1194 *Biochemistry* **2011,** *50,* 1194–1202
DOI: 10.1021/bi101585c

BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Hypoxic Induction of Human Erythroid-Specific δ-Aminolevulinate Synthase Mediated by Hypoxia-Inducible Factor 1†

Feng-Lin Zhang, Guo-Min Shen, Xiao-Ling Liu, Fang Wang, Hua-Lu Zhao, Jia Yu, and Jun-Wu Zhang*

National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

Received September 30, 2010; Revised Manuscript Received January 5, 2011

ABSTRACT: Hypoxia-inducible factor 1 (HIF1) is a heterodimeric basic helix−loop−helix transcription factor that regulates many key genes. δ-Aminolevulinate synthase (ALAS) catalyzes the first and rate-limiting reaction in the heme biosynthetic pathway. In this study, we show that hypoxia-induced expression of erythroid-specific ALAS2 is mediated by HIF1 in erythroid cells. Under hypoxic conditions, significantly increased *ALAS2* mRNA and protein levels were detected in K562 cells and erythroid induction cultures of CD34+ hematopoietic stem/progenitor cells. Enforced HIF1α expression increased the level of *ALAS2* expression, while HIF1α knockdown by RNA interference decreased the level of *ALAS2* expression. In silico analysis revealed three potential hypoxia-response elements (HREs) that are located 611, 621, and 741 bp downstream of the *ALAS2* gene. The results from reporter gene and mutation analysis suggested that these elements are necessary for a maximal hypoxic response. Chromatin immunoprecipitation and polymerase chain reaction showed that the HREs could be recognized and bound by HIF1α in vivo. These results demonstrate that the upregulation of *ALAS2* during hypoxia is directly mediated by HIF1. We hypothesize that HIF1-mediated *ALAS2* upregulation promotes erythropoiesis to satisfy the needs of an organism under hypoxic conditions. This may be accomplished via increased heme levels and an interaction between ALAS2 and erythropoietin.

Oxygen supply contributes significantly to many physiologic and pathophysiologic responses, including erythropoiesis, angiogenesis, glucose uptake, glycolysis, iron transport, and apoptosis. Mountain sickness and other diseases related to high altitude are normally caused by the insufficient availability of oxygen and have been considered to be public health problems. Hypoxia can be a severe hazard for people traveling to high-altitude areas. However, native people are able to adapt to high altitudes and develop the ability to tolerate environmental hypoxia. The detailed mechanisms related to these processes are not yet clear. Previous studies have shown that hypoxia-inducible factor (HIF)[1] plays a vital role in sensing and responding to hypoxia.

HIF, originally identified as a nuclear factor capable of binding a DNA sequence within the human erythropoietin (*EPO*) gene (*1*), is a heterodimeric transcription factor. It is composed of two basic helix−loop−helix subunits: HIFα, which is constitutively expressed but rapidly degraded under normoxic conditions, and HIF*β*, which is a constitutively expressed, stable protein (*2*). Under normoxic conditions, HIF1α is hydroxylated by prolyl hydroxylase (PHD), bound by the von Hippel−Lindau tumor suppressor protein (VHL), and targeted for degradation by the 28S proteasomal system (*3*−*5*). Moreover, the carboxy-terminal activation domain of HIF1α can be hydroxylated by asparaginyl hydroxylase, a factor inhibiting HIF (FIH). This blocks recruitment of the transcriptional coactivators p300 and CREB binding protein (p300/CBP) (*6*, *7*). The combination of these processes leads to a decreased level of transcription of HIF target genes. Under hypoxic conditions, as the activities of these hydroxylases become impaired because of the lack of oxygen, hydroxylation of HIF1α is inhibited. When HIF1α accumulates, it dimerizes with HIF1*β*, binds to hypoxia-response elements (HREs) in target genes, and recruits coactivator proteins, which leads to an increased level of gene transcription (*8*, *9*). HREs are associated with a broad range of transcriptional target genes, and the core DNA motif is RCGTG (R = G or A) (*10*).

Hypoxia induces erythropoiesis by stimulating erythropoietin production, mainly in the adult kidneys, and the cell−cell contacts of erythroblasts and endothelial cells in the bone marrow (*11*). However, it has also been suggested that hypoxia directly promotes hemoglobin production in erythroid cells (*12*). The prosthetic group of hemoglobin, heme, consists of a complex organic ring structure, protoporphyrin, and a single iron atom in the ferrous oxidation state (*13*). Iron in the ferrous state binds oxygen reversibly; in the ferric state, it does not bind oxygen (*14*). Ferrochelatase (FECH) catalyzes the insertion of the ferrous form of iron into protoporphyrin, and heme oxygenase 1 (HMOX1, also known as

†This research was supported by grants from the National Basic Research Program of China (2006CB504100) and the National Natural Science Foundation of China (30721063) to J.-W.Z.

*To whom correspondence should be addressed: National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College. Beijing 100005, China. Tel: 86-10-65296423. Fax: 86-10-65240529. E-mail: junwu_zhang@pumc.edu.cn.

