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Hypoxic regulation of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene family (PFKFB-1–4) expression in vivo

Oleksandr Minchenko[a,b], Iryna Opentanova[a], Jaime Caro[b,*]

[a] *Department of Molecular Biology, Institute of Biochemistry, National Academy of Science of Ukraine, Kyiv 01601, Ukraine*
[b] *Cardeza Foundation for Hematologic Research, Department of Medicine, Jefferson Medical College of Thomas Jefferson University, Curtis Bldg. 810, 1015 Walnut Street, Philadelphia, PA 19107, USA*

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Abstract When oxygen becomes limiting, cells shift primarily to a glycolytic mode for generation of energy. A key regulator of glycolytic flux is fructose-2,6-bisphosphate (F-2,6-BP), a potent allosteric regulator of 6-phosphofructo-1-kinase (PFK-1). The levels of F-2,6-BP are maintained by a family of bifunctional enzymes, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB or PFK-2), which have both kinase and phosphatase activities. Each member of the enzyme family is characterized by their phosphatase:kinase activity ratio (K:B) and their tissue-specific expression. Previous work demonstrated that one of the PFK-2 isozyme genes, PFKFB-3, was induced by hypoxia through the hypoxia-inducible factor-1 (HIF-1) pathway. In this study we examined the basal and hypoxic expression of three members of this family in different organs of mice. Our findings indicate that all four isozymes (PFKFB-1–4) are responsive to hypoxia in vivo. However, their basal level of expression and hypoxia responsiveness varies in the different organs studied. Particularly, PFKFB-1 is highly expressed in liver, heart and skeletal muscle, with the highest response to hypoxia found in the testis. PFKFB-2 is mainly expressed in the lungs, brain and heart. However, the highest hypoxia responses are found only in liver and testis. PFKFB-3 has a variable low basal level of expression in all organs, except skeletal muscle, where it is highly expressed. Most importantly, its hypoxia responsiveness is the most ample of all three genes, being strongly induced in the lungs, liver, kidney, brain, heart and testis. Further studies showed that PFKFB-1 and PFKFB-2 were highly responsive to hypoxia mimics such as transition metals, iron chelators and inhibitors of HIF hydroxylases, suggesting that the hypoxia responsiveness of these genes is also regulated by HIF proteins. In summary, our data demonstrate that PFK-2 genes are responsive to hypoxia in vivo, indicating a physiological role in the adaptation of the organism to environmental or localized hypoxia/ischemia.

Key words: Fructose-2,6-bisphosphate; Phosphofructokinase-2; Hypoxia; Hypoxia-inducible factor; Glycolysis

1. Introduction

The ability to respond to hypoxia is an essential evolutionary adaptation in higher vertebrates. Hypoxia could be caused by a generalized reduction in oxygen delivery, such as in altitude and pulmonary diseases, or by disruption in the local blood supply, such as in ischemic disorders. Important in the adaptations to hypoxia is the activation of genes that ameliorate or compensate for the oxygen deficit. At a systemic level, there is an increase in erythropoietin that increases hemoglobin production and the oxygen-carrying capacity of circulating blood. At the tissue level, hypoxia activates the genes of several angiogenic factors that promote local neo-circulation, and at the cellular level, the lack of oxygen stimulates the expression of glucose transporters and glycolytic enzymes that compensate for the reduced activity of the mitochondrial respiratory chain [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, a phenomenon described by Warburg more than 70 years ago [4]. The rate of glucose utilization via the glycolytic pathway is coordinated with other processes that also use glucose, notably gluconeogenesis and the pentose pathway. Phosphofructokinase (PFK-1) is the key regulatory enzyme that controls the glucose flux through the glycolytic pathway. It is allosterically activated by ADP and inhibited by ATP and citrate. However, recent evidence indicates that fructose-2,6-bisphosphate (F-2,6-BP) is the most potent allosteric activator of PFK-1 and an inhibitor of fructose-1,2-bisphosphatase [5]. Because of the antagonistic effects in these enzymes, F-2,6-BP plays a critical role in the opposing glycolytic and gluconeogenic pathways. Since glycolysis requires the presence of F-2,6-BP, it is not unexpected that this compound is present in all eukaryotic cells, from yeast to mammals. A single family of bifunctional 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB or PFK-2) enzymes is responsible for maintaining the cellular levels of F-2,6-BP by synthesizing and degrading this compound at distinctive active sites in each enzyme type. That is, the kinase site synthesizes F-2,6-BP from F-6-P and ATP whereas the phosphatase site degrades F-2,6-BP to F-6-P and Pi [6].

