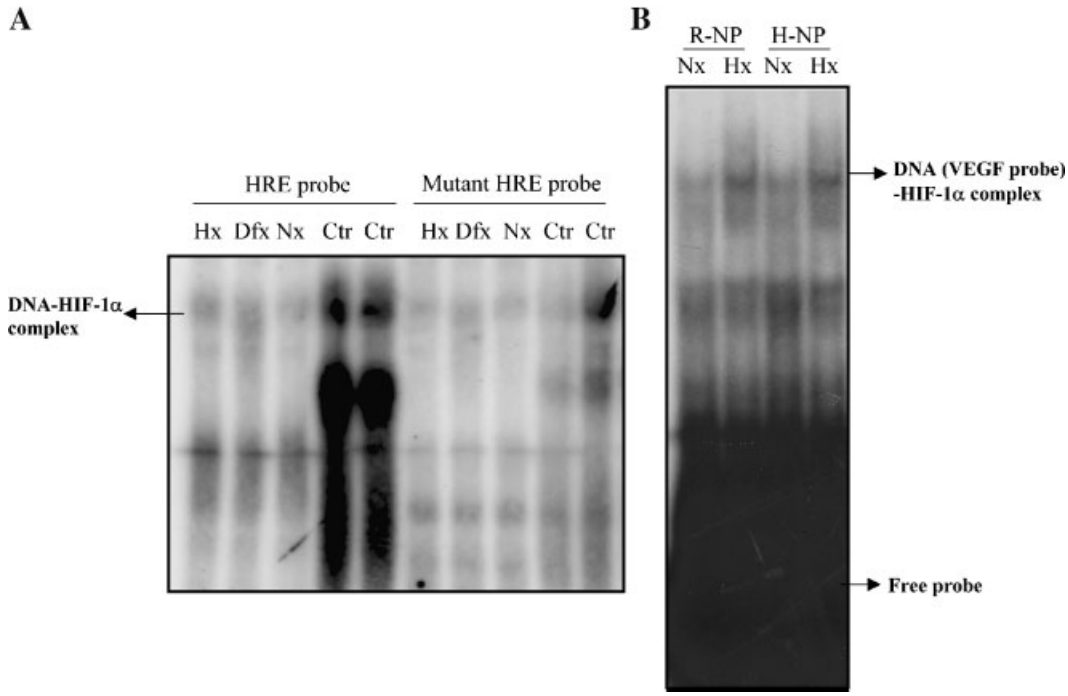

Fig. 4. Electromobility shift assay to examine the functional activity of HIF-1α protein. **A**: Nuclear protein was isolated from rat nucleus pulposus cells cultured at 21% and 2% oxygen tension. Ten micrograms of nuclear protein was incubated with 0.5–1 ng of ^{32}P-end-labeled double-stranded oligonucleotide probes containing HRE. For confirming the specificity of the binding, mutated HRE probe was used in the binding reaction. Binding reaction was resolved on polyacrylamide gel. Note that under normoxic condition (Nx), binding of HIF-1α protein to the DNA probe is evident. There is more pronounced binding under hypoxia (Hx) than in Nx or treated with Dfx. Control for this study included nuclear extracts of HTB5 cells (human chondrosarcoma) cultured under hypoxia (Ctr). Note that there is a significant reduction in binding when a mutant HRE probe is used. **B**: EMSA using nuclear extracts from rat and human nucleus pulposus cells. Both rat and human nucleus pulposus cells show binding under normoxic conditions. Note that increased binding is observed under hypoxia indicating a higher proportion of functional protein.

As a further evaluation of the effect of hypoxia on HIF-1α expression, transcriptional activity was assessed using luciferase reporter constructs containing wild-type and mutant HRE. Figure 5A shows high basal luciferase activity of nucleus pulposus cells in nomoxia with a twofold increase when they were cultured in hypoxia. In contrast, HeLa cells showed 70–100 fold induction in the luciferase activity over normoxia after exposure to hypoxia (Fig. 5B) with minimal basal luciferase activity under normoxic condition. Transfected with mutated control plasmid there was little difference in luciferase activity in normoxia and hypoxia (Fig. 5A).

DISCUSSION

The current study is an extension of our earlier work that was directed at identifying unique phenotypic markers of nucleus pulposus cells [Rajpurohit et al., 2002]. Previously, we have shown that HIF-1α, a key transcription factor, can be used as a phenotypic marker to distinguish nucleus pulposus cells from annulus fibrosus and cartilage endplate cells. However, it could be argued that since HIF-1α protein is sensitive to the environmental oxygen tension, its expression level provides an index of the current metabolic or oxemic status of the tissue. From this perspective, the HIF-1α is of limited use as a phenotypic marker protein. The results of the present study indicates that unlike cells of the annulus fibrosus or cells of other skeletal tissues, nucleus pulposus cells express functionally active HIF-1α protein under normoxic condition. More importantly, by culturing nucleus pulposus cells in a low-oxygen environment we show that at best there is a modest increase in HIF-1α transcriptional activity. These results emphasize that HIF-1α can be used as a phenotypic marker of the nucleus pulposus cells and lends strength to the notion that the cells mature in a unique microenvironment.

