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Expression and hypoxia-responsiveness of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 4 in mammary gland malignant cell lines*

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Recently, we have shown that PFKFB4 gene which encodes the testis isoenzyme of PFKFB is also expressed in the prostate and hepatoma cancer cell lines. Here we have studied expression and hypoxic regulation of the testis isoenzyme of PFKFB4 in several malignant cell lines from a female organ — the mammary gland. Our studies clearly demonstrated that PFKFB4 mRNA is also expressed in mammary gland malignant cells (MCF-7 and T47D cell lines) in normoxic conditions and that hypoxia strongly induces it expression. To better understand the mechanism of hypoxic regulation of PFKFB4 gene expression, we used dimethyloxalylglycine, a specific inhibitor of HIF-1α hydroxylase enzymes, which strongly increases HIF-1α levels and mimics the effect of hypoxia. It was observed that PFKFB4 expression in the MCF7 and T47D cell lines was highly responsive to dimethyloxalylglycine, suggesting that the hypoxia responsiveness of PFKFB4 gene in these cell lines is regulated by HIF-1 proteins. Moreover, desferrioxamine and cobalt chloride, which mimic the effect of hypoxia by chelating or substituting for iron, had a similar stimulatory effect on the expression of PFKFB mRNA. In other mammary gland malignant cell lines (BT549, MDA-MB-468, and SKBR-3) hypoxia and hypoxia mimics also induced PFKFB4 mRNA, but to variable degrees. The hypoxic induction of PFKFB4 mRNA was equivalent to the expression of PFKFB3, Glut1, and VEGF, which are known HIF-1-dependent genes. Hypoxia and dimethyloxalylglycine increased the PFKFB4 protein levels in all cell lines studied except MDA-MB-468. Through site-specific mutagenesis in the 5′-flanking region of PFKFB4 gene the hypoxia response could be limited. Thus, this study provides evidence that PFKFB4 gene is also expressed in mammary gland cancer cells and strongly responds to hypoxia *via* an HIF-1α dependent mechanism. Moreover, the PFKFB4 and PFKFB3 gene expression in mammary gland cancer cells has also a significant role in the Warburg effect which is found in all malignant cells.

Keywords: PFKFB4, hypoxia, HIF-1, mammary gland cancer cell lines, dimethyloxalylglycine

Hypoxia is an important component of many physiological and pathophysiological processes including tumor formation and growth. Tumors are usually exposed to a hypoxic microenvironment due to their irregular growth and abnormal vascular supply (Vaupel 1996; Hockel & Vaupel, 2001). This hypoxic microenvironment exerts selective pressure on cells, favoring those that can survive and proliferate in the absence of adequate oxygen supply (Brown & Giaccia, 1998). Hypoxia is one of the most potent inducers of gene expression, especially of genes involved in glycolysis for maintaining cellular energy (Gleade & Ratcliffe, 1998; Dang & Semenza, 1999). Furthermore, tumors have a high glycolytic activity even in normoxic conditions, a phenomenon described by Warburg more than 75 years ago (Warburg *et al.*, 1927). Fructose-2,6-bisphosphate is considered to be the major allosteric regulator of 6-phosphofructo-1-kinase, a key regulatory enzyme of glycolysis, and an inhibitor

*Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession No. AY734234).

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Abbreviations: HIF, hypoxia-inducible factor; PFKFB, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau disease.

