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Hypoxia induces transcription of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-4 gene via hypoxia-inducible factor-1α activation

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Abstract The PFKFB4 gene encodes isoenzyme of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB or PFK-2/FBPase-2) which originally was found in the testes. We have studied hypoxic regulation of PFKFB4 gene in prostate cancer cell line, PC-3, and several other cancer cell lines. It was shown that hypoxia significantly induced PFKFB4 mRNA levels in PC-3 as well as in HeLa, Hep3B and HepG2 cell lines. Hypoxia increased PFKFB4 protein levels also. Moreover, desferrioxamine and cobalt chloride, which are known to mimic hypoxia, also had a stimulatory effect on the expression of PFKFB4 mRNA. In order to investigate the mechanisms of hypoxic regulation of PFKFB4 gene expression, we used dimethyloxalylglycine, which has the ability to mimic effect of hypoxia by significant induction of hypoxia-inducible factor (HIF-1α) protein levels. Our studies showed that PFKFB4 mRNA expression in PC-3, HeLa, Hep3B and HepG2 cell lines was highly responsive to dimethyloxalylglycine, an inhibitor of HIF-1α hydroxylase enzymes, suggesting that the hypoxia responsiveness of this gene is regulated by HIF proteins. To better understand the hypoxic regulation of PFKFB4 gene expression, we isolated genomic DNA, which includes the promoter region of PFKFB4. Cell transfection, deletion and site-specific mutagenesis of the PFKFB4 promoter region indicates that hypoxic induction of PFKFB4 gene expression is mediated by the hypoxia-responsive element (HRE). These experiments identified a HRE 422–429 bp upstream from the translation start site. Thus, our results indicate that testis-specific form of PFKFB or PFK-2/FBPase-2 is also expressed in several cancer cell lines and that hypoxia induces transcription of PFKFB4 gene in these cell lines by HIF-1α dependent mechanism. HRE in 5′-promoter region of PFKFB4 gene mediates hypoxic induction of PFKFB4 gene transcription.

Keywords: 6-Phosphofructo-2-kinase 4; Hypoxia; Hypoxia-inducible factor-1; Cancer cell; Dimethyloxalylglycine

1. Introduction

Hypoxia is an important component of many pathophysiological processes including tumor formation and growth. Hypoxia is one of the most potent inducers of gene expression especially genes involved in glycolysis for maintaining cellular energy [1–3]. This change from aerobic respiration to glycolysis is essential for cell survival in hypoxic conditions. Most interestingly, tumors have a high glycolytic activity even in normoxic conditions that correlate with the increased expression of glycolytic enzymes. The fructose-2,6-biphosphate is the most potent allosteric activator of 6-phosphofructo-1-kinase, a key regulatory enzyme of glycolysis, and an inhibitor of fructose-1,2-biphosphatase [4–6]. Because of the antagonistic effects in these enzymes, fructose-1,2-biphosphatase plays a critical role in the opposing glycolytic and gluconeogenic pathways. A single family of bifunctional 6-phosphofructo-2-kinase/fructose-2,6-biphosphate (PFK-2/FBPase-2 or PFKFB) enzymes is responsible for maintaining the cellular levels of FBPase-2 by synthesizing and degrading this compound at distinctive active sites in each enzyme type and controls glycolysis [6,7]. Several tissue-specific mammalian PFKFB or PFK-2/FBPase-2 isoenzymes have been identified (reviewed in [8]). PFK-2/FBPase-2 isoenzymes are encoded by at least four different genes (PFKFB1–4) in human cells. These genes encode isoenzymes that differ not only in their tissue distribution but also in their kinetic and regulatory properties. The PFKFB4 gene encodes isozyme of PFK-2/FBPase-2 which originally was found in the testes. Importantly, tissue-specific isoforms are not completely exclusive and several tissues express more than one isoforms [9,10]. This multiple expression suggests that each isozyme plays a key role in different physiologic conditions or in response to different hormonal stimulation. It is possible that there is a cell specific expression of PFKFB isozymes in different type of cells inside one organ.

The regulation of gene expression by hypoxia appears to be linked to a common mechanism, which includes the activation of a transcriptional complex termed hypoxia-inducible factor-1 (HIF-1) that binds to specific enhancer elements in hypoxia-responsive genes [11–14]. Hydroxylation of specific prolyl and asparaginyl residues in the alpha subunits of HIF-1α by a series of non-heme iron-dependent dioxygenases has been defined as a novel mechanism of protein modification that

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Abbreviations: PFKFB or PFK-2/FBPase-2, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase; HIF, hypoxia-inducible factor; HRE, hypoxia responsive element

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transduces the oxygen-sensing signal [13,15]. Many genes which expression are regulated by hypoxia contains HIF-1 binding site (hypoxia-responsible element/enhancer) [3,13,16–20]. Transcription factor HIF-1 is a necessary mediator of the hypoxic effect as well as Pasteur effect in mammalian cells. HIF is central in coordinating many of the transcriptional adaptations to hypoxia.

