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Subcellular localization and regulation of hypoxia-inducible factor-2α in vascular endothelial cells

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Abstract

The hypoxia-inducible factors 1α (HIF-1α) and 2α (HIF-2α) have extensive structural homology and have been identified as transcription factors that mediate hypoxia-inducible gene expression through hypoxia-responsive element (HRE). They play critical roles not only in normal development, but also in tumor progression. Endothelial cells (EC) express both HIF-1α and -2α. In this study, we examined the subcellular localization of HIF-1α and -2α in bovine arterial EC (BAEC) by immunoblotting and immunocytostaining analysis and found that even under normoxic conditions, as with its heterodimeric partner ARNT, HIF-2α was stable, and was localized in the nucleus of BAEC differently than HIF-1α. HIF-2α might be regulated by a different mechanism than HIF-1α and might mediate the expression of some EC-specific genes under normoxic conditions. We further found that cardiovascular helix–loop–helix factor (CHF) 2, which had been identified as an ARNT-interacting protein, was expressed in BAEC and suppressed HRE-dependent gene expression both under normoxia and hypoxia. CHF2 might be one of the key regulators of HIF-2α-mediated gene expression in normoxic EC.

Keywords: Hypoxia-inducible factor; Hypoxia; Normoxia; Endothelial cells; Transcriptional repressor

Hypoxia induces a group of physiologically important genes such as erythropoietin and vascular endothelial growth factor (VEGF) [1]. These genes are transcriptionally upregulated by hypoxia-inducible factors (HIFs), which are heterodimeric transcriptional factors consisting of HIF-α and -β, both of which belong to basic helix–loop–helix (bHLH)/PAS domain transcription factors [2]. The HIF-β subunit is identical to the aryl hydrocarbon receptor nuclear translocator (ARNT) that also serves as a heterodimeric partner with the aryl hydrocarbon receptor (AhR) [3]. In contrast, it appears that the HIF-α subunit's sole but critical function is to mediate the response to hypoxia. The first identified isoform of HIF-α, HIF-1α, was originally discovered as a high-affinity DNA binding protein localized to the 3′ hypoxia-responsive element (HRE) on the erythropoietin gene [4]. Two additional HIF-α subunits have subsequently been cloned and named HIF-2α (independently identified as EPAS-1 [5], HLF [6], HRF [7] or MOP2 [8] by four laboratories) and HIF-3α [9,10].

HIF-1α and -2α have high sequence identity and their functional domains are similarly organized with a bHLH/PAS domain in their N termini as well as two transcription activation domains and an inhibitory domain in their C termini [5,6]. Like HIF-1α, HIF-2α is subjected to oxygen-dependent proteasomal destruction, mediated by the von Hippel–Lindau tumor suppressor protein [11,12] and the protein levels of HIF-2α are increased under hypoxic conditions [13]. However, the modes of expression of HIF-1α and -2α differ substantially in various tissues of adult mice and during different developmental processes [6,14]. HIF-1α is believed to be a universal master regulator for hypoxia-inducible gene expression along with its partner, ARNT/HIF-1β,

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as they are expressed in a wide range of cell types [15]. In contrast, HIF-2α is abundantly expressed in certain tissues and cell types. HIF-2α is expressed most prominently in the endothelial cells (EC) of various tissues, such as the brain, heart, kidney, and liver, and the HIF-2α mRNA is also observed in alveolar epithelial cells in the lung [6,7]. These observations indicate that HIF-1α and -2α have their own specific physiological functions in vivo. Gene targeting technology has been utilized to investigate the functions of HIF-1α and -2α, and has revealed that their complete deficiency results in developmental arrest and embryonic lethality. Histopathological analyses of homozygotic mutant embryos showed that HIF-2α deficiency either causes severe vascular defects in both the yolk sac and embryo proper [16] or displays pronounced bradycardia due to defective catecholamine production [17]. In contrast, HIF-1α-deficient mice manifested neural tube defects and cardiovascular malformations [18,19]. These results suggest that the two HIF-α isoforms play separate but essential roles during embryonic development.

The proliferation of vascular EC is a key step in the vascular growth involved in normal embryonic development [20]. EC proliferation is mediated primarily by VEGF signaling via its high-affinity tyrosine receptors, and it has been shown that the expression of VEGF and its receptors is regulated by HIFs [21]. Both HIF-1α and -2α mRNAs are expressed in vascular EC and have been postulated to play essential roles in EC proliferation, but the isoform-specific function and regulation of these two HIF-α isoforms in EC have not been fully elucidated.

