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Study of the hypoxia-dependent regulation of human *CYGB* gene

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Abstract

Cytoglobin (CYGB) is ubiquitously expressed in all tissues and has been characterized as a respiratory protein in connective tissues. CYGB is up-regulated during hypoxia, implicating its function in maintaining the homeostasis redox of the cell. Here, we study the underlying molecular mechanisms by which hypoxia regulates human *CYGB* gene expression. When cells were subjected to hypoxia, the expression of endogenous CYGB was up-regulated ~1.7-fold in BEAS-2B cells ($p \leqslant 0.05$) and ~1.6-fold in HeLa cells ($p \leqslant 0.05$). Dual-luciferase assays and site directed mutagenesis showed the presence of hypoxia responsive elements (HREs) at positions −141, −144 and −448 that were essential for activation of CYGB expression under hypoxic conditions. The binding of hypoxia inducible factor (HIF-1) protein to the HREs was confirmed by gel shift and chromatin immunoprecipitation (ChIP) assays. These results indicate that HRE motifs are directly involved in the activation of the CYGB transcription under hypoxia.

Keywords: Cytoglobin (CYGB); Hypoxia; Normoxia; Hypoxia responsive elements (HREs); Hypoxia inducible factor (HIF-1)

In the vertebrates, five types of globin have been identified so far, namely hemoglobin (HB), myoglobin (MB), neuroglobin (NGB), cytoglobin (CYGB) and globin X (GbX) [1–4]. Hemoglobin (HB) and myoglobin (MB) are well known pentacoordinated globin proteins that have been examined extensively [5–7]. Neuroglobin (NGB) and cytoglobin (CYGB) are new members of the globin superfamily, both of which are hexacoordinated globins discovered recently in human and other vertebrates. NGB is mainly expressed in the cytoplasm of neurons in the central and peripheral nervous systems [8], whereas CYGB is detected in the nucleus as well as in the cytoplasm of many types of tissues [9–11]. 'Globin X' (GbX) as the fifth type of vertebrate globin, is only found in fish and amphibians [12]. Although it is well know that all of the globin members are able to transport and store oxygen, to sustain the oxidative metabolism in cells [5,9,13,14], the physiological function of CYGB is still unclear.

CYGB is found in all tissues analyzed so far, suggesting a general function of this ubiquitously expressed protein [10,15,16]. The strict conservation of CYGB sequence among mouse, rat and human suggested an important function in metabolism [9,16]. Studies indicated that CYGB might act as scavenger of reactive oxygen species (ROS). Xu et al. [17] reported that overexpression of CYGB protected hepatic stellate cells (HSCs) against oxidative stress *in vitro*. Using real-time quantitative RT-PCR, Schmidt et al. [11] and Fordel et al. [15,18] showed that CYGB was up-regulated upon hypoxic conditions *in vitro* and *in vivo*. However, the detailed molecular mechanisms by which hypoxia regulates human *CYGB* gene expression is still unclear.

In this study, we demonstrate an increase of CYGB expression under hypoxia as compared to normoxia. We also report that the HRE motifs at positions −141, −144 and −448 are essential for up-regulation of CYGB expression under hypoxia conditions.

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Materials and methods

Cell culture. BEAS-2B, a transformed human bronchial epithelial cell line, and HeLa, a human cervix carcinoma cell line, were purchased from the American Type Culture Collection (ATCC). Cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum (HyClone) at 37 °C. Hypoxia conditions were established by placing the cells in a tissue culture chamber (Billups-Rothenberg, Inc., US) at reduced oxygen content (1% O_2, 5% CO_2 and 94% N_2).

Plasmids DNA. Cloning of the substitution mutants were constructed by PCR based site directed mutagenesis from human CYGB gene promoter plasmid pGL3 (−1113) [19]. In Table 1, primers were listed for mutagenesis of the HRE motifs in the promoter region of the human *CYGB* gene.

Luciferase assay. To determine whether the putative HREs found within the CYGB promoter region was indeed responsible for the transcription activity under hypoxia, the prepared mutant constructs were transfected into BEAS-2B and HeLa cells. The plasmids were transfected into the cells as described previously [19]. The cells were cultured for 24 h after transfection under hypoxia (1% O_2) and normoxia (21% O_2) conditions and then harvested. Luciferase activity was measured and calculated as described previously [19].