of fructose-1,6-bisphosphatase (Crepin *et al.,* 1989; Pilkis *et al.,* 1995; Hue *et al.,* 2003). Because of the antagonistic effects in these enzymes, fructose-2,6-bisphosphate plays a critical role in the opposing glycolytic and gluconeogenic pathways. A family of bifunctional 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (EC 2.7.1.105)/(EC 3.1.3.46) (PFKFB) enzymes is responsible for maintaining the cellular levels of fructose-2,6-bisphosphate by synthesizing and degrading this compound at distinctive active sites in each enzyme type, and controls glycolysis (Okar *et al.,* 2001). Several tissue-specific mammalian PFKFB isoenzymes have been identified. These isoenzymes are encoded by four different genes (PFKFB1, PFKFB2, PFKFB3 and PFKFB4) in mammalian cells. These genes encode isoenzymes that differ not only in their tissue distribution but also in their kinetic and regulatory properties (Pilkis *et al.,* 1995; Okar *et al.,* 2001). The human PFKFB4 gene is located in chromosome 3 (bands p21-p22) and encoded an isoenzyme which originally was found in the testes (Sakata *et al.,* 1991; Manzano *et al.,* 1998). Importantly, tissue-specific isoforms are not completely exclusive and several tissues express more than one isoform (Sakakibara *et al.,* 1999; Minchenko *et al.,* 2003). This multiple expression suggests that each isoenzyme plays a key role in different physiologic conditions or in response to different hormonal or other regulatory mechanisms.

The regulation of gene expression by hypoxia is linked to the activation of a transcriptional complex termed hypoxia-inducible factor-1 (HIF-1) that binds to a specific hypoxia-responsive element (Wang & Semenza, 1993). This element has been identified now in most hypoxia-responsive genes (Wenger, 2002). HIF-1 activation depends on the hydroxylation of specific prolyl and asparaginyl residues in the α subunit of HIF-1 that controls the survival and transcriptional activity of this protein (Ivan *et al.,* 2001; Epstein *et al.,* 2001; Schofield & Ratcliffe, 2004). These recently described HIF hydroxylases are a family of non-haem iron and oxoglutarate-dependent dioxygenases that define a novel mechanism of protein modification that transduces the oxygen-sensing signal and control hypoxic gene activation (Lando *et al.,* 2002; Masson & Ratcliffe, 2003). The hypoxia-responsible element/enhancer mediates hypoxic induction by recruiting the HIF complex and allowing its interaction with other trans-activators and the basal transcriptional machinery (Epstein *et al.,* 2001).

Previously, we have reported that the PFKFB1, PFKFB2, and PFKFB3 genes are induced by hypoxia in various cell lines and that the effect of hypoxia is reproduced by several hypoxia mimics in normoxic conditions (Sakakibara *et al.,* 1999; Minchenko *et al.,* 2002; 2003). However, regulation of the expression of these PFKFB isoenzymes following hypoxic treatment was different and occurred in a cell-specific manner. 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 (Min *et al.,* 2002). Moreover, the rapid activation of glycolysis by fructose-2,6-bisphosphate as well as by hypoxia in activated monocytes is regulated by phosphorylation/dephosphorylation of PFKFB isoenzymes by AMP-activated and several other protein kinases in different signaling pathways (Marsin *et al.,* 2002; Hue *et al.,* 2003; Rider *et al.,* 2004). We have shown that the expression of all four genes of PFKFB1-4 is responsive to hypoxia *in vivo* and that regulation of the expression of these PFKFB isoenzymes following hypoxic treatment is different and can occur in an organ-specific manner (Minchenko *et al.,* 2003). Recently, we have demonstrated that the PFKFB4 isoenzyme is expressed in PC-3 prostate cancer and hepatoma cell lines (Minchenko *et al.,* 2004). However, the expression and hypoxia-responsiveness of the testis isoenzyme of PFKFB has never been investigated in female cells.

This study provides evidence that the PFKFB4 gene is expressed in human mammary gland cancer cell lines strongly responds to hypoxia *via* an HIF-1α dependent mechanism and, together with the expression of PFKFB3 gene, has a significant role in the Warburg effect.

MATERIALS AND METHODS

Cobalt chloride and desferrioxamine were purchased from Sigma. Dimethyloxalylglycine was obtained from Frontier Scientific, Inc. (Logan, UT, USA). [α-^{32}P]UTP (800 Ci/mmol) and Hyperfilm ECL obtained from Amersham Biosciences.