We have found that bovine arterial EC (BAEC) also express both HIF-1α and -2α mRNAs [22]. In the present study, in order to investigate the isoform-specific regulatory mechanism of HIF-2α activity in EC, we examined the stability and subcellular localization of HIF-2α and compared them with those of HIF-1α using immunoblotting and immunocytostaining analysis. As one of the transcriptional regulators of HIFs in vascular growth, cardiovascular bHLH factors (CHFs) were recently identified by a yeast two-hybrid screen using ARNT as a bait [23]. Furthermore, we here examined the expression of CHFs in BAEC and the effects of CHFs on HIF-1α and -2α-mediated gene expression.

Materials and methods

Cell culture. BAEC were kindly provided by Dr. M. Masuda of the National Cardiovascular Research Institute (Osaka, Japan). BAEC were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS).

Reporter gene assay. BAEC were seeded in 6-well plates at 2×10^5 cells/well. The cells were transiently transfected with 1 μg HIF or the CHF expression plasmid, 1 μg pHRE-Luc plasmid or the pXRE-Luc plasmid [10], and 0.04 μg of *Renilla* luciferase-expressing plasmid (as an internal transfection efficiency control) by the LipofectAmine method according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). For the reporter gene assay of the pHRE-Luc plasmid, after 5 h of incubation with the LipofectAmine–DNA complex, the cells were washed and cultured for 24 h, and then cultured under normoxia (21% O_2) or hypoxia (1% O_2) for an additional 16 h. For the pXRE-Luc plasmid, after 5 h of incubation with the LipofectAmine–DNA complex, the cells were washed and then cultured with or without 3-methylcholanthrene (3MC) (10 μg/ml) for 40 h. The cells were then lysed and assayed for firefly and *Renilla* luciferase activities using the Dual-Luciferase Reporter Assay system (Promega, Madison, WI).

Construction of FLAG-tagged bovine ARNT expression plasmid. Using two primers, ARNTfw (5′-GGCCATGGCGGCGACTACTG C-3′) and ARNTrv (5′-CCCAATAGTTCTATTCTGAAAA-3′), designated on the basis of the sequences of human (M69238) and mouse (U10325) ARNT cDNA, bovine ARNT cDNA was amplified with BAEC cDNA as a template. Approximately 2.3 kb cDNA fragment was cloned into pGEM-T Easy vector (Promega) and then sequenced. A typical clone was designated as pBARNT. The nucleotide sequence of bovine ARNT has been deposited in the GenBank database with Accession No. AB053954. Using pBARNT as a template, bovine ARNT cDNA was re-amplified with the primers ARNTrv and FLAG-tagged-ARNTfw (5′-ATGGCCGACTACAAGGACGACGACGAC AAGGCTAGCATGGCGGCGACTACTG-3′; the start codon of bovine ARNT is underlined) and then cloned into pcDNA3.1 (Invitrogen) for construction of the FLAG-tagged expression plasmid.

Visualization of subcellular localization of HIF-1α, HIF-2α, and FLAG-tagged ARNT. BAEC were seeded onto poly-L-lysine-coated coverslips at 4×10^4 cells/ml and cultured for 24 h, and then cultured under normoxia (21% O_2) or hypoxia (1% O_2), or in the presence of 150 μM $CoCl_2$ for an additional 16 h. For analysis of FLAG-tagged ARNT, BAEC on coverslips were transiently transfected with FLAG-tagged ARNT expression plasmid. Cells were fixed with 2% paraformaldehyde for 15 min in phosphate-buffered saline (PBS). After three washes with PBS, the fixed cells were sequentially treated with 0.1% Triton X-100 for 5 min in PBS (for permeabilization), with 1% skim milk for 1 h in PBS (for blocking), with anti-HIF-1α (BD Transduction Laboratories, San Diego, CA), anti-HIF-2α [10] or anti-FLAG (Sigma, St. Louis, MO) antibody for 2 h in PBS containing 1% skim milk, and then with fluorescein isothiocyanate-conjugated anti-mouse Igs or anti-rabbit-IgG, or Cy3-conjugated anti-rabbit IgG antibody (Sigma) for 1 h in PBS containing 1% skim milk. After six washes with PBS, the cells on coverslips were mounted on glass slides with Aqua-Poly/Mount (Polysciences, Warrington, PA). The fluorescent signals were visualized using a laser scanning confocal microscope IX70 (Olympus Optical, Tokyo, Japan), as described previously [24].