Previously we have shown that one isozyme of PFKFB or PFK-2/FBPase-2, PFKBF3, is highly induced by hypoxia in vitro in several cell lines and that cobalt and desferrioxamine has the ability to mimic effect of hypoxia by chelation or substitution of iron [21]. This induction could be replicated by the use of an inhibitor of the prolyl hydroxylase enzymes responsible for the VHL-dependent destabilization and tagging of HIF-1α. Marsin et al. [22] have shown that the stimulation of glycolysis by hypoxia in activated monocytes requires the phosphorylation and activation of inducible 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-3 (iPFK-2) enzyme, a well-known stimulator of glycolysis, by AMP-activated protein kinase [22–25]. Recently, we have shown that the expression of all four genes of PFKFB or PFK-2/FBPase-2 (PFKFB1–4) are responsive to hypoxia in vivo but regulation of the expression of these PFKFB isozymes following hypoxic treatment is different and can occurs in an organ-specific and possibly cell-specific manner [10]. However, the expression of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-4 isozyme, which originally was found in the testes, in other cells as well as mechanisms of hypoxic regulation of the PFKFB4 gene transcription has not been addressed.

In the present study, we have characterized the effect of hypoxia on the expression of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-4 isozyme in the human prostate cancer cell line, PC-3, as well as in HeLa, Hep3B and HepG2 cell lines and the role of HRE in the transcriptional response of this gene to hypoxia.

2. Materials and methods

2.1. Cell cultures

Human prostate cancer cell line, PC-3, human hepatoma Hep3B and HepG2 cell lines and HeLa cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and grown according to manufacturer protocols. The cells were incubated at 37 °C before harvesting under normoxic (21% oxygen) or hypoxic conditions (1% oxygen) or exposed for 6 h to 0.13 mM desferrioxamine, 0.1 mM cobalt chloride or 1 mM dimethyloxalylglycine, a specific inhibitor of the prolyl hydroxylase enzymes, which protects HIF-1α from degradation and significantly increases HIF-1α protein levels.

Chemicals were obtained from Sigma, except dimethyloxalylglycine (Frontier Scientific, Inc., Logan, UT, USA).

2.2. RNA isolation

Total RNA was extracted from cells using Trizol reagent according to manufacturer protocols (Invitrogen, Carlsbad, CA, USA). RNA pellets was washed with 75% ethanol and dissolved in nuclease-free water.

2.3. Plasmid construction

The cDNA probe of human 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-4 was created by RT-PCR of total RNA from human prostate cancer (PC-3) cells using forward primer (5′-GGGATGGCGTCCCCACGGG-3′) and reverse primer (5′-CGCTCTCCGTTCTCGGGTG-3′). These oligonucleotides correspond to nucleotide sequences 15–33 and 434–416 of human PFKFB4 cDNA, respectively (GenBank™ Accession No. NM_004567). The PCR fragment was cloned into plasmid pCR II-TOPO (Invitrogen). A *Bgl*II–*Eco*RI fragment of cDNA for human PFKFB4 cDNA was recloned into pBluescript II SK$^+$ (Stratagene, La Jolla, CA, USA). Following digestion with *Xba*I, this plasmid was utilized to generate a radiolabeled antisense probe for the human PFKFB4 probe generation using T7 RNA-polymerase and [α^{32}P]UTP. PFKFB4 construct used in this work was verified by sequencing the insert in the plasmid.

The 18S ribosomal RNA antisense probe was used to evaluate total RNA. The plasmids for synthesis of mouse 18S ribosomal RNA probe for ribonuclease protection assays were described previously [21].

2.4. In vitro transcription to prepare antisense probes for ribonuclease protection assay

Synthesis of radiolabeled probes for ribonuclease protection assay was carried out according to BD Biosciences protocol using T7 RNA polymerase (BD Biosciences Pharmingen, San Diego, CA, USA) and [α^{32}P]UTP (Amersham Biosciences). For ribonuclease protection assays, water solutions of total RNA were dried under vacuum and dissolved in 25 μl of 80% formamide hybridization buffer containing labeled probes. Samples were preincubated for 5 min at 85 °C and then incubated for 16 h at 45 °C as described previously [26]. The extracted, protected probe fragments were run on a 6% polyacrylamide sequencing gel in 1× Tris–borate–EDTA buffer for two hours at 50 mA. The gel was then dried and expression of mRNA was determined using Fujix BAS 2000 Bio-Image Analyzer (Fuji Photo Film Co.). Intensity of each mRNA band was normalized for 18S ribosomal RNA level.