Western blotting analysis. BEAS-2B and HeLa cells were exposed to hypoxia (1% O_2) for 1 and 3 h, with reference to the normoxia (21% O_2) maintained cells as control. Cells were lysed and the Western blot analyses for HIF-1α protein were performed by using anti-HIF-1α monoclonal antibody (Santa Cruz Biotechnology). The signal was visualized with the ECL (enhanced chemiluminescence system) according to the manufacturer's instruction (PIERCE).

Electrophoretic mobility shift assay (EMSA). Electrophoretic mobility shift assays were carried out with nuclear extracts from BEAS-2B cells, which had been grown under hypoxia (1% O_2) and normoxia (21% O_2) conditions, respectively. Nuclear extract preparation and DNA binding reaction were performed according to the procedures mentioned previous [19]. Double-stranded DNA probes were obtained by annealing the individually synthesized single-stranded oligonucleotides shown in Table 2.

Chromatin immunoprecipitation (ChIP) assay. BEAS-2B and HeLa cells were exposed to hypoxia (1% O_2) for 1 and 3 h, respectively, using normoxia (21% O_2) maintained cells as control. 1% formaldehyde was added to the culture medium and incubated for 10 min to cross-link histones to DNA. ChIP assays were carried out with a commercial kit (Upstate Biotechnology) according to the manufacture's manual. The extracted DNA was used for PCR using primers: HIF-448(ChIP) forward: 5′-AGTTCCCCGCCGCGGGAAGGG-TCCG-3′ and reverse: 5′-GC CGCCCGCCCACCCGCAG-GCCACG-3′ and HIF-144/-141 (CHIP) forward: 5′-TTTAAACATTTTCCAGCAGACCACA-3′ and reverse 5′-TGCTCGGCGGCGGC-GGTGGCGGGGC-3′. The PCR (30 cycles) was performed on a PTC-200TM Programmable Thermal Controller (MJ Research, Inc.) and the conditions of the PCR program were as follows: the samples were first heated at 95 °C for 3 min, and then 30 PCR cycles were applied: 95 °C for 15 s, 59 °C for 15 s, 72 °C for 15 s, the samples were further held at 72 °C for 10 min and cooled down to 15 °C.

Reverse transcription real-time PCR. Total RNA was isolated from cultured cells by the use of TRIzol® reagent (Invitrogen) according to the manufacturer's protocol. First-strand cDNA synthesis was performed with 2 μg of total RNA using oligo$(dT)_{18–20}$ primers and Superscript II reverse transcriptase (Invitrogen). 1% of the cDNA products were used for quantitative real-time PCR amplification with SYBR® Green PCR Master Mix (Applied Biosystems, Inc.). To normalize the input cDNA amount in each set of real-time PCR, the house keeping gene *β-actin* was chosen as an internal control. Primers (listed in Table 3) were designed to determine the expression level of CYGB, VEGF and β-actin under hypoxia as well as normoxia. The PCRs were carried out on iCycler Real-time PCR detection system (Bio-Rad) under the following conditions (40 cycles): DNA denaturation (15 s) at 95 °C, primer annealing (15 s) at 61 °C and extension of double-stranded DNA at 72 °C (15 s). SYBR Green analyses were followed by dissociation curves in a temperature range from 55 to 90 °C to analyze the specificity of the amplification reactions. Quantification was performed by dividing the mean value of expression of the hypoxia samples through that of the normoxia samples.

Table 1
Oligonucleotides used for mutagenesis of HRE motifs

Oligonucleotides	Sequence (5′–3′)
Common forward primer (CF)	CCGACGCGTGCCCTTGGTGGCGGCTGAGAT
Common reverse primer (CR)	CGAAGCTTCAAGCCCAGCCCGGCTTTGCTC
h-CYGB-pGL3 (Δ −448)	(F) GCGCCGAGCGACCGCCCATTCCTCCC
	(R) CGGTCGCTCGGCGCTGGGCGGCGCG
h-CYGB-pGL3 (Δ −144)	(F) GGCGCGCGAAGACACACGCTCCCTC
	(R) GTGTGTCTTCGCGCGCCGGGTGTGT
h-CYGB-pGL3 (Δ −141)	(F) AGACACGGGCTCCCTCCCTCCGCGC
	(R) AGGGAGCCCGTGTCTGTGCGCGCCG