Western blot analysis of nuclear HIF-2α protein in BAEC. BAEC were seeded in 10 cm dishes at 2×10^5 cells/ml and cultured for 24 h, and then cultured under normoxia (21% O_2) or hypoxia (1% O_2), or in the presence of 150 μM $CoCl_2$ for an additional 16 h. Nuclear proteins were prepared from BAEC as described previously [25], and 50 μg of nuclear protein was loaded on a 10% SDS–polyacrylamide gel. After electrophoresis, the proteins were electrotransferred to polyvinylidene difluoride membrane, probed with anti-HIF-2α antibody [10], and detected by chemiluminescence.

RT-PCR analysis of the expression of CHFs in BAEC. Four degenerated oligonucleotide primers, Fw1 (5′-GC(AC)AG(AG)AA(AG) A(AG)A(AC)GGAGAGG-3′), Fw2 (5′-AA(AG)A(AG)(AC)GGAGA GG(ATGC)AT(ATGC)AT-3′), Rv1 (5′-TTA(AG)AAAGCTCC(AG) A(CT)(CT)TC(CT)GT-3′), and Rv2 (5′-GCTCC(AG)A(CT)(CT)TC (CT)GTCCCCCA-3′), were designated on the basis of the sequences of bHLH domain and the C-terminal region of human and mouse CHF1 (AF173901 and AF173902, respectively) and CHF2 (AF176422 and AF176423, respectively) [23]. We first performed RT-PCR using the primers Fw1 and Rv1 and BAEC cDNA or human cerebellum cDNA (BioChain Institute, San Leandro, CA) as templates, and then the

second stage amplification was carried out with the primers Fw2 and Rv2 using the first PCR products as templates. The PCR was run for 30 cycles in the following cycle profile: 94 °C for 45 s, 47 °C for 1 min, and 72 °C for 1 min. Approximately 750 bp PCR products were extracted, ligated into pGEM-T Easy vector (Promega, Madison, WI), and then subjected to sequence analysis. The sequence analysis revealed that all of the sequenced clones derived from BAEC encoded bovine CHF2. One of these clones was designated as pBCHF2A. On the other hand, all of the sequenced clones derived from human cerebellum encoded human CHF-1.

We next tried to isolate cDNA encoding the entire open reading frame of bovine CHF2. To isolate the 5′-upstream terminal region of bovine CHF2, a 5′-RACE technique was used. The protocol for 5′-RACE is essentially the same as that described previously [26]. In brief, homometric dA tails were added to the randomly primed first strand cDNA, and the second strand cDNA was synthesized with dT_{17} adaptor primer, 5′-GACTCGAGTCGACATCGA$(T)_{17}$-3′. Two specific primers, 5R1 (5′-GAAGAGGGTCCGAGGCATCC-3′) and 5R2 (5′-GCTCAGGTAACGGGCGACTT-3′), were synthesized on the basis of the sequences of pBCHF2A. The first-stage PCR was performed using the adaptor primer and the specific primer 5R1 and the second-stage PCR was carried out with the first PCR products as templates using the adaptor primer and the specific primer 5R2. The PCR products were cloned into pGEM T-Easy vector and sequenced. A typical clone was designated as pBCHF2B. Next, for isolation of the 3′-downstream terminal region of bovine CHF2, we first tried to use a 3′-RACE technique as described previously [26,27]. Briefly, the first-stage PCR was carried out with dT_{17} adaptor primer-primed first strand cDNA as a template using the adaptor primer and the specific primer 3F1 (5′-CACCTCCGCCTCCAAACTCT-3′). We further performed the second PCR using the adaptor primer and the specific primer 3F2 (5′-CTCTCCACCCCTGCTCTCCT-3′). The PCR products were cloned into pGEM T-Easy vector and sequenced. A typical clone was designated as pBCHF2C. The cDNA inserts of the three clones, pBCHF2A, pBCHF2B, and pBCHF2C, as a whole covered bovine CHF2 cDNA. The nucleotide sequence of bovine CHF2 has been deposited in the GenBank database with Accession No. AB118750.