2.5. Reporter plasmid constructs and transient transfection assays

A 3 kb nucleotide sequence containing the promoter and 5′-flanking region of human PFKFB4 gene was isolated using DNA from pancreatic cancer cell line Panc-1. Fragments of PFKFB4 gene were cloned into the pGL3-basic or pGL3-promoter vector (Promega, USA). A series of deletion mutants was prepared by PCR or by restriction endonuclease digestion and religation. Mutant was generated with QuickChange site-directed mutagenesis kit (Stratagene), by using nucleotides containing the desired mutation as described previously [27]. All constructs and mutant were sequenced to verify that the correct sequences or mutation were present. The dominant-negative HIF-1α construct was generously provided by Dr. M. Kobayashi, Institute of Genetic Medicine, Hokkaido University, Sapporo, Japan. The reporter plasmid constructs (1 μg) were transfected into HeLa cells in a six wells tissue culture plate with 8 μl of Plus reagent and 3 μl of lipofectamine (Invitrogen). Equal parts of transfected cells then were seeded on six 35 mm tissue culture plates, grown and incubated before harvesting under normoxic (21% oxygen) or hypoxic conditions (1% oxygen) or exposed for 6 hours to 1 mM dimethyloxalylglycine. Cell extracts were performed using Lysis buffer (Promega).

Luciferase activity was determined in a luminometer (Luminescencer-JNR; ATTO, Tokyo, Japan) using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instruction. Results were expressed as the percent of the control (untreated cells) value.

2.6. Western blot analysis

Cells were incubated at 37 °C before harvesting under normoxic (21% oxygen) or hypoxic conditions (1% oxygen) or exposed for 6 h to 1 mM dimethyloxalylglycine. Cell extracts were prepared using buffers A, as previously described [28]. The proteins were resolved using sodium dodecyl sulfate–polyacrylamide gel (10% acrylamide) electrophoresis and transferred to a polyvinylidene difluoride membrane (Immobilon-P Transfer Membrane; Millipore, Chelmsford, MA, USA) by a semi-dry blotting system. Excess sites on the membrane were saturated with 5% non-fat dried milk in TPBS (PBS containing 0.1% Tween 20). The membrane was incubated for 16 h at 4 °C with a 1:5000 dilution of rabbit polyclonal anti-PFKFB4 antibody. Rabbit polyclonal anti-PFKFB4 antibody was generated by immunization with a synthetic peptide from human PFKFB4 (MASPRELTQNPLKK-Cys-NH_2) conjugated with Keyhole Limpet Hemocyanin (KLH) which we received from Asahi Techno Glass Corp. (Japan). Peptide was certified by AnaSpec, Inc. (San Jose, CA, USA). Rabbits were immunized subcutaneously once with mixture peptide-carrier conjugate and complete Freund's adjuvant (Sigma) and four times with a mixture of peptide-carrier conjugate and incomplete Freund's adjuvant (Sigma). Horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a secondary antibody with 1:5000 dilutions. The bands were visualized by enhanced chemilumi-

nescence's reagents (Amersham Biosciences). Actin was used for normalization.

2.7. Statistical analysis

The results are expressed as means ± standard error of the mean (S.E.M.) of three or more independent experiments. Comparison of two means was performed by the use of unpaired Student's t test. Statistical significance was assumed at a value of $P < 0.05$.

3. Results

In this study, we used human prostate cancer cell line, PC-3, and several other cancer cell lines to study mechanisms of hypoxic regulation of PFKFB4 gene expression in various cancer cells.

For this aim, the cells were incubated at 37 °C before harvesting under normoxic (21% oxygen) or hypoxic conditions (1% oxygen) or exposed for 6 h to hypoxia mimics (0.13 mM desferrioxamine, 0.1 mM cobalt chloride or 1 mM dimethyloxalylglycine), total RNA was extracted from cells and PFKFB4 mRNA was readily quantified by ribonuclease protection assay or RT/PCR analysis.