Cell lines and culture conditions. Human MCF7, T47D, MDA-MB-468, BT549, and SKBR-3 mammary gland cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA) and grown according to the supplier's protocols. The cells were incubated at 37°C before harvesting under normoxic (21% oxygen and 5% carbon dioxide) or hypoxic (1% oxygen and 5% carbon dioxide) conditions or exposed for 6 h to 0.13 mM desferrioxamine, 0.1 mM cobalt chloride or 1 mM dimethyloxalylglycine.

RNA isolation. Total RNA was extracted using Trizol reagent according to the manufacturer's protocols (Invitrogen, Carlsbad, CA, USA). RNA pellet was washed with 75% ethanol and dissolved in nuclease-free water.

Plasmid construction. For synthesis of an antisense probe for analysis of PFKFB4 transcript levels we created cDNA using forward primer (5′-GGGCAAGACCTACATCTCC-3′) and reverse

primer (5′-TCAGGATCCACACAGATGG-3′). These oligonucleotides correspond to nucleotide sequences 167–185 and 505–487 of human PFKFB4 cDNA, respectively (GenBank accession No. NM_004567). The plasmids used for the determination of VEGF, Glut1, and PFKFB3 mRNA and 18S ribosomal RNA have been described previously (Minchenko *et al.*, 2002; 2003). The 18S rRNA antisense probe was used to evaluate total RNA. A 1567 bp nucleotide sequence containing the 5′-region of mRNA, promoter and 5′-flanking region of human PFKFB4 gene was isolated by PCR using DNA from human cancer cells and cloned into the pGL3-basic vector (Luc-1567). Mutation in the hypoxia responsive element of Luc-1567 (Luc-1567-mut) was generated with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) using oligonucleotides containing the desired mutation, as described previously (Mueller *et al.*, 2000). The Luc-1567-mut construct contains a mutation (CTAG instead of CGTG) in the HIF-1 binding site (the octonucleotide CGCGTGCC corresponds to nucleotide sequence 1136–1143 of the promoter region of PFKFB4 gene (293–300 bp upstream from the GATA site); GenBank accession No. AY734234). All constructs were sequenced.

***In vitro* transcription to prepare antisense probes for ribonuclease protection assay.** The synthesis of radiolabeled probes for ribonuclease protection assay was according to the BD Biosciences protocol using T7 RNA polymerase (BD Biosciences Pharmingen, San Diego, CA, USA) and [α-^{32}P]UTP. 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 (Minchenko & Caro, 2000). The extracted, protected probe fragments were run on a 6% polyacrylamide sequencing gel. Expression of mRNA was determined using a Fujix BAS 2000 Bio-Image Analyzer (Fuji Photo Film Co.). The intensity of each mRNA band was normalized to the 18S ribosomal RNA level.

Transient transfection assays. The reporter plasmid constructs containing luciferase cDNA and 5′-regions of the PFKFB4 gene (Luc-1567) and Luc-1567-mut (1 μg) were transfected into human mammary gland malignant cell line MCF7 in a 35 mm tissue culture plate with 9 μl of Plus reagent and 2.5 μl of lipofectamine (Invitrogene, Rockville, MD, USA). Equal parts of transfected cells were then seeded on six 35 mm tissue culture plates, grown and incubated before harvesting under normoxic or hypoxic conditions. Cell lysis was performed using Lysis buffer from Promega Corporation (Madison, WI, USA). Luciferase activity was determined in a luminometer (Luminescencer-JNR; ATTO, Tokyo, Japan) using the duel luciferase assay kit (Promega) according to the manufacturer's instruction. Results were expressed as the percent of the control (untreated cells) value.