Results and discussion

Identification of the putative HRE motifs in the human CYGB gene promoter region

Analysis of the human CYGB promoter region showed that there are hypoxia responsive elements (HREs) in the 5′ UTR region of the *CYGB* gene, which suggests that it may have an oxygen-dependent regulation. There are two hypoxia inducible factors (HIF-1) binding sites at −141 and −448, and one erythropoietin (EPO) binding site at −144 (Fig. 1A). Potential transcriptional binding sites of HREs are identified using the programs MatInspector 7.4 (http://www.genomatix.com) and Transcription Element Search System (TESS http://www.cbil.upenn.edu/tess/).

Transcriptional regulation of the CYGB gene promoter by hypoxia

To determine whether the putative HRE motifs found within the CYGB promoter were indeed responsible for the transcriptional activation under hypoxia, specific muta-

Table 2
Oligonucleotides used for gel shift assays on the HRE motifs binding

Oligonucleotides	Sense (5′–3′)	Antisense (5′–3′)
h-cygb(E)-448	CCAGCGCCGCGTGACCGCCCA	TGGGCGGTCACGCGGCGCTGG
h-cygb(E)-448Muta	CCAGCGCCGAGCGACCGCCCA	TGGGCGGTCGCTCGGCGCTGG
h-cygb(E)-144/141	CGCGCACAGACACACGCTCCC	GGGAGCGTGTGTCTGTGCGCG
h-cygb(E)-144Muta	CGCGCGAAGACACACGCTCCC	GGGAGCGTGTGTCTTCGCGCG
h-cygb(E)-141Muta	CGCGCACAGACACGGGCTCCC	GGGAGCCCGTGTCTGTGCGCG
Consensus-HIF	CCGTGGAGACGTGCGCGTCCT	AGGACGCGCACGTCTCCACGG

Table 3
Primers used in real-time PCR experiments

Oligonucleotides	Sequence (5′–3′)
h-cygb (RT-PCR)	TGGCCATCCTGGTGAGGTT
	CAGGCGTGCTTCCGCAGCT
h-beta-actin(RT-PCR)	CCGTCTTCCCCTCCATCGT
	ACTTCAGGGTGAGGATGCC
h-VEGF(RT-PCR)	ACTTCAGGGTGAGGATGCC
	TCTGCTGTCTTGGGTGCATT

tions were introduced within the HRE motifs. The effects on transcription were analyzed by dual-luciferase assay. The hypoxia induced activation of transcription was markedly abrogated by the mutation of the HREs in BEAS-2B cells (Fig. 1B). The promoter activity of pGL3 (−1113) increased by 72.4% upon exposure to hypoxia compared with normoxia. Site directed mutagenesis of HRE sites at −141, −144 and −448 reduced the increase in promoter activity by 35%, 26.5% and 17.5%, respectively, under hypoxia as compared to normoxia conditions. When all three HREs were mutated, hypoxia was unable to induce CYGB promoter activity. Qualitatively similar observations were made in HeLa cells transfected with the same reporter constructs (data not shown). The data indicate that HRE motifs are directly involved in the activation of the CYGB transcrip-