Construction of bovine CHF2 expression plasmid. Using two primers CHF2fw (5′-CCCGGCTAGCCAGCATGAAGCGAGCC-3′, *Nhe*I site is underlined) and CHF2rv (5′-CGGGATCCTTAAAAAGCTCC GATCTC-3′, *Bam*HI site is underlined) designated on the basis of the sequences of pBCHF2B and pBCHF2C, ORF of bovine CHF2 cDNA was amplified with BAEC cDNA as a template. An approximately 0.9 kb cDNA fragment was cloned into pGEM-T Easy vector and then sequenced. A typical clone was designated as pBCHF2. pBCHF2 was cut by *Nhe*I/*Bam*HI and then cloned into pcDNA3.1 (Invitrogen) to construct CHF2 expression plasmid (pBCHF2wt). For the construction of the expression vector for YRPW-motif deleted CHF2 mutant (pBCHF2mut), pBCHF2 was cut by *Nhe*I/*Nar*I and then cloned into pcDNA3.1.

Results

Effects of ectopic overexpression of HIF-1α and -2α on HRE-driven transcription in BAEC

In several kinds of cultured cells other than EC, ectopic overexpression of HIF-2α stimulated HRE-mediated reporter gene expression under hypoxic conditions, but the hypoxic induction was lower than that induced by HIF-1α [5,8,10,13]. We first examined the ectopic overexpression of HIF-1α and -2α on HRE-mediated reporter gene expression in BAEC, which express both HIF-1α and -2α [22]. As shown in Fig. 1, the transient transfection of the HIF-1α expression vector upregulated HRE-driven gene expression in normoxic BAEC, and exposure to hypoxia further enhanced the HRE-driven gene expression observed in normoxic cells. This effect of HIF-1α overexpression on HRE-driven transcription in BAEC was similar to that in cultured cells other than EC as described previously [5,10,13,28]. On the other hand, the effect of HIF-2α overexpression on HRE-driven transcription in BAEC was significantly different from that of HIF-1α. The transient transfection of HIF-2α expression vector also upregulated HRE-driven transcription, but exposure to hypoxia did not enhance the HRE-driven transcription in HIF-2α-transfected BAEC. The hypoxic induction of HRE-driven transcription in HIF-2α-transfected cells other than EC was lower than that in HIF-1α-transfected cells, but it was significant [5,10,13]. The transcriptional activity of HIF-2α might be regulated by a mechanism different from HIF-1α in BAEC, and the mechanism might be EC-specific.

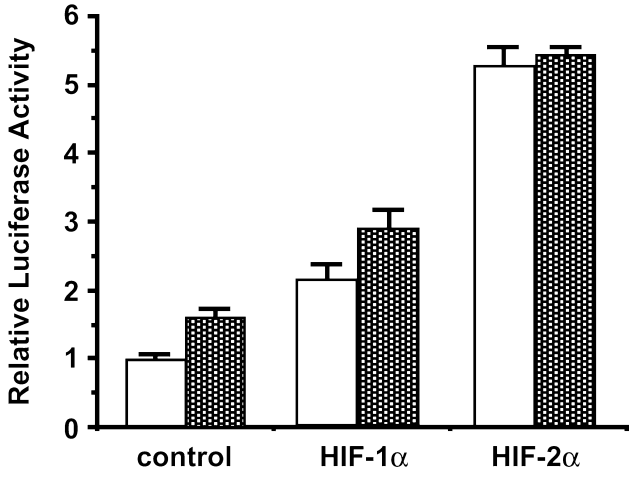

Fig. 1. Effects of ectopic overexpression of HIF-1α or -2α on HRE-driven transcription. BAEC were transiently transfected with the HIF expression plasmid (1 μg), the pHRE-Luc plasmid (1 μg), and a plasmid containing SV40 promoter and sea pansy luciferase (0.04 μg). The transfected cells were cultured under normoxia (open columns) or hypoxia (shaded columns) for 16 h and then assayed for luciferase activities.