Since the isolation of PFKFB-1 from rat liver and skeletal muscle tissues, several other mammalian isozymes have been identified: in heart (PFKFB-2), in testis (PFKFB-4), and a ubiquitous form (PFKFB-3), present in placenta, brain and most tumor cells (reviewed in [6]). These isozymes are encoded by four different genes, PFKFB-1–4, located in different chro-

*Corresponding author. Fax: (1)-215-955 2101.
E-mail address: jaime.caro@jefferson.edu (J. Caro).

Abbreviations: F-2,6-BP, fructose-2,6-bisphosphate; PFK-1, phosphofructokinase-1; PFKFB or PFK-2, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; HIF, hypoxia-inducible factor; Glut-1, glucose transporter-1

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mosomes [7]. The level of expression and activity of these enzymes is controlled by hormones and metabolites [8,9]. Importantly, tissue-specific isoforms are not completely exclusive and several tissues express more than one isoform. This multiple expression suggests that each isozyme plays a key role in different physiologic conditions or in response to different hormonal stimulation. Despite a great structural similarity of all isoforms, there are significant functional differences in their kinase and phosphatase activities. For example, the PFKFB-3 form, which is highly expressed in placenta and in tumors, has a very high kinase:phosphatase ratio, thus favoring the accumulation of F-2,6-BP and enhancing glycolysis [10]. Two forms (constitutive and inducible) that differ in 23 nucleotides in the C-terminal domain of the PFKFB-3 gene have been described [11]. These two variants result from alternative splicing of the PFKFB-3 gene. The increased levels of PFKFB-3 expression in tumors, specially its inducible form, may explain the Warburg phenomenon present in malignant cells [12,13]. Possibly, many more splice variants of PFKFB-3 exist; Kessler and Eschrich [14] have shown the occurrence of six splice variants of PFKFB-3 in human brain. Watanabe et al. [15,16] reported on the existence of eight splice isoforms of ubiquitous PFKFB-3 in rat brain and heart. The role of these variants in different tissues is not yet clear.

When oxygen is limiting, cells must rely on glycolysis to meet their energy demands (Pasteur effect). This adaptation from aerobiosis to anaerobiosis involves the activation of genes that facilitate glucose transport and utilization [17]. The regulation of gene expression by hypoxia is linked to the activation of a hypoxia-inducible transcriptional complex (HIF-1) that binds to specific enhancer elements in hypoxia-responsive genes [18]. Recently we reported that one member of the PFK-2 family, PFKFB-3, is highly induced by hypoxia in various cell lines. The hypoxic activation of the PFKFB-3 gene was found to be absolutely dependent on HIF-1 activity, since HIF-1α-negative cells showed no hypoxic activation of this gene [12]. Furthermore, transition metals, iron chelators and oxoglutarate analogs, which mimic hypoxia by inhibiting hydroxylases that negatively regulate both the level and activity of HIF-1α [19–22], also induced the expression of PFKFB-3. Here, we present animal studies that show the hypoxic induction of the PFKFB-3 gene in vivo. Furthermore, we extend our observation of the effect of hypoxia on the expression of the other members of the PFKFB family in vitro and in vivo.

2. Materials and methods

2.1. Animals and cell cultures

All animal studies were performed in accordance with the National Institute of Health guidelines for the use of experimental animals and all animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Thomas Jefferson University. C57BL/6 mice (10–12 weeks old and 20–25 g body weight) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). For hypoxia in vivo, mice were placed for 6 h in a modular incubator chamber (Billup-Rothenburg, Forma Scientific, Marietta, OH, USA) and flushed with a gas mixture, containing 7.5% oxygen and 92.5% nitrogen. Animal were killed immediately following extraction from the hypoxic chamber and their tissues frozen in liquid nitrogen for total RNA extraction. Hep3B, A_{549} and HeLa cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and grown according to ATCC protocols. For hypoxic treatment, the culture plates were placed for 6 h in a modular incubator chamber (Billup-Rothenburg) and flushed with a gas mixture, containing 0.5% oxygen, 5% carbon dioxide and 94.5% nitrogen.

Chemicals were obtained from Sigma, except dimethyloxalylglycine, which was provided by Dr. P. Ratcliffe (Oxford, UK).

2.2. RNA isolation

Total RNA was extracted from cells or mouse organs using the acid guanidinium–phenol–chloroform extraction method described by Chomczynski and Sacchi [23]. Cells or mouse tissue were extracted with 2 ml of guanidine isothiocyanate solution (Ultra Pure) consisting of 4 M guanidine isothiocyanate, 50 mM Tris–HCl (pH 7.5), 25 mM EDTA and 0.1 M 2-mercaptoethanol directly in the plates. Sequentially, 0.2 ml of 2 M sodium acetate, pH 4.0, 2 ml of phenol (water-saturated) and 0.4 ml of chloroform–isoamyl alcohol mixture (49:1) were added to cell lysate, with thorough mixing after the addition of each reagent. RNA was precipitated with an equal volume of 2-propanol. RNA pellets were washed with 75% ethanol and dissolved in nuclease-free water.