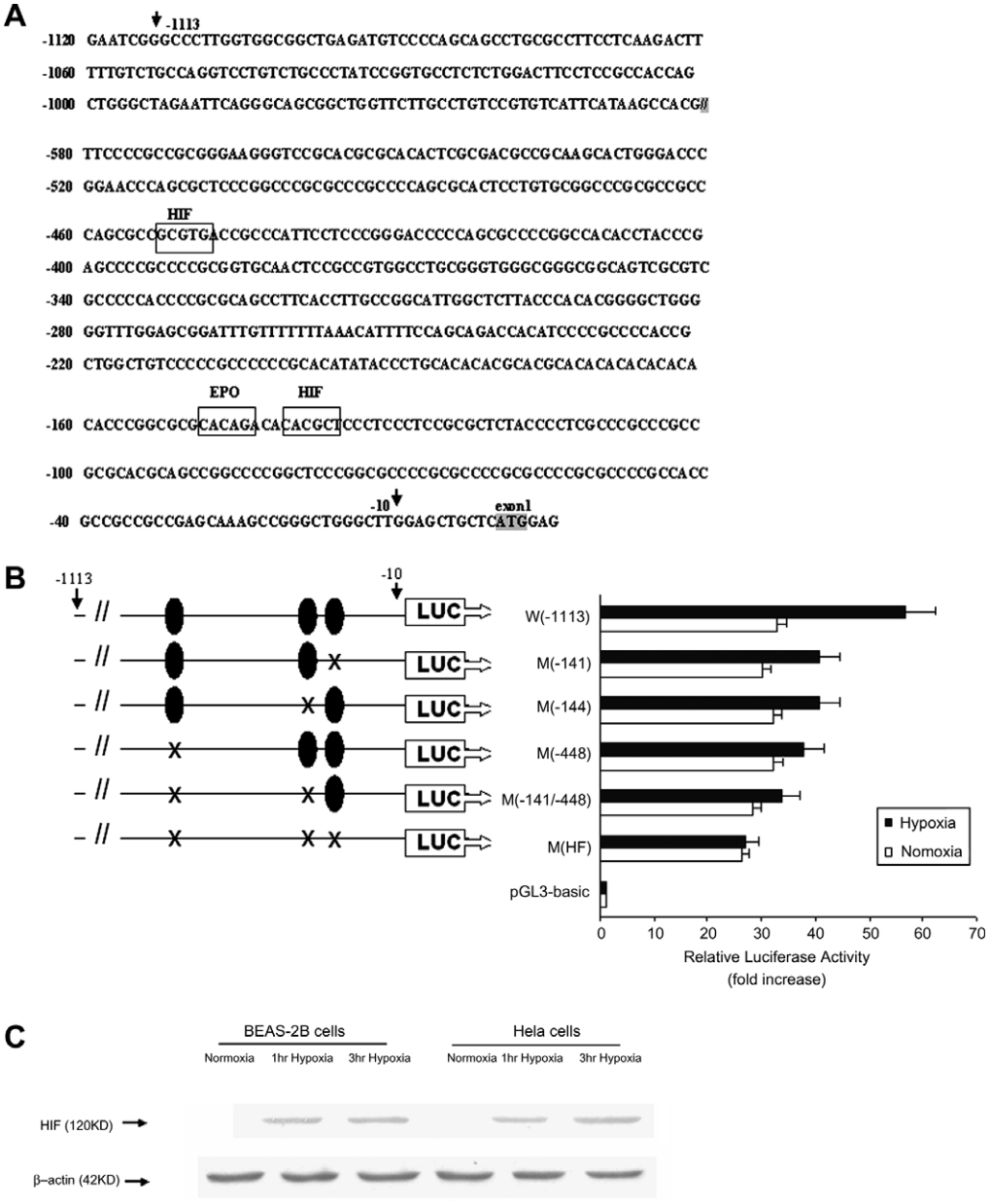

Fig. 1. Identification of the HRE elements responsible for the hypoxia-induced activation of the CYGB promoter. (A) Nucleotide sequence of the 5′ flanking region of the human CYGB gene. Boxed sequences indicate the potential HRE motifs. The ATG translation start site is indicated by highlight. (B) Schematic diagram of luciferase reporter construct fused to human CYGB promoter proximal sequence. Each bar represents the relative luciferase activity under hypoxia (solid bars) and normoxia (open bars) conditions. Error bars represent SD. (C) Expression profiles of HIF-1α by Western blotting. BEAS-2B and HeLa cells were exposed to 1% O_2 for 1 and 3 h, cells maintained at 21% O_2 were used as the reference. β-Actin was used as internal control.

tion under hypoxia. However, HRE sites are not crucial to the basal promoter activity under normoxia as mutation at the HRE sites does not significantly affect this activity.

We have previously shown that the c-Ets-1 site at −1008 and three Sp1 sites at −400, −230 and −210 are crucial for the regulation of *CYGB* gene expression under normoxia [19]. To identify whether these *cis* elements are responsible for the hypoxia induced activation of the CYGB promoter, mutations of the c-Ets-1 site at −1008 and Sp1 sites at −400, −230 and −210 were analyzed for their promoter activities under hypoxia. The results showed that mutations at the c-Ets-1 and Sp1 sites did not change the response to hypoxia, suggesting that there was no synergistic interaction between the HREs and c-Ets-1 protein or Sp1 factors that affected the promoter activity under hypoxia conditions (data not shown).