Western blot analysis. Mammary gland cancer cells were incubated at 37°C before harvesting under normoxic or hypoxic conditions or exposed for 6 h to 1 mM dimethyloxalylglycine. Cell extracts were prepared as previously described (Armstead *et al.*, 1999). 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. The membrane was incubated for 16 h at 4°C with rabbit polyclonal anti-PFKFB4 antibody with a dilution of 1:10000. Rabbit anti-PFKFB4 antibody was generated with a synthetic peptide corresponding to the N-terminus of human PFKFB4. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG (Santa Cruz Biotechnology; Santa Cruz, CA, USA) were used as a secondary antibody with a dilution of 1:5000. The protein complexes were visualized by enhanced chemiluminescence reagents and Hyperfilm ECL (Amersham Biosciences). β-actin was used to normalize the loading of the samples.

Statistical analysis. The results are expressed as mean ± S.E.M. of four or more independent experiments. Comparison of two means was performed by the use of unpaired Student's *t*-test. *P* value of < 0.05 was regarded as significant.

RESULTS

Effect of hypoxia and dimethyloxalylglycine on PFKFB4 mRNA expression

To examine the effects of hypoxia, dimethyloxalylglycine, desferrioxamine and cobalt chloride on the expression of PFKFB4 gene, mRNA and protein levels were measured by ribonuclease protection assays and Western blotting, respectively. As shown in Fig. 1, mRNA of the PFKFB4 isoenzyme is visible in the human mammary gland cancer cell lines MCF7 and T47D growing under normoxic conditions. Moreover, the levels of PFKFB4 mRNA were greatly stimulated by hypoxia in these cell lines. To better understand the mechanism of hypoxic regulation of PFKFB4 gene expression, we used dimethyloxalylglycine (an oxoglutarate analog), a specific inhibitor of HIF-1α hydroxylases, which protects HIF-1α protein from proteasomal degradation, strongly increases HIF-1α levels and mimics the effect of hypoxia under normoxic conditions. PFKFB4 expression in the MCF7 and T47D cell lines was highly responsive to dimethyloxalylglycine, suggesting that the hypox-

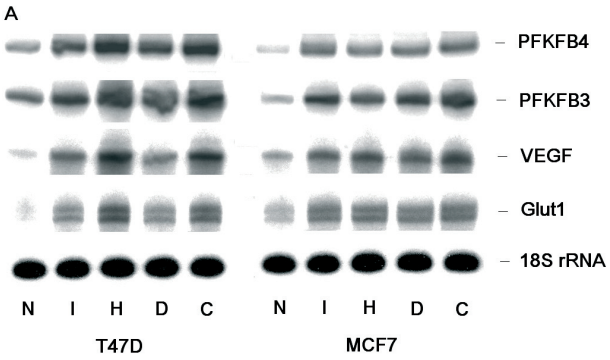

Figure 1. Effect of hypoxia and hypoxia mimics on the expression of PFKFB4, PFKFB3, Glut1 and VEGF mRNA in human mammary gland cancer cell lines T47D and MCF7, measured by ribonuclease protection assay.
The cells were exposed under hypoxia (H) or treated with dimethyloxalylglycine (I), desferrioxamine (D) or cobalt chloride (C) for 6 h. N, control (normoxia) cells. Intensities of the PFKFB4 mRNA bands were normalized to 18S rRNA.

ia responsiveness of the PFKFB4 gene in these cell lines is regulated by HIF-1 proteins (Fig. 1). Moreover, desferrioxamine as well as cobalt chloride, which mimic some effects of hypoxia by chelating or substituting for iron, had a similar stimulatory effect on the expression of PFKFB4 mRNA (Fig. 1). To compare the hypoxia responsiveness of the PFKFB4 gene with known HIF-1 dependent genes we also studied the effect of hypoxia and hypoxia mimics on the expression of PFKFB3, Glut1 and VEGF genes. Hypoxia as well as dimethyloxalylglycine, desferrioxamine, and cobalt chloride significantly, although to a variable degree, induced PFKFB3, Glut1, and VEGF mRNA levels in the MCF7 and T47D mammary gland malignant cell lines (Fig. 1).