2.3. Plasmid construction

The plasmid for synthesis of rat or human PFKFB-1 probes were made by synthesis of a cDNA using rat liver or A_{549} cell total RNA and specific primers as follows: human and rat PFKFB-1 cDNAs were amplified using forward primer 5′-ACAGGAACTATGAATTCTTTC-3′ and reverse primer 5′-ACGTCGAAGATCTTGATGTAG-3′. These oligonucleotides correspond to nucleotide sequences 465–485 and 870–850 of rat PFKFB-1 cDNA, respectively (GenBank™ accession number NM_012621). These fragments of cDNA for rat and human PFKFB-1 mRNA were digested with *Eco*RI and *Bgl*II and cloned into plasmid pBluescript II KS$^+$ (Stratagene, La Jolla, CA, USA) for riboprobe preparation. The plasmids contained a 387-base protected fragment of rat or human PFKFB-1 cDNA, were digested with *Xho*I and used as a template for in vitro transcription of radiolabeled antisense probe using T7 RNA polymerase and [α-^{32}P]UTP. The rat probe was suitable for detecting PFKFB-1 expression in both rat and mouse.

The plasmids for synthesis of mouse or human PFKFB-2 probe were made by synthesis of a cDNA using mouse heart or HeLa cell total RNA and primers as follows: mouse and human PFKFB-2 cDNA was amplified using forward primer 5′-CTTGACCCAGACAACTATG-3′ and reverse primer 5′-ACTCCCCACCAGGGTATCG-3′. These oligonucleotides correspond to nucleotide sequences 655–673 and 1138–1119 of mouse PFKFB-2 cDNA, respectively (GenBank™ accession number NM_008825). For human PFKFB-2 cDNA these oligonucleotides correspond to nucleotide sequences 696–714 and 1179–1160, respectively (GenBank™ accession number NM_052034). These polymerase chain reaction (PCR) fragments of cDNA for PFKFB-2 mRNA were cloned into plasmid pCR II-TOPO using TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). An *Eco*RI-*Kpn*I fragment from plasmid containing the human or mouse fragment of PFKFB-2 cDNA was subcloned into plasmid pBluescript II KS$^+$ for RNase protection assays. These plasmids digested with *Xba*I and used as a template for in vitro transcription of a radiolabeled antisense probe contained a 424-base protected fragment using T3 RNA polymerase and [α-^{32}P]UTP.

The plasmids for synthesis of mouse ubiquitous PFKFB-3, mouse glucose transporter-1 (Glut-1) and mouse 18S ribosomal RNA probe for RNase protection assays have been described previously [12]. The 18S ribosomal RNA antisense probe was used to evaluate total RNA. All constructs were confirmed by sequencing analysis.

For the PFKFB-4 probe, mouse PFKFB-4 cDNA was amplified from total testis RNA using forward primer 5′-GGGATGGCGTCTCCACGGG-3′ and reverse primer 5′-GGCACTGGAAACAGCTCGG-3′. The nucleotides correspond to sequences 23–41 and 439–421 of the published mouse cDNA (GenBank™ accession number AK079023). The cDNA fragment was cloned in pBluescript II SK$^+$ vector and utilized for RNase protection assays.

2.4. Preparation of antisense probes for ribonuclease protection assay

Radiolabeled probes for RNase protection assay were synthesized using T7 or T3 RNA polymerase kit (Roche Molecular Biochemicals) and [α-^{32}P]UTP, according to the manufacturer's instructions. For RNase protection assays water solutions of total RNA were dried in speed 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 [11]. The extracted, protected probe fragments were run on a 6% polyacrylamide sequencing gel in 1×Tris–borate–EDTA buffer for 2 h at 50 mA. The gel was then dried and exposed to X-ray film (Hyperfilm MP, Amersham, Arlington Heights, IL, USA) at −70°C. Expression of mRNA was quantified using storage phosphor technology (Molecular Dynamics, Sunnyvale, CA, USA). The intensity of each mRNA band was normalized to the 18S ribosomal RNA level.