Induction of HIF-1α by hypoxia

HIF-1α mRNA is constitutively expressed and is not significantly induced under hypoxia. HIF-1α is regulated at the protein level, where HIF-1α is subjected to rapid degradation during normoxia by proteasome proteolysis [20]. Western blotting was employed to detect the presence of HIF-1α protein expression under hypoxia and normoxia. Our data showed that HIF-1α was undetectable in either BEAS-2B or HeLa cells lysates under normoxia conditions. However, under hypoxia conditions, HIF-1α was rapidly induced after cells were subjected to hypoxia (1% O_2) treatment for 1 and 3 h (Fig. 1C). The literature showed that at the protein level, HIF-1α is subjected to rapid degradation during normoxia by the process of pVHL-mediated ubiquitin–proteasome pathway [21–23]. Under hypoxic conditions, prolyl hydroxylation of HIF-1α is blocked, which permits HIF-1α protein to be stabilized so that HIF-1α is free to bind with HIF-1β to form the HIF-1 transcription complex. The HIF-1 heterodimer then binds to hypoxia responsive elements (HREs) of the target gene.

Analysis of nuclear proteins which bind to the HIF-1 motifs by EMSA and ChIP assay

To confirm the binding of nuclear proteins to the HRE motifs, EMSA (electrophoretic mobility shift assay) was performed. With HRE probes (Table 2), we analyzed BEAS-2B cell nuclear extracts for protein factors that specifically recognize HIF-1. We found that nuclear proteins from BEAS-2B were able to bind to the oligonucleotides containing the wild type HIF-1 binding motif at −448, −141/−144 of the CYGB promoter and to HIF-1 consensus sequence to form complex 1 (Fig. 2). Competition assays showed that a 50-fold molar excess of unlabeled wild type oligonucleotides (self competition) as well as HIF-1 consensus oligonucleotides prevented the formation of complex 1. Mutant oligonucleotides failed to compete for the binding of the complex. When HIF-1α antibody was incubated with the ^{32}P-labeled HIF-1 oligonucleotides at −448 and −141/−144, the DNA–protein complex 1 was supershifted to a slower-migrating band denoted as complex 2 (Fig. 2A, lane 9 and Fig. 2B, lane 10, complex 2).

To show the binding of HIF-1 protein to HRE motifs *in vivo*, we performed ChIP experiments using HIF-1α antibody (Fig. 3). Positive signals were found in BEAS-2B and HeLa cells subjected to hypoxia conditions (1% O_2), dem-