3.1. Effect of hypoxia on the PFKFB4 gene in PC-3 cells

We have found that 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-4 isoenzyme which originally was found in the testes is expressed also in the human prostate cancer cell line PC-3. As shown in Fig. 1A, hypoxia significantly induced 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-4 mRNA levels in this prostate cancer cell line. Moreover, desferrioxamine (an iron-chelating agent), cobalt chloride (transition metal) and dimethyloxalylglycine (oxoglutarate analog), which are known to mimic hypoxia, also had a stimulatory effect on the expression of PFKFB4 mRNA in these cells. High responsiveness of the PFKFB or PFK-2/FBPase-2 gene expression to inhibitor of HIF-hydroxylase enzymes dimethyloxalylglycine in these cells suggests that hypoxic regulation of PFKFB4 gene expression is mediated via HIF proteins. We have studied also effect of hypoxia and hypoxia mimics on the expression of PFKFB3 and VEGF genes to compare hypoxia responsiveness of PFKFB4 with known HIF-1 dependent genes. Hypoxia as well as dimethyloxalylglycine, desferrioxamine and cobalt chloride significantly induced PFKFB3 and VEGF mRNA levels in this cancer cell line but induction of PFKFB3 was much stronger than VEGF. Quantification of the effect of hypoxia and hypoxia mimics on 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-4 mRNA expression is shown in Fig. 1B. Intensity of each PFKFB4 mRNA band was normalized to those of 18S rRNA level. The PFKFB4 transcript levels in prostate cancer cells were increased by hypoxia in sixfold ($P < 0.001$), by dimethyloxalylglycine in sevenfold ($P < 0.001$), desferrioxamine in fivefold ($P < 0.01$) and cobalt chloride in threefold ($P < 0.05$). Strong induction of PFKFB4 mRNA levels in response to hypoxia and dimethyloxalylglycine in PC-3 cells was shown by RT/PCR also (Fig. 1C).

3.2. Effect of hypoxia on the PFKFB4 gene in a HeLa cells

We have shown that hypoxia significantly induced PFKFB/FBPase-2 mRNA levels in the HeLa cells (Fig. 2A). PFKFB4 transcript levels were significantly increased in these cells also by desferrioxamine, cobalt chloride and dimethyloxalylglycine. We have compared effect of hypoxia and hypoxia mimics on

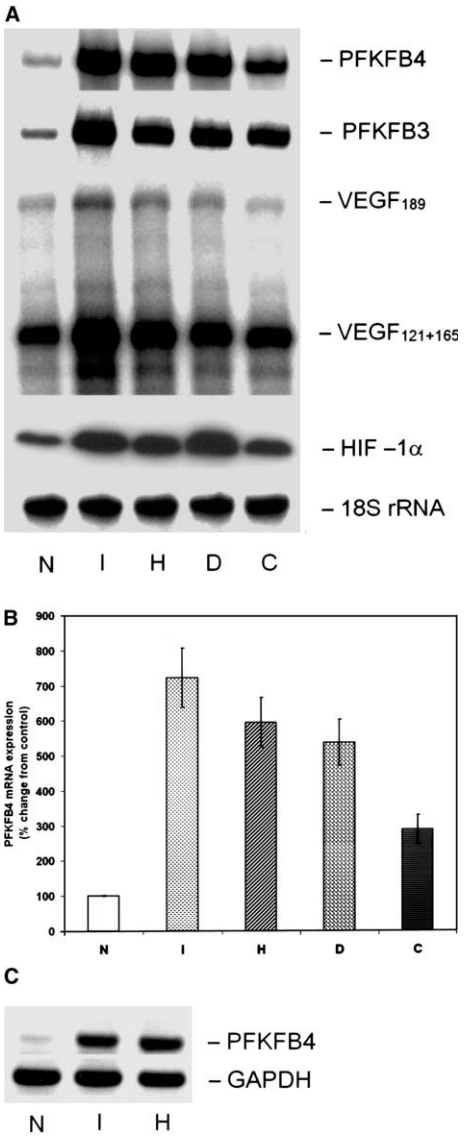

Fig. 1. Representative polyacrylamide gel autoradiograph employed in a typical ribonuclease protection assay of PFKFB4, PFKFB3, and VEGF mRNA from human prostate cancer cell line PC-3 and Western analysis of HIF-1α protein (A). The cells exposed under hypoxia (H) or treated with dimethyloxalylglycine (I), desferrioxamine (D) or cobalt chloride (C) for 6 h. N: control (normoxia) cells. Quantification of the effect of hypoxia, dimethyloxalylglycine, desferrioxamine and cobalt chloride on PFKFB4 mRNA levels in PC-3 cell line (B). Intensities of the PFKFB4 mRNA bands were normalized to 18S rRNA. Bar heights are mean values obtained from a series of four independent experiments ± standard errors of the mean. Representative ethidium bromide stained agarose gel employed in a typical RT/PCR analysis of PFKFB4 mRNA expression in human prostate cancer cell line PC-3 (C).

the expression of PFKFB4 in the HeLa cells with induction of PFKFB3 and Glut1, known HIF-1 dependent genes. However, as shown in Fig. 2A, hypoxia as well as desferrioxamine and dimethyloxalylglycine slightly induce PFKFB3 mRNA levels but hypoxia responsiveness of Glut1 gene in this cell line is much stronger as compared to PFKFB3.