2.5. Statistical analysis

Data comparing all PFKFB and Glut-1 mRNA expressions were subjected to analysis of variance followed by Fisher's method for evaluation of differences between groups. Comparison of PFKFB and Glut-1 mRNA was subjected to Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effect of hypoxia on PFKFB gene expression in vivo

Mice were maintained for 6 h in a chamber flushed continuously with a gas mixture containing 7.5% oxygen and 92.5% nitrogen (environmental hypoxia), total RNA was extracted from different organs and PFKFB-1, 2 and 3 mRNAs were readily quantified by RNase protection assays.

3.1.1. PFKFB-1. Exposure of mice to environmental hypoxia moderately induced PFKFB-1 mRNA levels in the liver and skeletal muscle as compared to control mice. Surprisingly, the strongest response was found in the testis, whereas hypoxia had insignificant effect in brain, heart or kidneys (Fig. 1A). It is important to note that basal expression was highly variable, being intense in liver, heart and muscle. Densitometric quantification of the effect of hypoxia on PFKFB-1 mRNA expression is shown in Fig. 1A. The intensity of each PFKFB-1 mRNA band was normalized to the 18S rRNA level in each sample. In a mean of four experiments, the PFKFB-1 transcript levels were significantly increased in the testis (+230%; $P < 0.01$), liver (+33%; $P < 0.05$) and skeletal muscle (+27%; $P < 0.05$). The levels of PFKFB-1 mRNA

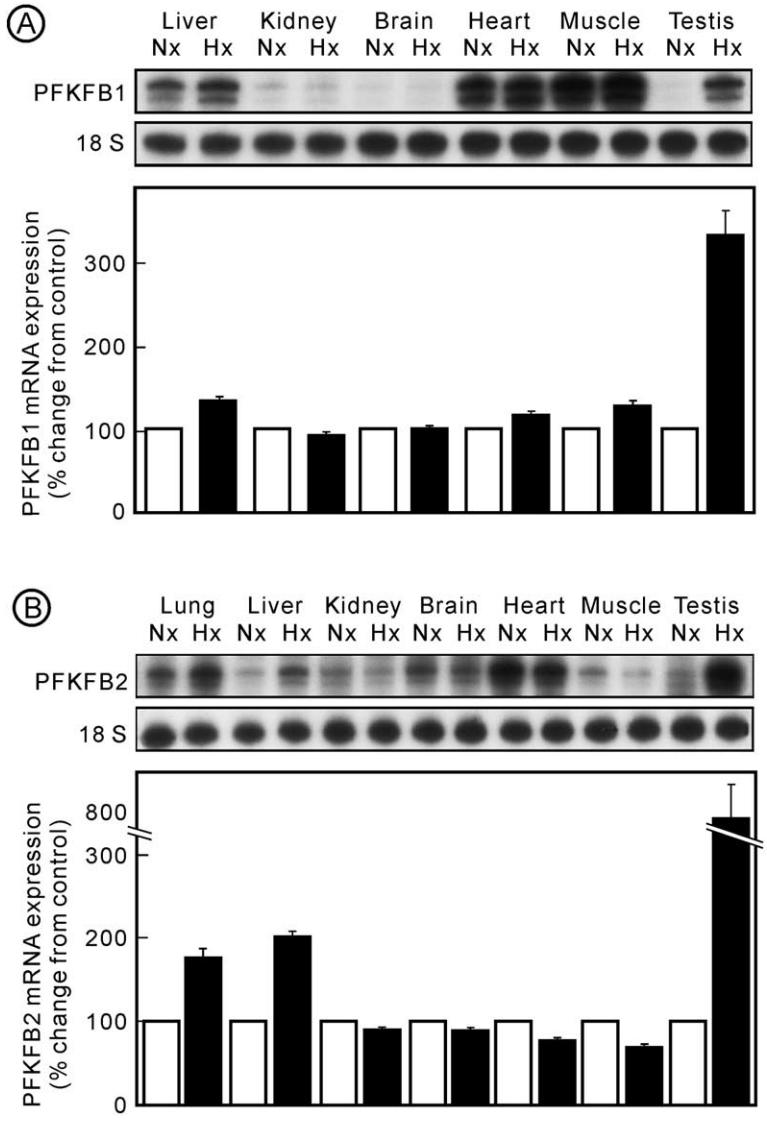

Fig. 1. Hypoxic expression of PFKFB-1 and PFKFB-2 mRNA in mouse organs. Mice were exposed to hypoxia and their RNA analyzed by RNase protection assays. Bands are autoradiographs of representative assays utilizing total organ RNA from normal (Nx) and hypoxic (Hx) mice. Bands represent autoradiographs utilizing specific PFKFB-1 (A) or PFKFB-2 (B) probes. Densitometry measurements with S.D. are ratios between each organ-specific mRNA and their 18S ribosomal RNA.

in the lungs of control and hypoxia-treated mice were undetectable (data not shown).