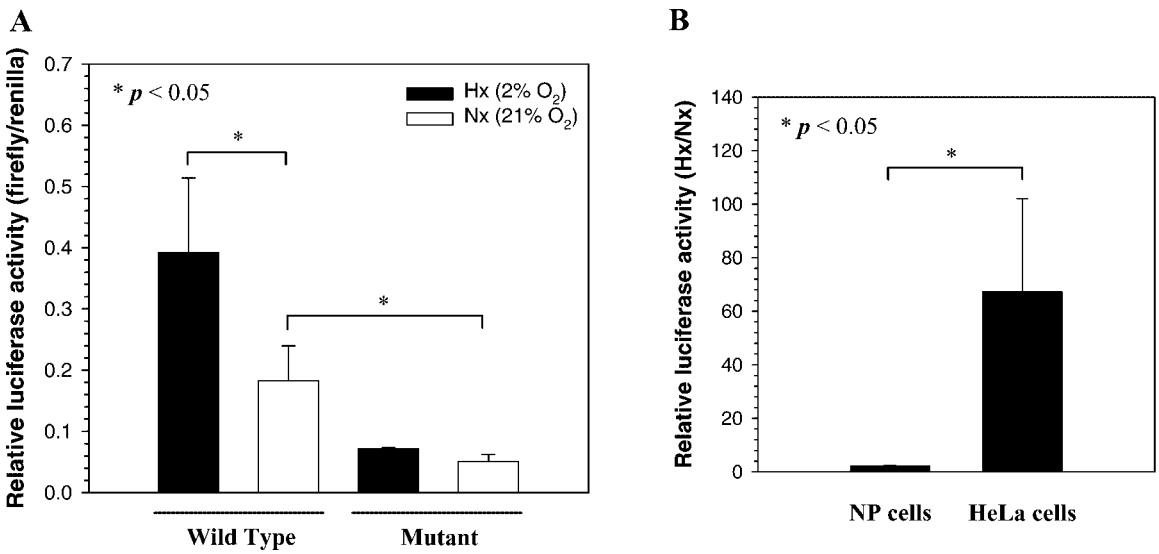

Fig. 5. Evaluation of transcriptional activity of normoxic HIF-1α protein by dual-luciferase assay. **A**: Reporter plasmids harboring a wild-type (p2.1) or a mutant (p2.4) HRE, respectively, driving SV40 promoter and firefly luciferase were transfected into rat nucleus pulposus cells along with pRL-TK vector containing renilla luciferase gene. Cells were cultured under normoxia (Nx) or hypoxia (Hx). Note that nucleus pulposus cells show high luciferase activity under normoxia with approximately twofold increase in activity in hypoxia. Cells transfected with mutant plasmid (p2.4) showed very low luciferase activity. **B**: Relative expression of luciferase in hypoxia compared to normoxic levels by rat nucleus pulposus and HeLa cells. HeLa cells show a significantly higher induction (70–100 fold) in luciferase activity over nucleus pulposus cells ($P < 0.05$).

Since the vascular architecture of the spine varies slightly across animal species, it was important to determine if there was a singular pattern of HIF-1α expression in nucleus pulposus cells. Predictably, at 21% oxygen, Western blot and immunofluorescence analysis showed that HIF-1α was expressed in nucleus pulposus cells isolated from rat, sheep, and human disc. However, in contrast to other skeletal cells, including annulus fibrosus, chondrocytes and osteoblasts, where a decrease in the pO_2 caused a strong induction of HIF-1α protein, there was at best a little change in nucleus pulposus HIF-1α expression level. This finding was surprising as HIF-1α protein is labile in an oxygen-rich environment and elicits a rate of degradation that is proportional to the oxygen tension [Wang et al., 1995]. Thus, conservation of HIF-1α expression under normoxic condition probably reflects a specialized metabolic need of this cell type within the intervertebral disc environment.

Using Western blot analysis, we further evaluated the expression of HIF-1α protein and its sub-cellular localization. Both at 2% and 21% oxygen, HIF-1α was mostly localized to the nuclear fraction of nucleus pulposus cells. This observation was consistent across the number of animal species. In contrast, both Western blot and immunofluorescence analysis indicated that annulus fibrosus cells, chondrocytes and osteoblasts showed low-level expression of HIF-1α at 21% oxygen, with significant protein induction at 2% oxygen. This observation is in agreement with our previous work demonstrating differential HIF-1α expression by rat nucleus pulposus and annulus fibrosus cells in vivo [Rajpurohit et al., 2002]. These findings point to significant metabolic differences between nucleus pulposus cells and other cell types which is probably linked to differences in developmental cues as well as vascularization status [Coventry, 1969; Hassler, 1969; Rudert and Tillmann, 1993].

The transcriptional activity of HIF-1α was evaluated using the electromobility gel shift assay. HIF-1α expressed by normoxic nucleus pulposus cells was bound to the HRE. Specificity was confirmed by the mutant HRE probe and by supershift using anti-HIF-1α antibody. In hypoxia, there was increased binding to the HRE suggesting increased transcriptional activity of HIF-1α protein [Mukhopadhyay et al., 2000]. Likewise, the luciferase reporter assay driven by binding of HIF-1 protein to the HRE suggested that when cells were hypoxic there was increased transcriptional activity [Zhong et al., 2000; Slomiany and Rosenzweig,

2004]. From the perspective of transcriptional activity, hypoxia upregulated HIF-1 functional activity. While at first glance, this result may seem to be in contradiction with the Western blot or immunofluorescence analysis, scrutiny of the results show that the magnitude of increase in transcriptional activity was very limited. For example, HeLa cells evidenced approximately 10-fold increase in protein expression and about 100-fold increase in transcriptional activity. Likewise, annulus fibrosus and other skeletal cells evidenced significant increase in HIF-1α protein expression in hypoxia. Thus, while little discrepancies exist in both protein expression and transcriptional activity, the results emphasize that significant differences exist in the response of disc cells (nucleus pulposus versus annulus fibrosus) to the environmental oxygen tension. In summary, results of this study point to unique characteristics of nucleus pulposus cells and their exquisite adaptation to the hypoxic microenvironment.

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