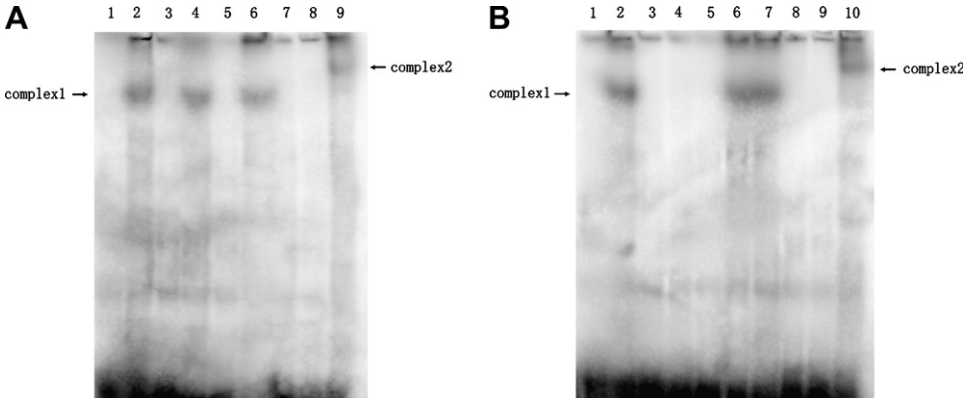

Fig. 2. EMSA analysis of the HIF-1 binding sites of the human CYGB promoter. Gel shift assays were performed with BEAS-2B nuclear extracts and HIF-1 sites ^{32}P-labeled probes (Table 2). (A) HIF-1 binding site at −448 in the promoter of the human CYGB gene. Lane 1, free probe without nuclear extract; lane 2, wild type −448 probe; lane 3, mutant probe; lane 4, HIF-1 consensus probe; lane 5, 50-fold cold "self" competition; lane 6, 50-fold cold "mutant" competition; lane7, 50-fold HIF-1 consensus cold competition (lanes 2–7, with 5 μg of nuclear extract from BEAS-2B cells exposed to hypoxia); lane 8, 5 μg of nuclear extract from BEAS-2B cells exposed to normoxia, incubated with wild type −448 probe; lane 9, 5 μg of nuclear extract from BEAS-2B cells exposed to hypoxia with wild type −448 probe and 2 μg of HIF-1α antibody. (B) HIF-1 binding site at −141/−144 in the promoter of the human CYGB gene. Lane 1, free probes without nuclear extract; lane 2, wild type −141/−144 probe; lane 3, −141 mutant probe; lane 4, −144 mutant probes; lane 5, 50-fold cold "self" competition; lane 6, 50-fold cold −141 mutant competition; lane 7, 50-fold cold −144 mutant competition; lane 8, 50-fold HIF-1 consensus cold competition (lanes 2–8, with 5 μg of nuclear extract from BEAS-2B cells exposed to hypoxia); lane 9, 5 μg of nuclear extract from BEAS-2B cells exposed to normoxia with wild type −141/−144 probe; lane 10, 5 μg of nuclear extract from BEAS-2B cells exposed to hypoxia incubated with wild type −141/−144 probe and 2 μg of HIF-1α antibody. Samples were analyzed on a 6.5% polyacrylamide gel in 0.5× Tris–borate–EDTA (pH 8.4) at 4 °C using constant voltage 250 V for 2.5 h.

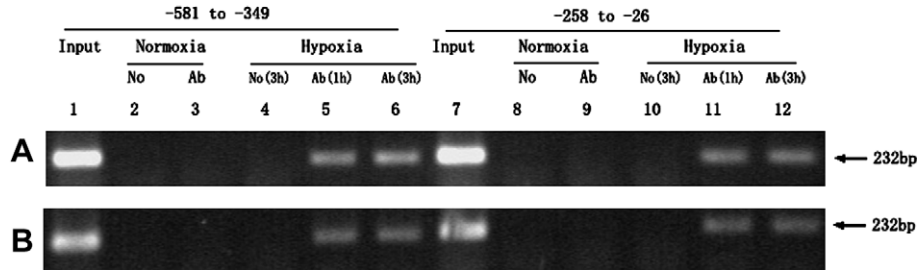

Fig. 3. ChIP assay. Genomic DNA and proteins from BEAS-2B (panel A) and HeLa (panel B) cells were cross-linked by formaldehyde and immunoprecipitated with a HIF-1α antibody. After immunoprecipitation, the human CYGB gene promoter region of HIF-1 motifs at −448 (lanes 1–6) and −141/−144 (lanes 7–12) were amplified by PCR using specific primers. Positive signals were found in BEAS-2B and HeLa cells subjected to hypoxia conditions (lanes 5,6,11,12). No signals were observed when BEAS-2B and HeLa cells exposed to normoxia conditions (lanes 3 and 9). Immunoprecipitation without an antibody (No) were carried out as negative controls (lanes 2,4,8,10).

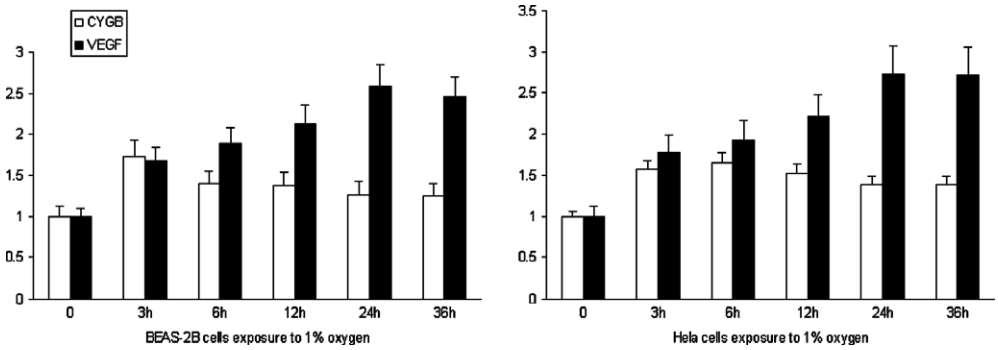

Fig. 4. Real-time quantitative RT-PCR analysis of the expression of CYGB and VEGF in BEAS-2B and HeLa cells when exposed to 1% O_2 during 36 h. After normalization with β-actin, results were statistically analyzed taking cells maintained at 21% O_2 as the reference. Error bars represent SD.