3.1.2. PFKFB-2. Similar analysis as above was conducted in the organs of normoxic and hypoxic mice for the expression of the PFKFB-2 gene. As shown in Fig. 1B, induction of PFKFB-2 mRNA expression was observed in the lungs, liver and testis, with the highest induction occurring in the testis. Basal levels were the highest in heart, lungs and brain. Quantification of the effect of hypoxia on the expression of PFKFB-2 mRNA in different organs is shown (bottom). In a mean of four experiments, the PFKFB-2 transcript levels were significantly increased in the testis (9-fold; $P<0.001$), lungs (+76%; $P<0.05$) and liver (+101%; $P<0.05$). No significant changes were found in the brain and kidneys, whereas a small but significant down-regulation of expression was observed in the heart (−23%; $P<0.05$) and skeletal muscle (−31%; $P<0.05$).

3.1.3. PFKFB-3. The ubiquitously expressed isoform PFKFB-3 was reported to be highly responsive to oxygen deprivation in various cell lines [12]. The in vivo studies shown in Fig. 2A confirmed its stimulation by hypoxia in all tissues tested with the exception of skeletal muscle. As quantified in Fig. 2A (bottom), the mean PFKFB-3 transcript levels from four experiments were significantly increased in the lungs (+91%; $P<0.01$), liver (+94%; $P<0.01$), kidney (+115%; $P<0.05$), brain (+210%; $P<0.05$), heart (+84%; $P<0.01$) and testis (+230%; $P<0.05$). In the skeletal muscle, expression of PFKFB-3 mRNA was decreased (−50%; $P<0.05$).

3.1.4. PFKFB-4. The hypoxia responsiveness of the testis isoform PFKFB-4 was tested using RNase protection assay with a specific testis probe and total RNA obtained from normal and hypoxic mouse testis. As shown in Fig. 2B, the testicular isoform has a significant response to hypoxia in vivo. The expression of PFKFB-4 in other tissues was not investigated.

We also studied the effect of environmental hypoxia on a control gene, Glut-1, a gene well characterized for its hypoxia response [16]. Hypoxia strongly increased expression of Glut-1 mRNA in the lungs, brain, heart and testis, slightly induced its expression in the liver and kidneys, but did not change significantly in skeletal muscle (Fig. 2C).