The results of four independent ribonuclease protection experiments were quantified using a Fujix BAS 2000 Bio-Image Analyzer and expressed as the ratio between PFKFB4 mRNA and 18S rRNA. The increase of the PFKFB4 transcript levels in the T47D cells by hypoxia was +306% ($P < 0.01$), by dimethyloxalylglycine it was +225% ($P < 0.01$), by desferrioxamine +249% ($P < 0.01$), and by cobalt chloride was +183% ($P < 0.05$). The increase of PFKFB4 mRNA levels in the MCF7 cell line by hypoxia was +312% ($P < 0.01$), by dimethyloxalylglycine it was +386% ($P < 0.01$), by desferrioxamine +351% ($P < 0.01$), and by cobalt chloride +140% ($P < 0.05$).

Furthermore, as shown in Fig. 2, PFKFB4 mRNA accumulation is visible by 2 h following treatment with dimethyloxalylglycine in the MCF7 cells and is maximal at 6 h. Exposure of cells under hypoxia for 6 h and 16 h led to a similar increase in PFKFB4 mRNA levels. A similar pattern of induction is seen for VEGF mRNA, but PFKFB4 mRNA expression is more sensitive to treatment by hypoxia or the hypoxia mimic dimethyloxalylglycine at the same experimental conditions (Fig. 2).

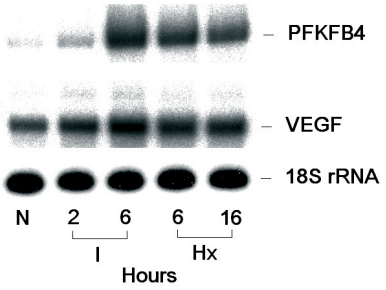

Figure 2. Effect of hypoxia and dimethyloxalylglycine on the expression of PFKFB4 and VEGF mRNA in human mammary gland cancer cell line MCF7.
The cells were exposed under hypoxia (Hx) or treated with dimethyloxalylglycine (I), for 2, 6 or 16 h. N, control (normoxia) cells.

We also observed that hypoxia significantly induced PFKFB4 mRNA levels in other mammary gland cancer cell lines (Fig. 3). Quantification of the effect of hypoxia and hypoxia mimic on PFKFB4 mRNA expression in the SKBR-3, MDA-MB-468, and BT549 cancer cell lines showed that the expression of PFKFB4 mRNA was increased more than two times ($P < 0.05$) by hypoxia and dimethyloxalylglycine in these cell lines. Moreover, as shown in Fig. 3, hypoxia as well as dimethyloxalylglycine induced the transcript levels of Glut1, PFKFB3, and VEGF in the SKBR-3, MDA-MB-468, and BT549 cancer cell lines, but the hypoxia responsiveness of the PFKFB4 gene was much stronger than of the PFKFB3 and VEGF genes.

Effect of hypoxia and dimethyloxalylglycine on PFKFB4 protein levels

To test the hypoxic regulation of the PFKFB4 protein levels, we used Western blot analysis with a rabbit polyclonal anti-PFKFB4 antibody. As shown

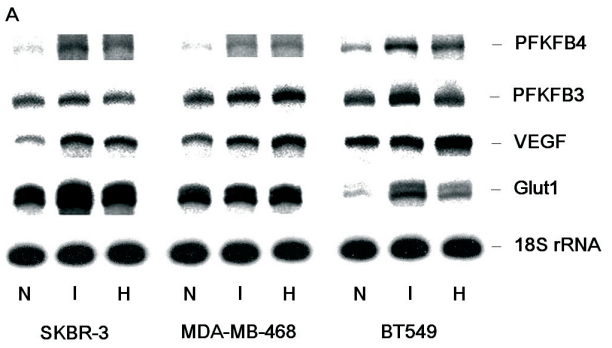

Figure 3. Effect of hypoxia and hypoxia mimics on the expression of PFKFB4, PFKFB3, VEGF and Glut1 mRNA from human mammary gland cancer cell lines SKBR-3, MDA-MB-468 and BT549.
The cells were exposed under hypoxia (H) or treated with dimethyloxalylglycine (I) for 6 h. N, control (normoxia) cells. Intensities of the PFKFB4 mRNA bands were normalized to 18S rRNA.