Subcellular localization of HIF-1α and -2α in BAEC

We previously found that in BAEC, transfected GFP-tagged HIF-2α was localized in the nucleus both under normoxia and hypoxia, although GFP-tagged HIF-1α was localized in the cytoplasm under normoxia and translocated into the nucleus in response to hypoxia [29]. However, Park et al. [30] recently found that in mouse embryo fibroblasts, ectopic overexpressed HIF-2α was accumulated in the nucleus, but endogenous

HIF-2α was localized in the cytoplasm. Thus, we examined the subcellular localization of endogenous HIF-1α and -2α in BAEC by immunocytostaining analysis (Fig. 2A). Under normoxic conditions, endogenous HIF-1α was not detected in BAEC. HIF-1α protein could accumulate and translocate to the nucleus only when BAEC were exposed to hypoxia or $CoCl_2$ known to mimic hypoxic induction, as well as under the conditions seen in previous reports using several kinds of human and mouse cell lines [31]. On the other hand, HIF-2α protein could escape from oxygen-dependent protein degradation in BAEC and, even under normoxic conditions, HIF-2α was localized in the nucleus of BAEC. The staining pattern of HIF-2α in the nucleoplasm was extranucleolar and appeared in prominent nuclear dots. Subcellular localization of HIF-2α protein was not affected by hypoxia or $CoCl_2$. Western blot analysis using nuclear proteins revealed that the expression level of HIF-2α protein was also not affected by hypoxia or $CoCl_2$ (Fig. 2B). It was recently reported that endogenous HIF-2α protein in mouse embryo fibroblasts also could escape from oxygen-dependent protein degradation, but it was localized in the cytoplasm [30]. The subcellular localization of HIF-2α protein might be regulated by an EC-specific mechanism.

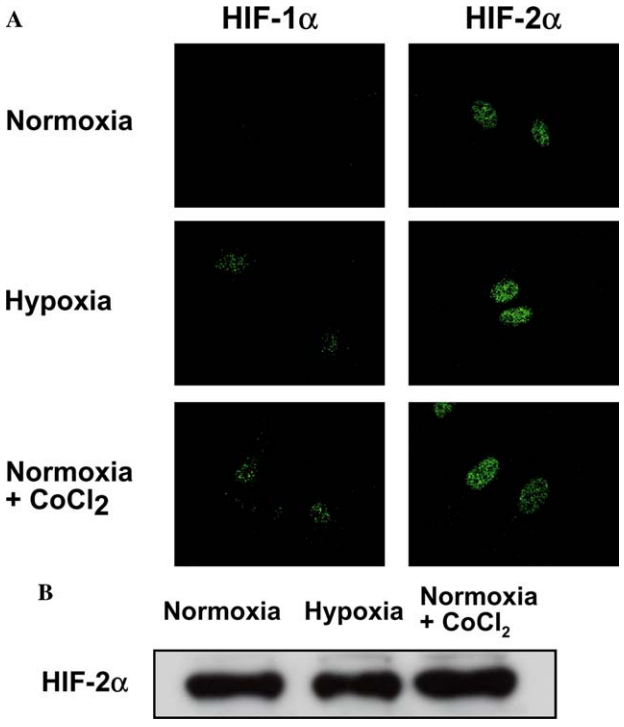

Fig. 2. Expression of HIF-2α in BAEC. (A) Subcellular localization of endogenous HIF-1α and -2α in BAEC. BAEC were cultured under normoxia or hypoxia, or in the presence of 150 μM $CoCl_2$ for 16 h, and then immunostained with anti-HIF-1α or anti-HIF-2α antibody. (B) Protein levels of HIF-2α in the nucleus of BAEC. BAEC were cultured under normoxia or hypoxia, or in the presence of 150 μM $CoCl_2$ for 16 h, and then nuclear proteins were extracted. Fifty micrograms of nuclear proteins was separated on a 10% SDS–polyacrylamide gel, electrotransferred to polyvinylidene difluoride membrane, and then probed with anti-HIF-2α antibody.

Colocalization of HIF-2α and ARNT in BAEC

Both HIF-1α and -2α heterodimerized with ARNT/HIF-1β. In order to examine the subcellular localization of ARNT in BAEC, we next constructed the expression vector for FLAG-tagged bovine ARNT, transfected the vector into BAEC, and then examined the subcellular localization of FLAG-tagged ARNT in BAEC using immunocytostaining with anti-FLAG antibody. As shown in Fig. 3, in normoxic BAEC, the immunostaining pattern of FLAG-tagged ARNT appeared in prominent nuclear dots characteristic of nuclear and splicing bodies. Neither hypoxia nor $CoCl_2$ affected the subcellular localization of FLAG-tagged ARNT. Furthermore, double immunocytostaining analysis revealed that HIF-2α was colocalized with FLAG-tagged ARNT in the nuclear dots. HIF-2α might heterodimerize with ARNT and mediate the expression of some EC-specific genes in BAEC both under normoxia and hypoxia.