3.3. Effect of hypoxia on the PFKFB4 gene in hepatoma cell lines

As shown in Fig. 2B, hypoxia as well as desferrioxamine and dimethyloxalylglycine significantly induced PFKFB4 mRNA

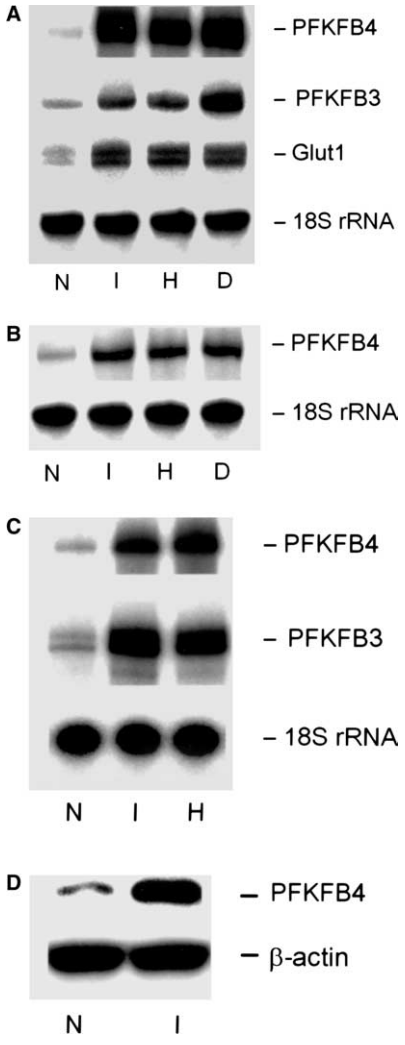

Fig. 2. Representative polyacrylamide gel autoradiograph employed in a typical ribonuclease protection assay of PFKFB4, PFKFB3 and Glut1 mRNA from HeLa (A), HepG2 (B) and Hep3B (C) cell lines. (D) Western blot analysis of PFKFB4 protein levels in Hep3B cells. The actin was used to ensure equal loading of the sample. The cells exposed under hypoxia (H) or treated with dimethyloxalylglycine (I) or desferrioxamine (D) for 6 h. N: control cells.

expression in the HepG2 cells. Significant increase of PFKFB4 transcript levels was observed also in other hepatoma cell line, Hep3B, after 6 h exposure under hypoxia (Fig. 2C). This hypoxic induction of PFKFB4 mRNA expression was compatible with induction of Glut1 mRNA in these cells by hypoxia. Further studies showed (Fig. 2C) that PFKFB4 gene expression was highly responsive to inhibitor of HIF-hydroxylase enzymes dimethyloxalylglycine in Hep3B cells also. Previously, we have reported that the PFKFB3 gene was highly induced by hypoxia and dimethyloxalylglycine in Hep3B cells [21]. As shown in Fig. 2D, hypoxia mimics dimethyloxalylglycine strongly induced PFKFB4 protein levels in Hep3B cells. The actin was used to ensure equal loading of the sample.