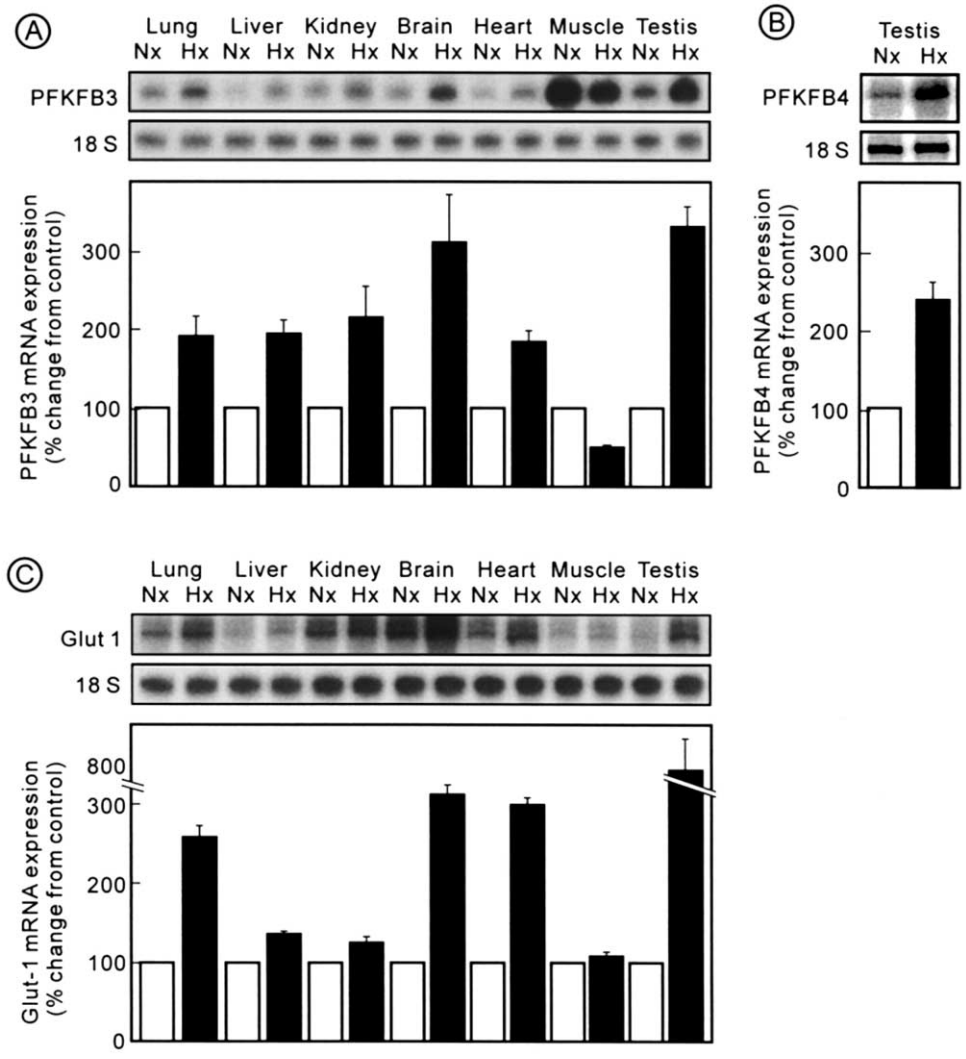

Fig. 2. Hypoxic expression of PFKFB-3, PFKFB-4 and Glut-1 in mouse organs. Autoradiographs of representative RNase protection assays of PFKFB-3 (A), PFKFB-4 (B) and Glut-1 (C) mRNA (upper) and 18S ribosomal RNA (bottom) using total RNA extracted from organs of normoxic (Nx) or hypoxic (Hx) animals. Densitometry measurements with S.D. of each specific mRNA are expressed as ratios between individual organs and their respective 18S RNA.

3.2. Effect of hypoxia on PFKFB-1 and PFKFB-2 in cultured cells

To investigate further the mechanisms of hypoxia-induced up-regulation of PFKFB-1 and PFKFB-2 mRNA expression, we studied the effect of hypoxia and hypoxia mimics on the expression of these genes in various cell lines. As shown in Fig. 3A, hypoxia significantly induced PFKFB-1 mRNA expression in HeLa and A_{549} cells, while no expression was observed in Hep3B cells (not shown). Moreover, desferroxamine (an iron-chelating agent), cobaltous chloride (transition metal) and dimethyloxalylglycine (oxoglutarate analog), which are known to mimic hypoxia, also had a stimulatory effect on the expression of PFKFB-1 mRNA in these cells. These agents inhibit HIF hydroxylase enzymes that are involved in the oxygen-dependent hydroxylation of residues that control either the proteasomal degradation or the transactivation activity of HIF proteins. Similar analysis was conducted for the expression of the PFKFB-2 gene. As shown in Fig. 3B, hypoxia and dimethyloxalylglycine significantly induced PFKFB-2 mRNA expression in HeLa and MCF-7 cells while minimal or no effect was observed in Hep3B and A_{549} cells. The Glut-1 control mRNA was induced by all stimuli, with variable intensity, in all cell lines tested (not shown).

4. Discussion

During hypoxia, when oxygen is limiting, cells rely on glycolysis to sustain energy expenditure. The key step of glycolysis commitment is controlled by PFK-1 (6-phosphofructo-1-kinase) that phosphorylates fructose-6-P to produce fructose-1,6-bisphosphate. This enzyme is allosterically regulated by the AMP:ATP ratio. However, since 1980 it has become apparent that the most potent allosteric activator of PFK-1 is F-2,6-BP, the product of phosphorylation of fructose-6-P by a family of enzymes known generically as phosphofructokinase-2 (PFK-2) [5,6]. These enzymes contain both kinase and phosphatase activities at distinct active sites of the molecule. Thus, F-2,6-BP levels are regulated by the relative kinase and phosphatase activities which depend on various physiologic or pathologic conditions of the cell. The K:B ratio (kinase/bisphosphatase) in a cell is ultimately determined by the types of isoforms present, the levels of several glycolytic or gluconeo-