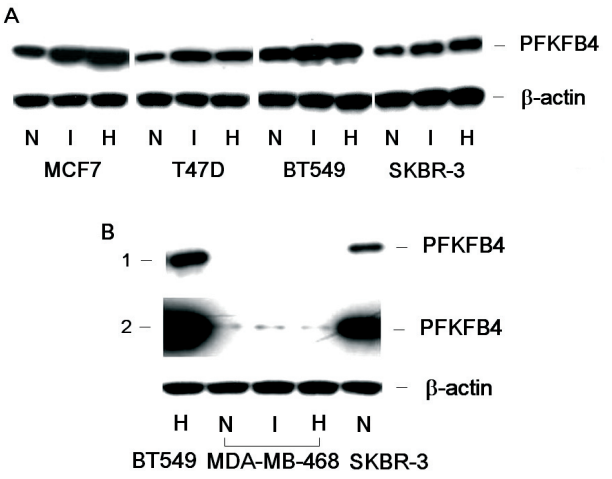

Figure 4A. Western blot analysis of PFKFB4 protein levels in the human mammary gland cancer cell lines: MCF7, T47D, BT549, and SKBR-3. B. Western blot analysis of PFKFB4 protein levels in the human mammary gland malignant cell lines BT549, MDA-MB-468, and SKBR-3 at different detection time (1 = 1 min and 2 = 10 min).

β-Actin was used to confirm equal loading of samples. The cells were exposed under hypoxia (H) or treated with dimethyloxalylglycine (I) for 6 h. N, control cells.

in Fig. 4A, hypoxia as well as dimethyloxalylglycine induced PFKFB4 protein levels in the MCF7, T47D, BT549, and SKBR-3 mammary gland cancer cell lines. Unexpectedly, no significant levels of PFKFB4 protein were observed in the MDA-MB-468 cell line at the experimental conditions used for the other cell lines (Fig. 4B). Only upon longer exposure could a weak band of PFKFB4 protein be detected in the MDA-MB-468 mammary gland cancer cell line, which did not increase significantly under hypoxic conditions.

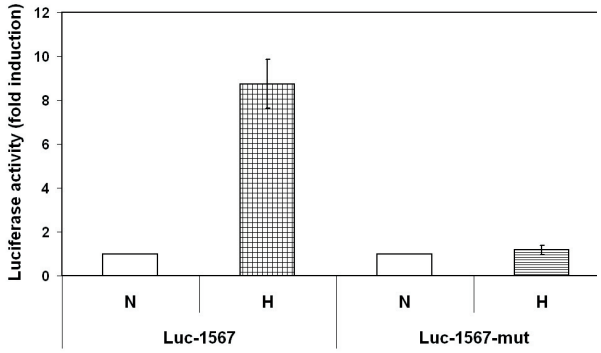

Figure 5. The expression of luciferase reporter constructs Luc-1567 and Luc-1567-mut in human mammary gland malignant cell line MCF7.

The cells were exposed under hypoxia (H) for 6 h and luciferase activity was measured. Results are expressed as the ratio of the levels of luciferase activity in hypoxic cells to that in parallel normoxic (N) control cells. Bar heights represent mean values obtained from a series of 5 experiments ± S.E.M., each done in triplicate.

Hypoxic stimulation of PFKFB4 gene transcription is mediated by the hypoxia responsive element

A reporter plasmid construct containing a 1567 bp fragment of the 5′-flanking region of the PFKFB4 gene (Luc-1567) was highly hypoxia responsive in a transfection assays using MCF7 mammary gland cancer cells (Fig. 5). However, the Luc-1567-mut construct containing a mutation in the HIF-1 binding site (hypoxia responsive element) transfected into MCF7 cells was unresponsive to hypoxia treatment. Thus, the hypoxia-responsive element in the promoter region of the PFKFB4 gene, located 293–300 bp upstream from the GATA site, mediates hypoxic induction of PFKFB4 gene transcription.