onstrating that HIF-1 interacts with the human CYGB gene *in vivo* (Fig. 3, lanes 5, 6, 11, 12). No signals were observed when BEAS-2B and HeLa cells exposed to normoxia conditions were used for ChIP analysis (Fig. 3, lanes 3 and 9).

HREs are usually characterized by the conserved consensus hypoxia-inducible factor (HIF-1) binding motif 5′-RCGTG-3′ [24]. Typically, the *cis*-acting binding sites for HIF consist of at least one HIF-1 core motif plus a second HIF-1 core motif or a co-stimulatory sequence, such as the EPO box [25–27]. In this study, we report the presence of two HIF-1 binding sites located at −448 and −141. Although the sequences of these two HIF-1 motif are not exactly the same as HIF-1 consensus sequence, gel shift assays showed that nuclear proteins from hypoxia treated BEAS-2B were able to bind to the oligonucleotides containing the HIF-1 binding motifs at −448, −141/−144 of the CYGB promoter to form the same complexes as that with HIF-1 consensus motif (Fig. 2). We also found that nuclear proteins from hypoxia treated BEAS-2B were not able to bind to the oligonucleotides containing the mutant HRE motifs, at −448, −141/−144 of the CYGB promoter. However, no binding was observed under normoxia condition. Competition assays and supershifted assays further confirmed the specificity of the binding of HIF-1 protein to HRE motifs. Moreover, mutation at the EPO site at −144 also affected the binding of HIF-1 with nuclear protein (Fig. 2B), suggesting that the EPO motif played an important synergistic role with HIF-1 factor resulting in a combined effect on the promoter activity under hypoxia. This proposal is supported by luciferase assays where site directed mutagenesis of the EPO site at −144 resulted in only small increase in promoter activity (26.5%) under hypoxia versus the wild type construct pGL3 (−1113) where the activity increased by 72.4% under hypoxia (Fig. 1B). This trend was also observed in the HeLa cells transfection (data not shown). These data indicated that the EPO motif at −144 was also directly involved in the activation of the CYGB transcription under hypoxia.

Induction of CYGB expression by hypoxia

Quantitative real-time RT-PCR was used to analyze the effects of hypoxia on CYGB mRNA expression in BEAS-2B and HeLa cells at different time points under hypoxia condition (exposure to 1% O_2 as compared to 21% O_2 under normoxia). VEGF (vascular endothelial growth factor), a target gene of HIF-1 and known to be up-regulated by hypoxia, was used as a positive control to confirm the effect of hypoxia treatment.

During hypoxia, the expressions of CYGB as well as VEGF were increased and both were up-regulated in a time-dependent manner during the first 24 h in BEAS-2B and HeLa cells (Fig. 4). For *CYGB* gene, the expression

was up-regulated to a maximum of 1.73-fold in BEAS-2B cells at 3 h and 1.65-fold in HeLa cells at 6 h. However, the expression of the VEGF increased continuously during the first 24 h in both BEAS-2B and HeLa cells ($p \leqslant 0.05$, Fig. 4).

Our data show that the up-regulation of CYGB is a quick response, in which the maximum expression of CYGB is observed after 3 h in BEAS-2B cells and 6 h in HeLa cells, and subsequently decreasing gradually. Jewell et al. [28] reported that the induction of HIF-1α in response to hypoxia was instantaneous reaching a maximum level after 1 h and maintained for up to 4 h. Our Western blotting data show that HIF-1α is rapidly induced after cells are subjected to hypoxia (1% O_2) treatment for 1 and 3 h, whereas under normoxic conditions, HIF-1α is undetectable in both BEAS-2B and HeLa cell lysate (Fig. 1C). These results suggested that the up-regulation of CYGB was an acute response to hypoxia which was mediated by HIF-1 transcription factor.

Acknowledgments

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