3.4. Hypoxic stimulation of the PFKFB4 gene transcription is HIF-1 dependent

In order to understand the transcriptional regulation of the PFKFB or PFK-2/FBPase-2 gene under hypoxic conditions, we isolated its 3 kb genomic DNA sequence, which includes the promoter region of this gene. The nucleotide sequence of a 1610 bp of the 5′ flanking region of PFKFB4 gene containing PFKFB4 promoter was subcloned into pGL3-basic vector and a distal fragment of 5′ flanking region of this gene (from −2964 to −1529, where +1 is translation start site) was subcloned into pGL3-promoter vector. In order to determine the *cis*-acting HIF-1 responsive element (HRE) that control the response to hypoxia of the PFKFB or PFK-2/FBPase-2 gene, several constructs which contained a luciferase reporter cDNA and variable 5′-regions of the PFKFB4 gene were utilized in transient transfection assays using HeLa cells. After transfection, the cell cultures were exposed to hypoxia or incubated with dimethyloxalylglycine and the results expressed as percent induction of luciferase activity by hypoxia or dimethyloxalylglycine of untreated control cells. As shown in Fig. 3A, a construct containing 1610 bp of 5′ flanking region fragment, which included the putative promoter and transcription and translation start sites of human PFKFB4 gene (from −1564 to +46, where +1 is translation start site), was highly hypoxia and dimethyloxalylglycine responsive. Equivalent response was found with a smaller fragment (from −560 to +46) cloned into basic pGL3 (Fig. 3A). Much smaller fragment of 5′ flanking region of the PFKFB4 gene (from −560 to −365) was created using synthetic oligonucleotides and cloned upstream of SV40 promoter into pGL3 promoter plasmid. As shown in Fig. 3A, this construct retained almost full hypoxia and dimethyloxalylglycine responsiveness. However, 5′-flanking sequence (from −560 to −438) of the human PFKFB or PFK-2/FBPase-2 gene was unresponsive to either hypoxia or dimethyloxalylglycine. These results suggested that a hypoxia responsible element was located within the −437 to −365 segment of the PFKFB4 5′-flanking region. Within this segment we identified an octonucleotide CGCGTGCC (from −429 to −422), which has homology with a hypoxia responsible element described in other hypoxia responsive genes. After nucleotide mutation (CTAG instead CGTG) in the HIF-1 binding site produced complete ablation of the response to hypoxia and dimethyloxalylglycine. A distal fragment of 5′ flanking region of PFKFB4 gene (from −2964 to −1529) was isolated also and cloned in promoter luciferase vector pGL3. As shown in Fig. 3A, a distal fragment of 5′ flanking region of PFKFB4 gene (from −2964 to −1529) was unresponsive to either hypoxia or dimethyloxalylglycine.

To confirm the HIF-1 dependent mechanism of hypoxic stimulation of PFKFB4 transcription, we used dominant-negative HIF-1α construct [29] in transient transfection experiments. This construct decrease the HIF-1α expression and eliminate HIF-1 dependent gene expression induced by hypoxia [29]. As shown in Fig. 3B, dominant-negative HIF-1α construct abrogated the enhanced expression of the PFKFB or PFK-2/FBPase-2 promoter constructs in HeLa cells.

4. Discussion

Recently, we have shown that tissue-specific isoforms of PFKFB or PFK-2/FBPase-2 are not completely exclusive and several tissues express more than one isoforms [10]. The PFKFB4 gene encodes the PFKFB or PFK-2/FBPase-2 isozyme originally found in the rat [32] and human testes [33,34] but it is not unexpected that this isozyme is present in other cell types. Using ribonuclease protection assays and RT-PCR we