DISCUSSION

Using ribonuclease protection assay we found detectable constitutive levels of PFKFB4 mRNA in several mammary gland cancer cell lines. The transcript level of PFKFB4 was strongly induced under hypoxic conditions or under normoxic conditions in the presence of hypoxia mimics. Thus, this study provides clear evidence that the testis isoform of PFKFB is also expressed in cells from a female organ. We recently found that the testis isoform of PFKFB (PFKFB4) mRNA is also expressed in the HeLa, prostate and hepatoma cancer cell lines (Minchenko *et al.*, 2004). Thus, our studies demonstrate that the PFKFB4 gene is express in cancer cell lines from different tissues, not only in the testis.

Importantly, all the mammary gland malignant cell lines tested also constitutively express another member of the PFKFB gene family, PFKFB3, but the transcript level of this isoenzyme is variable under normoxic conditions and depends on the cell line type. The results of this investigation have also shown that the expression of PFKFB3 mRNA in mammary gland cancer cells is hypoxia responsive and that the hypoxic induction of PFKFB3 mRNA is much stronger in MCF7 and T47D breast cancer cells (estrogen receptor-positive cell lines) as compared to SKBR-3 and MDA-MB-468 cells (estrogen receptor-negative cell lines). The different sensitivity of the PFKFB3 isoenzyme to hypoxic induction was shown recently for many other cell lines (HeLa, Hep3B, RPE, and fibroblasts), while hypoxic induction of Glut1 in these cell lines was similar (Minchenko *et al.*, 2002). Previously, it has been reported that the mammary gland cancer cell line MCF7 constitutively expresses mRNA of the PFKFB2 isoenzyme and that hypoxia significantly induces the transcript level of PFKFB2 in this cell line (Minchenko *et al.*, 2003). Thus, our previous and current data clearly demonstrates that several

isoenzymes of the PFKFB gene family are expressed in mammary gland cancer cells.

Moreover, here we have shown that both desferrioxamine and cobalt chloride had a similar effect on the expression of PFKFB4 and PFKFB3 mRNA in the MCF7 and T47D malignant cell lines from mammary gland. Earlier observations have shown that desferrioxamine and cobalt chloride are potent stimulators of the expression of PFKFB3 and PFKFB1 mRNA in different cell lines, similarly to the effect on other hypoxia-responsible genes (Gleadle *et al.,* 1995; Minchenko *et al.*, 1994; 2002; 2003). Our results showed that the hypoxic induction of PFKFB4 gene expression was replicated by dimethyloxalylglycine in the MCF7, T47D, SKBR-3, MDA-MB-468, and BT549 cancer cell lines, suggesting that the hypoxia responsiveness of this gene is regulated by HIF proteins. Dimethyloxalylglycine (an oxoglutarate analog) is a specific inhibitor of the prolyl hydroxylases, protects the HIF-1α protein from proteasomal degradation and significantly increases its protein level (Epstein *et al.,* 2001; Schofield & Ratcliffe, 2004). It is known that oxygen sensing is mediated by 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 hydroxylases that utilize oxoglutarate as a co-substrate (Epstein *et al.,* 2001; Lando *et al.,* 2002; Mole *et al.,* 2003; Schofield & Ratcliffe, 2004). Inhibition of these enzymes can induce the level and transcription activity of HIF-1α under normoxic conditions and mimics hypoxic conditions (Metzen & Ratcliffe, 2004). Here we have shown that the induction of PFKFB4 mRNA expression by dimethyloxalylglycine is visible in two hours. A similar pattern of induction was shown for PFKFB3 mRNA (Minchenko *et al.*, 2002).