Expression of CHF2 in BAEC

Chin et al. [23] identified CHF1 and CHF2 by a yeast two-hybrid screen using ARNT as a bait, and found that CHF1 was highly expressed in developing cardiomyocytes and vascular smooth muscle cells and inhibited ARNT and HIF2α-mediated HRE-driven transcription. However, the expression and the function of CHFs in EC have not been fully elucidated. We next examined the

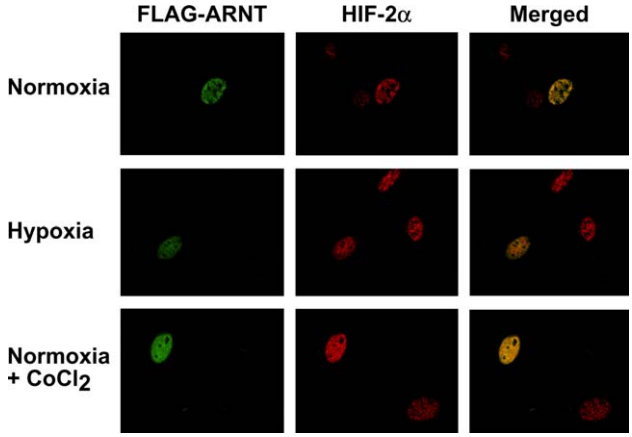

Fig. 3. Subcellular localization of transfected FLAG-tagged ARNT and endogenous HIF-2α in BAEC. BAEC were transiently transfected with FLAG-tagged ARNT expression vectors and then cultured under normoxia or hypoxia, or in the presence of 150 μM $CoCl_2$ for 16 h. The cells were immunostained with anti-FLAG and anti-HIF-2α antibody, and the immunostaining signals were visualized with fluorescein isothiocyanate-conjugated anti-mouse Igs and Cy3-conjugated anti-rabbit IgG antibodies.

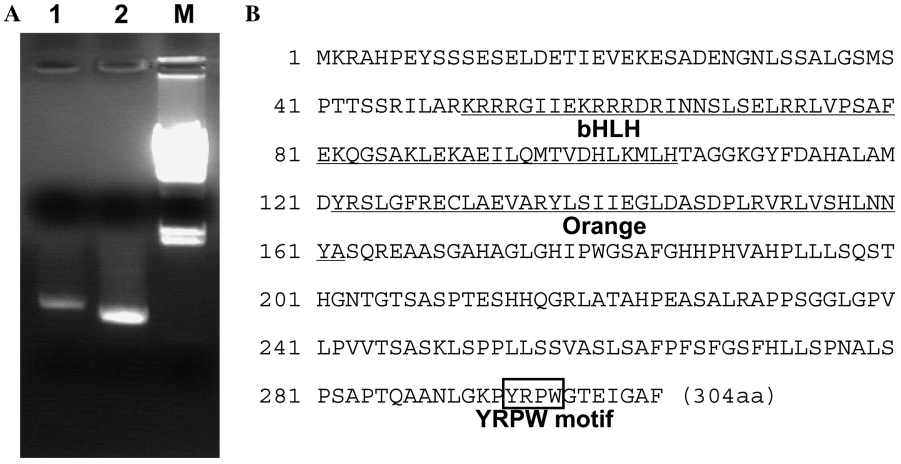

Fig. 4. Expression of CHF2 in BAEC. (A) RT-PCR analysis of expression of CHFs. The cDNA fragments of CHFs were amplified using BAEC cDNA (lane 2) or human cerebellum cDNA (lane 1) as templates and then visualized on 1.5% agarose gels. M, molecular weight marker. (B) Predicted amino acid sequence of bovine CHF2. The bHLH region (53–105 amino acids) and Orange domain (122–163) are underlined, and the YRPW motif (294–297) is boxed. The nucleotide sequence of bovine CHF2 has been deposited in GenBank as Accession No. AB118750.

expression of CHF isoforms by degenerated oligonucleotide primers designated on the basis of the sequences of the bHLH domain and the C-terminal region of human and mouse CHF1 and CHF2 [23]. As shown in Fig. 4A, approximately 750 bp of cDNA was amplified, and the sequence analysis revealed that these PCR products encoded bovine CHF2. Human CHF1 cDNA fragments were amplified by these primers using human cerebellum cDNA as a template, but CHF1 was not amplified using BAEC cDNA. These results indicated that among CHF isoforms, only CHF2 was expressed in BAEC.