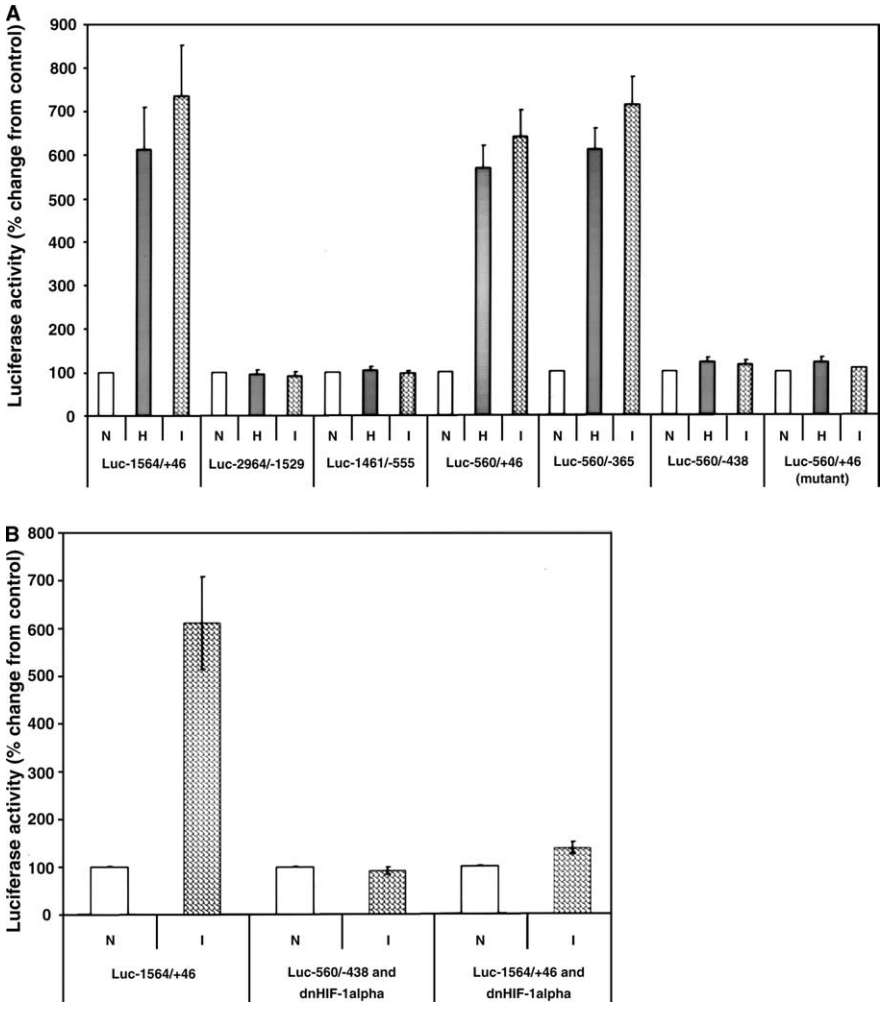

Fig. 3. Specific deletions of the 5′-UTR and 5′-flanking sequence of the human PFKFB4 gene promoter were obtained by digestion with restriction enzymes or by PCR and gene fragments were subcloned into pGL3-basic or pGL3-promoter vectors, where pGL3-promoter contains a minimal SV40 promoter sequence (SV40). HeLa cells transfected with luciferase expression vector containing different fragments of 5′-flanking region of the human PFKFB4 gene (A). HeLa cells transfected with luciferase expression vector containing of 5′-flanking region of the human PFKFB4 gene or cotransfected with dominant-negative HIF-1α construct (dnHIF-1α) (B). All calculations of 5′ flanking region were made from translation start site because transcription start site is not detected. The cells exposed under hypoxia (H) or treated with dimethyloxalylglycine (I) for 6 h and luciferase activity was measured. N: control (normoxia) cells. Results are shown as percent induction of luciferase activity by hypoxia or dimethyloxalylglycine of control value. Bar heights represent mean values obtained from a series of 3–5 experiments ± standard errors of the mean. Each luciferase experiment was conducted in duplicate.

found detectable basal levels of the PFKBF4 mRNA expression in the prostate cancer cell line PC-3. Moreover, PFKFB4 transcript was found in HeLa and hepatoma cell lines growing under normoxic conditions. Thus, this study provides the first clear evidence that the PFKFB4 gene express in the cells from other organs, in a different malignant cell lines. Recently, we have shown that the expression of all four genes of PFKFB or PFK-2/FBPase-2 (PFKFB1–4) are responsive to hypoxia in vivo but regulation of the expression of these PFKFB isozymes following hypoxic treatment is different and can occurs in an organ-specific and possibly cell-specific manner [10]. Previously, we have reported also that the PFKFB1–3 genes were induced by hypoxia in various cell lines and that the effect of hypoxia was reproduced by hypoxia mimics but regulation of the expression of these PFKFB isozymes following hypoxic treatment was different and can occurs in a cell-specific manner [10,21]. Further studies showed that PFKFB4 gene expression was highly responsive to hypoxia in the prostate cancer cells as well as in HeLa and hepatoma cell lines. Previously, we have reported that the PFKFB4 gene was induced in testis by hypoxia in vivo [10]. The major finding reported here is that one isozyme of PFKFB or PFK-2/FBPase-2 which originally was found in the testes is also expressed in cancer cells from different organs and that hypoxia highly induces PFKFB4 gene transcription in all cell lines tested. Expression of PFKFB4 in malignant cells and overexpression under hypoxic conditions suggests its possible role in the Warburg effect which was found in tumor cells [39].

Moreover, our results showed that an iron-chelating agent desferrioxamine and cobalt chloride (which are known to mimic hypoxia) have similar effect on the expression of PFKFB4 mRNA in these malignant cell lines. Importantly, cobalt chloride as well as desferrioxamine have been found to be potent stimulators of the expression of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-3 and 1 mRNA in different cell lines as well as other hypoxia-responsible genes [10,20,21,24,25,35].

The above results suggested that the HIF-1 complex was involved in the hypoxia responsiveness of the PFKFB4 gene.

We have also studied the effect of dimethyloxalylglycine (specific competitive inhibitor of prolyl-hydroxylase enzymes) on the expression of PFKFB4 mRNA for investigating the role of HIF-1α in hypoxic induction of this mRNA. Oxygen sensing is mediated by an oxygen-dependent hydroxylation of proline-564 in the oxygen dependent degradation domain of HIF-1α protein. This reaction is mediated by specific iron-dependent prolyl hydroxylase enzymes that utilize oxoglutarate as a co-substrate [13,14,36]. Inhibition of these enzymes can induce HIF-1α under normoxic conditions and mimics hypoxic conditions [13]. Our results showed that PFKFB4 mRNA expression in PC-3, HeLa, Hep3B and HepG2 cell lines was highly responsive not only to hypoxia but also to dimethyloxalylglycine, an inhibitor of HIF-1α hydroxylase enzymes, suggesting that the hypoxia responsiveness of this gene is regulated by HIF proteins. Thus, our results suggest that the 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-4 gene expression is induced in hypoxic conditions in several different cancer cell lines via HIF-1α dependent mechanism.