Because the glucose transporter-1 and VEGF genes are known HIF-1-dependent genes we also analyzed the expression of Glut1 and VEGF mRNA as a positive control for hypoxia-responsive genes in the mammary gland cancer cell lines. Our results clearly indicate that hypoxia as well as dimethyloxalylglycine enhance Glut1 and VEGF mRNA expression in the MCF7, T47D, SKBR-3, and BT549 mammary gland cancer cell lines and that the nature of this induction is similar to the expression of PFKFB4 mRNA in these cell lines. This data substantiates a HIF-dependent mechanism of PFKFB4 gene expression in mammary gland cancer cells.

The results of Western blot analysis with specific anti-human PFKFB4 antibodies revealed that the constitutive levels of PFKFB4 protein in the MCF7, T47D, SKBR-3, and BT549 mammary gland cancer cell lines are different and significantly increased by hypoxia and dimethyloxalylglycine. These data agree with our previous results that dimethyloxalylglycine significantly induces of PFKFB4 protein levels in Hep3B cells (Minchenko *et al.,* 2004). Despite the similar levels of PFKFB4 mRNA in the MDA-MB-468 and other mammary gland malignant cell lines, the expression of PFKFB4 protein in these cell lines is different. Unexpectedly, no significant levels of PFKFB4 protein were observed in the MDA-MB-468 cancer cell line at the experimental conditions used for MCF7 and other cell lines. It is possible that this discrepancy between PFKFB4 mRNA and protein levels, which were found in the MDA-MB-468 cells, is related to the instability of PFKFB4 protein. However, the precise molecular mechanism for this discrepancy between PFKFB4 mRNA and protein levels in the MDA-MB-468 mammary gland malignant cell line warrants further investigation.

There is data that the PFKFB3 isoenzyme, which has the highest kinase/phosphatase ratio, is highly expressed in transformed cells and malignant tissues, suggesting that it may contribute to the high glycolytic rate observed in tumors (Chesney *et al.,* 1999; Atsumi *et al.*, 2002). A previous study has shown that sustained hypoxia up-regulates the expression of the PFKFB3 gene and that this activation is mediated by a mechanism that depends on the activation of the HIF-1 transactivation complex (Minchenko *et al.,* 2002). Cancer cells show elevated glycolytic rates, produce high levels of lactate and pyruvate (the Warburg effect), and this correlates with an increased expression of glycolytic enzymes and glucose transporters *via* an HIF-1 dependent mechanism (Warburg, 1956; Lu *et al.,* 2002; Hopfl *et al.,* 2004). Since PFKFB isoenzymes catalyze the synthesis and degradation of fructose-2,6-bisphosphate, they control glycolysis and play a significant role in the Warburg effect, which is typical for tumor cells (Warburg *et al.,* 1927; Chesney *et al.,* 1999; Rider *et al.,* 2004). Our study provides evidence that the PFKFB4 gene is expressed in mammary gland cancer cells, is strongly responsive to hypoxia, and may have a significant role in the Warburg effect, much like PFKFB3.

Cell transfection and site-specific mutagenesis of the human PFKFB4 promoter region indicate that hypoxic induction of PFKFB4 gene transcription is mediated by the hypoxia responsive element located in the 5′-promoter region 293–300 bp upstream from the GATA site of this gene. This hypoxia responsive element has homology with the HRE described in other hypoxia responsive genes (Gleade & Ratcliffe, 1998; Minchenko & Caro, 2000; Wenger, 2002).

In summary, our results indicate that the PFKFB4 gene-encoded isoenzyme of PFKFB, which originally was found in the testis, is also constitutively expressed in different mammary gland cancer cell lines and that hypoxia induces transcription of this gene by an HIF-1α dependent mechanism *via* the hypoxia-responsive element located in the pro-

moter region. Expression of PFKFB4 in mammary gland cancer cells may have significance in the adaptation and survival of tumor cells in their hypoxic microenvironment.

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