To determine the full-length cDNA sequence of CHF2 from BAEC, we used a RACE technique and cloned 5′-upstream and 3′-downstream regions of bovine CHF2 cDNA. As shown in Fig. 4B, bovine CHF2 cDNA encoded 304 amino acid residues. Bovine CHF2 exhibited a 95.1%, 92.8%, 52.8%, and 55.0% identity to the human and mouse CHF2 and human and mouse CHF1, respectively. Three putative functional domains in CHF isoforms, namely the bHLH domain, Orange domain, and YRPW motif, were conserved in bovine CHF2.

Suppression of HIF-mediated gene expression by CHF2 in BAEC

We examined the effects of the overexpression of CHF2 on HRE-driven gene expression in BAEC. As shown in Fig. 5A, the transfection of CHF2 vector suppressed HRE-driven gene expression both under normoxia and hypoxia. On the other hand, the C-terminal deletion mutant of CHF2, which lacked the YRPW motif, did not suppress HRE-driven transcription. These results indicate that the YRPW motif is necessary for the repression of HRE-driven transcription. CHFs were identified as ARNT-interacting proteins [23], and ARNT heterodimerizes with AhR as well as HIF-1α and -2α [3]. We next examined the effect of the transfection of CHF2 on AhR-mediated xenobiotic responsive element (XRE)-driven gene expression. As shown in Fig. 5B, the transfection of CHF2 did not suppress XRE-driven transcription. CHF2 might be a sequence-specific transcription repressor of HRE-driven gene expression and might be one of the key regulators of HIF activity in BAEC.

Discussion

HIF-2α has a high identity with HIF-1α in its functional domains [5,6], and the expression level and transcriptional activity of HIF-2α are regulated by hydroxylation of its specific proline and asparagine residues as well as those of HIF-1α [32,33]. Like HIF-1α, HIF-2α, under hypoxic conditions, is stabilized and translocated into the nucleus, heterodimerizes with ARNT and then activates HRE-mediated gene expression [5,6,13,34]. However, the gene targeting studies have revealed that the phenotypes of HIF-1α- and -2α-deficient mice are different, and that HIF-1α and -2α cannot compensate for the lack of the other isoform [16–19]. In the present study, we found that, even under normoxic conditions, HIF-2α was stable and was localized in the nucleus of BAEC differently than HIF-1α (Fig. 2). The difference in the stability and subcellular localization of HIF-1α and -2α in vascular EC may contribute to the difference of the phenotypes of HIF-1α- and -2α-deficient mice. At present, the mechanism by which the stability and subcellular localization of HIF-2α are regulated remains unknown, but Hogenesch et al. reported that HIF-2α did not interact with the heat shock protein Hsp90, which tightly interacted with HIF-1α [8]. The lower affinity of HIF-2α for Hsp90 might enable HIF-2α to