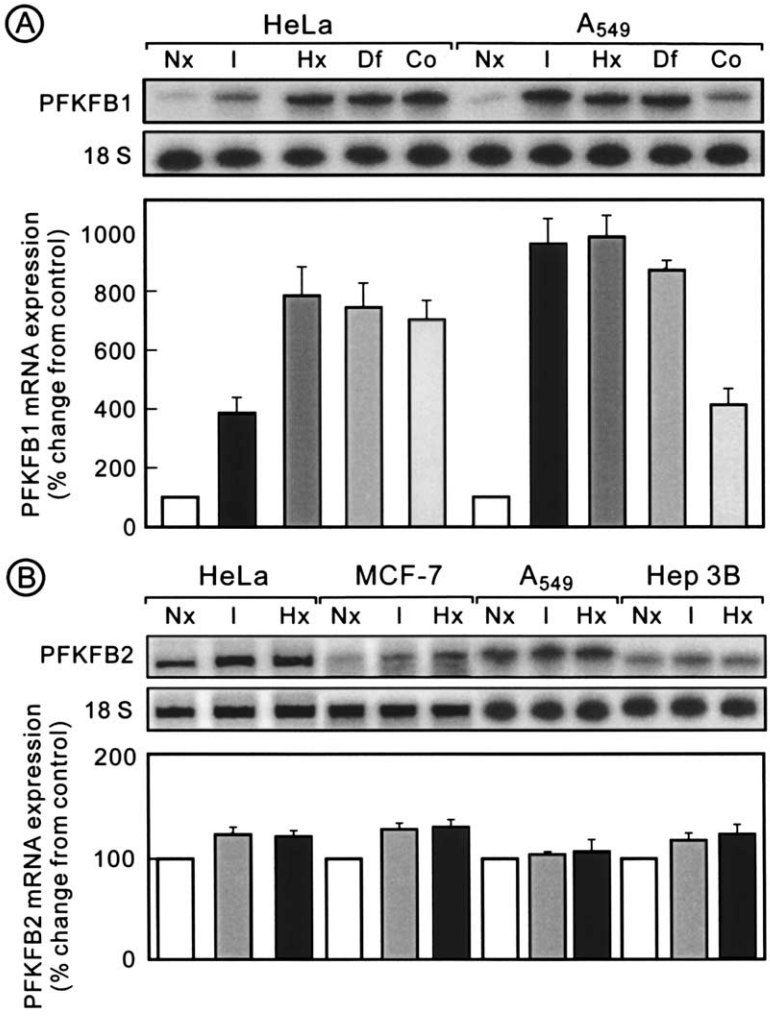

Fig. 3. A: Hypoxic expression of PFKFB-1 and PFKFB-2 mRNA in cultured cell lines. Autoradiographs of representative RNase protection assays of PFKFB-1 mRNA (A) and PFKFB-2 (B) using total RNA extracted from HeLa, A_{549}, MCF-7 and Hep3B cells in the presence of dimethyloxalylglycine (I), hypoxia (Hx), desferroxamine (Df) or cobalt (Co). Densitometry measurements of PFKFB mRNAs are expressed as ratios between individual cell cultures and their respective 18S RNAs. Bar represents S.D. of the mean.

genic metabolites and post-translational modifications of the enzymes, mainly phosphorylation. In the heart, acute ischemia induces rapid activation of AMP-dependent kinase that phosphorylates Ser-466 leading to a two-fold increase in the V_{max} of PFKFB-2 [24]. Previous studies by our laboratory had shown that sustained hypoxia up-regulates the expression of the PFKFB-3 gene and that this activation was mediated by a mechanism that depended on the activation of the HIF-1 transactivation complex [12]. Importantly, because PFKFB-3 lacks a serine phosphorylation residue that is critical for the down-regulation of its kinase activity, it has the highest K:B ratio of all PFKFB isozymes and greatly promotes glycolysis under conditions of limited oxygen supply [6]. The studies presented here were designed to investigate the expression of the PFKFB-1–4 isozymes under hypoxic conditions in vivo and to determine the mechanisms of hypoxia-induced up-regulation of these genes.

The PFKFB-1 gene encodes one form of PFK-2 isozyme originally found in liver and muscle tissues [25,26]. Using RNase protection assays we found detectable basal levels of expression of PFKFB-1 mRNA in the heart and skeletal muscle, with slightly lower levels in the liver. Low or undetectable basal levels were found in lungs, kidneys, brain and testis. Exposure of mice to environmental hypoxia induced moderate PFKFB-1 expression in the liver and skeletal muscle and, surprisingly, a strong induction of expression in the testis. These results indicate that the PFKFB-1 gene is hypoxia-inducible in vivo. However, the intensity of the hypoxic induction appears to vary in an organ-, tissue- or cell-specific manner. To investigate further the mechanisms of hypoxia-induced up-regulation of the PFKFB-1 gene we utilized several hypoxia mimics that are known to stimulate the formation of HIF-1 complex. Cobalt, desferroxamine and dimethyloxalylglycine showed a similar potency as hypoxia in the induction of PFKFB-1 mRNA expression in HeLa and A_{549} cells. However, no effect was found in Hep3B cells, which conforms with the variability of expression found in vivo. The induction of the HIF-1 complex is mediated by stabilization of its α-subunits that are rapidly degraded by the proteasomal system under oxic conditions. Oxygen sensing is mediated by a group of oxygen- and iron-dependent HIF hydroxylases that utilize oxoglutarate as co-substrate. These HIF hydroxylase enzymes regulate both the survival and the transcriptional activity of the HIF proteins. The effect of hypoxia can be mimicked by transition metals such as cobalt, iron chelators such as desferroxamine and by competitive inhibitors of oxoglutarate, such as dimethyloxalylglycine, all of which inhibit the activity of hydroxylase enzymes [19–22]. Thus, inhibition of these enzymes induces HIF-α stability and induces the transcriptional activity of the HIF-1 complex under normoxic conditions. The finding that treatment of HeLa and A_{549} cells with oxoglutarate analogs, cobalt and desferroxamine significantly induces PFKFB-1 mRNA strongly suggests the involvement HIF-α proteins in the hypoxia inducibility of the PFKFB-1 gene.