We also analyzed expression of glucose transporter-1, VEGF and PFKFB3 mRNA as a positive control for a hypoxia-responsive gene to compare with expression of PFKFB4 mRNA in these cell lines. Our results indicate that hypoxia enhances Glut1 and VEGF mRNA expression in PC-3, HeLa and Hep3B cell lines. Character of this induction was similar to PFKFB4 mRNA expression in these cell lines. Hypoxic induction of other member of the PFKFB or PFK-2/FBPase-2 gene family (PFKFB3) mRNA expression was significant and compatible to the expression of PFKFB4 mRNA in all cell lines studied, except HeLa cells. In these cells expression of Glut1 and PFKFB4 are strongly induced by hypoxia and dimethyloxalylglycine but expression of PFKFB3 mRNA did not show significant responses to hypoxia and dimethyloxalylglycine. It is possibly that molecular mechanisms leading to increase of the expression of PFKFB4 and PFKFB3 genes by hypoxia are similar but intensity of the hypoxic induction of PFKFB4 mRNA expression is variable and depends on type of cells. In contrast to the HeLa cells, in Hep3B cells hypoxia and dimethyloxalylglycine highly induced the PFKFB4 and Glut1 genes as well as PFKFB3 gene [21].

The PFKFB3 isozyme, which has the highest kinase/phosphatase ratio, is highly expressed in transformed cells, suggesting that it may contribute to the high glycolytic rate observed in tumors [37]. Previous study have shown that sustained hypoxia upregulates the expression of the PFKFB3 gene and that this activation is mediated by a mechanism that depends on the activation of the HIF-1 transcription complex [21]. There are data that iPFK-2 (inducible isoforms of PFKFB3) protein uniformly increased in the malignant tissues when compared with corresponding control tissues [38]. Cancer cells show elevated glycolytic rates, produce high levels of lactate and pyruvate (the Warburg effect) that correlate with the increased expression of glycolytic enzymes and glucose transporters via HIF-1 dependent mechanism [39–41]. Since PFKFB or PFK-2/FBPase-2 catalyzes the synthesis and degradation of FBPase-2, its controls glycolysis and has a significant role in the Warburg effect, especially PFKFB3 isozyme [21,42]. This study provides evidence that PFKFB4 gene also expressed in cancer cells, strongly response to hypoxia and possibly has a significant role in the Warburg effect also. This isozyme like PFKFB3 lacks a serine phosphorylation residue that is critical for the down-regulation of its kinase activity, it should has high K:B ratio and greatly promotes glycolysis under conditions of limited oxygen supply [8].

The present study clearly demonstrated that hypoxia and dimethyloxalylglycine strongly induces PFKFB4 gene expression by activation of transcription through the HRE located in 5′-region of this gene because mutation in the HRE which we identified in the promoter region of PFKFB4 gene loss of regulation by hypoxia. This study provides the first clear evidence that hypoxic induction of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-4 gene transcription is dependent on the transcription factor HIF-1α and mediated by the HRE located in 5′-region of this gene like many other hypoxia inducible genes [2,11,18,20,30,31]. Thus, the regulation of PFKFB4 gene expression by hypoxia is linked to a common mechanism, which includes the activation of a transcriptional complex termed hypoxia-inducible factor that binds to specific enhancer element. To confirm the HIF-1 dependent mechanism of hypoxic stimulation of PFKFB4 transcription, we also used dominant-negative HIF-1α construct [29] in transient transfection experiments. This construct significantly decrease the HIF-1α expression and eliminate HIF-1 dependent genes expression (Glut1 and aldolase A mRNA levels) induced by hypoxia [29]. Also it reduced the expression of Glut1 and the glucose uptake in the tumor tissues and consequently in vivo tumorogenesis [29]. Our results indicate that this dominant-negative HIF-1α construct abrogated the enhanced by dimethyloxalylglycine expression of the PFKFB or PFK-2/FBPase-2 promoter constructs in HeLa cells.

Thus, the major finding reported here is that one isozyme of PFKFB or PFK-2/FBPase-2 which originally was found in the testes is also expressed in cancer cells from different organs and that hypoxia highly induces 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-4 gene transcription by HIF-1α dependent mechanism. Moreover, hypoxic induction of the PFKBF4 gene transcription is mediated by the HRE located in the promoter region of this gene. Expression of PFKFB4 in malignant cells and overexpression under hypoxic conditions suggests its possible role in the Warburg effect which was found in tumor cells.

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