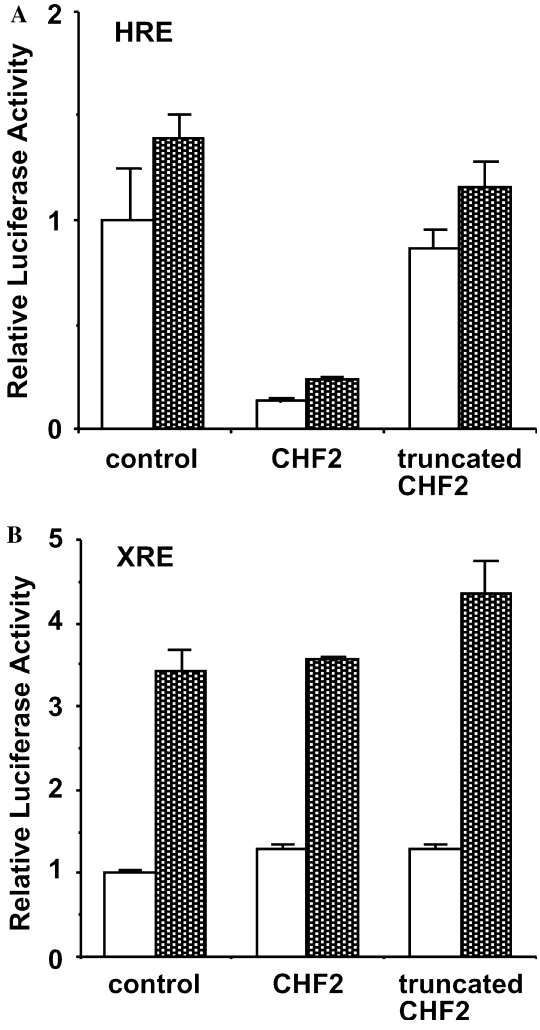

Fig. 5. Effects of overexpression of CHF2 on HRE- and XRE-driven transcription. (A) Effects of overexpression of CHF2 on HRE-driven transcription. BAEC were transiently transfected with the full-length or truncated CHF2 expression plasmid (1 μg), the pHRE-Luc plasmid (1 μg), and sea pansy luciferase plasmid (0.04 μg). The transfected cells were cultured under normoxia (open columns) or hypoxia (shaded columns) for 16 h and then assayed for luciferase activities. (B) Effects of overexpression of CHF2 on XRE-driven transcription. BAEC were transiently transfected with the full-length or truncated CHF2 expression plasmid (1 μg), the pXRE-Luc plasmid (1 μg), and sea pansy luciferase plasmid (0.04 μg). The transfected cells were cultured with (shaded columns) or without (open columns) 3MC (10 μg/ml) for 40 h and then assayed for luciferase activities.

escape from oxygen-dependent protein degradation in the cytoplasm and translocate into the nucleus in BAEC. However, it was recently reported that endogenous HIF-2α protein in mouse embryo fibroblasts also could escape from oxygen-dependent protein degradation, but it was localized in the cytoplasm [30]. The subcellular localization of HIF-2α protein might be regulated by an unknown EC-specific mechanism.

As shown in Fig. 3, in BAEC, HIF-2α was colocalized with ARNT in the nuclear dots both under normoxia and hypoxia. These results suggested that HIF-2α might heterodimerize with ARNT and mediate the expression of some EC-specific genes both under normoxia and hypoxia. It has been shown that HIF-2α, but not HIF-1α, stimulated the expression of *Tie-2* and *Flk-1*, both of which are EC-specific tyrosine kinase genes [5,35]. Elvert et al. [36] recently reported that HIF-2α, but not HIF-1α, interacted and cooperated with another transcriptional factor Ets-1 in activating the transcription of *Flk-1* gene. A heterodimer composed of HIF-2α and ARNT might interact with some of the other transcriptional factors and mediate several EC-specific genes in normoxic EC.

In the present study, we further showed that transcriptional repressor CHF2 was expressed in BAEC and suppressed HRE-dependent gene expression in a sequence-specific manner (Fig. 4). CHFs (CHF1 and CHF2) are bHLH proteins related to the hairy/enhancer of split (E(spl)) family and were identified as ARNT-interacting proteins by Chin et al. [23]. CHF1 and CHF2 were also known as HERP1/Hesr2/Hey2/HRT2/Gridlock and HERP2/Hesr1/Hey1/HRT1, respectively [37]. Although Chin et al. [23] reported that CHF1 was highly expressed in developing cardiomyocytes and vascular smooth muscle cells, CHF1 was not expressed in BAEC (Fig. 4A). As shown in Fig. 5A, transfected CHF2 suppressed HRE-dependent gene expression both under normoxia and hypoxia. CHF2 might be one of the key regulators of HIF-2α-mediated gene expression in normoxic EC. Henderson et al. [38] reported that overexpression of CHF2/Hesr1 in EC down-regulated *Flk-1* gene expression and blocked EC proliferation, migration, and network formation, and that reduction of the expression of CHF2/Hesr1 by antisense oligonucleotides also blocked EC network formation. CHF2 might regulate the tubular network formation of EC and the maintenance of the mature vessel by its ability to suppress HIF-2α-mediated gene expression.

In conclusion, our findings suggested that in vascular EC, HIF-2α might be regulated by an EC-specific mechanism different from that which regulates HIF-1α, and that HIF-2α and its suppressor CHF2 might cooperatively regulate the expression of EC-specific genes and the maintenance of the mature vessel.

Acknowledgments

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