The PFKFB-2 gene encodes the PFK-2 isoform originally found in the heart [27,28]. Our results indicate that the heart form was expressed at high levels not only in the heart but also in the brain and lungs. In vivo, hypoxia induced moderate expression in the lung and liver and very strong stimulation in the testis. No induction or even mild inhibition was found in the heart, kidney, brain and skeletal muscle. Thus, as shown for the PFKFB-1 gene, PFKFB-2 was also hypoxia-inducible in vivo in a tissue-specific manner. We also studied the effect of hypoxia on the expression of PFKFB-2 in cultured cells. Our results showed that hypoxia and hypoxia mimics (oxoglutarate analogs) induced PFKFB-2 in HeLa and MCF-7 cells with minor or no effect in Hep3B or A_{549} cells. Unfortunately, these genes were not expressed in HIF-1α-deficient cells and the direct role of HIF complexes in hypoxia induction could not be tested in HIF nullizygous cell lines, as shown for the PFKFB-3 gene [12].

Previously, we have reported that the PFKFB-3 gene was highly induced by hypoxia in various cell lines and that the effect of hypoxia was reproduced by hypoxia mimics. In this study we show that environmental hypoxia induces the PFKFB-3 gene in vivo in all tested organs with the exception of skeletal muscle. Thus, the observed hypoxic regulation of PFKFB-3 mRNA in cell cultures has now been extended to an in vivo situation, indicating the physiological significance of this particular isoform in the adaptive response to hypoxia.

Analysis of tissue distribution of PFKFB type 1, 2 and 3 mRNAs showed that liver, lungs, kidneys, heart, brain, testis and skeletal muscle have different levels of PFK-2 isozymes in normoxic and hypoxia-treated mice. The lungs have high basal levels of PFKFB-2 and PFKFB-3 mRNA. In addition, both isozymes were induced by hypoxia. The liver has higher basal levels of PFKFB-1 and much lower PFKFB-2 and PFKFB-3 mRNA. All three isozymes were induced by hypoxia. The brain and kidneys have medium basal levels of PFKFB-3, low levels of PFKFB-2 and much lower PFKFB-1 mRNA. Only PFKFB-3 was induced by hypoxia. The heart has high basal levels of PFKFB-2 and PFKFB-1 and low levels of PFKFB-3 mRNA. Hypoxia induced expression of PFKFB-3 and had almost no significant effect in the other two isoforms. In contrast to the heart, skeletal muscle has low levels of PFKFB-2 and high levels of PFKFB-1 and PFKFB-3 that were not responsive to hypoxia or even slightly inhibited. Testis had low basal levels of all three enzymes but surprisingly, it was the organ that showed the strongest response to hypoxic stimulation, including the specific PFKFB-4 isoform. The explanation for the strong hypoxic induction in this organ is unclear. In general, our findings confirm the earlier observations that tissue-specific PFKFB enzyme expression is not completely exclusive [5,6,29]. Of interest, analysis of Glut-1, a gene well characterized for its hypoxia responsiveness [17], also showed great variations with respect to basal expression and hypoxia responses in vivo. Furthermore, its hypoxia responsiveness parallels that of the PFKFB-3 gene, having stimulated expression in most organs except skeletal muscle. No correlation was found between the expression of the different isoforms and the in vivo expression of HIF-1, as reported by Stroka et al. [30].

In summary, our study provides evidence that all four tested PFK-2 genes are hypoxia-responsive in vivo but the regulation of their expression following hypoxic treatment is different and appears to occur in a cell-specific manner. Moreover, the hypoxic induction of PFKFB-1, 2 and 4, as already shown for PFKFB-3, is most likely HIF-1-dependent. The mechanisms underlying the expression of each isoform in different tissues remain unclear.

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