[1]Abbreviations: HIF, hypoxia-inducible factor; ALAS, δ-aminolevulinate synthase; HRE, hypoxia-response element; ChIP, chromatin immunoprecipitation; PHD, prolyl hydroxylase domain; VHL, von Hippel−Lindau tumor suppressor protein; FIH, factor inhibiting HIF; CBP, CREB binding protein; FECH, ferrochelatase; HMOX1, heme oxygenase 1; ALA, δ-aminolevulinic acid; UCB, umbilical cord blood; MNC, mononuclear cell; IMDM, Iscove's Modified Dulbecco's Medium; BSA, bovine serum albumin; 2-ME, 2-mercaptoethanol; IL-3, interleukin 3; SCF, stem cell factor; EPO, erythropoietin; RNAi, RNA interference; siRNA, short interfering RNA; HPC, hematopoietic progenitor cell; TBS-T, Tris-buffered saline with Tween; PBS, phosphate-buffered saline; IRE, iron regulatory element; IRP, IRE binding protein.

 Published on Web 01/05/2011

HO-1) cleaves heme to yield biliverdin, carbon monoxide (CO), and iron. δ-Aminolevulinate synthase (ALAS) catalyzes the condensation of glycine with succinyl-CoA to form δ-aminolevulinic acid, which is the first and rate-limiting reaction in the mammalian heme biosynthetic pathway. ALAS1 is a housekeeping enzyme, whereas ALAS2 is an erythroid-specific mitochondrial enzyme. Here, we show that induction of human *ALAS2* gene expression is mediated by HIF1 under hypoxic conditions.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The K562 (human myelogenous leukemia) and MCF-7 (human breast adenocarcinoma) cell lines were used in this study. The cells were grown in RMPI 1640 medium or Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS) and a penicillin/streptomycin mixture, at 37 °C in an incubator with a controlled humidified atmosphere containing 5% CO_2. For hypoxic stimulation, cells were placed in an incubator chamber that was tightly sealed and thoroughly flushed with a gas mixture containing 5% CO_2, 1% O_2, and 94% nitrogen and incubated at 37 °C for various periods of time. Where indicated, cobalt chloride (Sigma-Aldrich, Deisenhofen, Germany) was added to the medium at a final concentration of 100 μM.

Isolation and Erythroid Induction Cultures of CD34+ Hematopoietic Stem/Progenitor Cells (*HPCs*). Human umbilical cord blood (UCB) was obtained from normal full-term deliveries after informed consent as approved by the Research Ethics Committee of the Military General Hospital of Beijing (Beijing, China). Mononuclear cell (MNC) fractions were isolated from UCBs by Percoll density gradient (d = 1.077; Amersham Biotech). CD34+ HPCs were enriched from MNCs through positive immunomagnetic selection (CD34 MultiSort kit, Miltenyi Biotec, Bergisch-Glad-bach, Germany). The CD34+ cells were cultured in IMDM supplemented with 30% fetal bovine serum, 1% BSA, 100 μM 2-ME, 2 ng/mL recombinant human IL-3, 100 ng/mL recombinant human SCF (Stem Cell Technologies, Vancouver, BC), 2 units/mL recombinant human EPO (R&D Systems), 60 mg/mL penicillin, and 100 mg/mL streptomycin. Five days later, the cells were cultured in normoxia or hypoxia for the indicated time before being harvested.

Plasmid Constructs. The cDNA encoding HIF1α was obtained by polymerase chain reaction (PCR) amplification using the HA-HIF1α plasmid (*15*) (a gift from Y. Jung, Pusan National University, Busan, Korea) as a template. The primers HIF-1α-F (5′-ACCGGTACCATGGAGGGCGCCGGCGGC-3′) and HIF-1α-R (5′-ACGGGCCCGCGTTAACTTGATCCAAAGCTC-3′) were used for PCR amplification. The amplified fragment (2480 bp) was inserted, in-frame, into the KpnI/ApaI sites of FLAG-tagged pcDNA6/V5-His B (Invitrogen, Carlsbad, CA), yieding the pcDNA6 V5HisB/HIF1α construct (pHIF1α).

To specifically silence the gene encoding HIF1α, we constructed plasmid pSilencer 2.1U6-HIF1α. Plasmid pSilencer 2.1-U6 neo (Ambion, Austin, TX) was linearized using both BamHI and HindIII to facilitate directional cloning. The target sequences of HIF1α RNAi were consistent with that described by Berchner-Pfannschmidt et al. (*16*). To create the target sequences, we synthesized two oligonucleotides, 5′-AGCTTTTCCAAAAAACTAACTGGACACAGTGTGTTCTCTTGAAACACACTGTGTCCAGTTAGCG-3′ (sense) and 5′-GATCCGCTAACTGGACACAGTGTGTTTCAAGAGAACACACTGTGTCCAGTTAGTTTTTTGGAAA-3′ (antisense). The two oligonucleotides were annealed and inserted into the pSilencer 2.1-U6 vector to create plasmid pSilencer 2.1U6-HIF1α-RNAi (pSiHIF1α).

To construct recombination plasmid pGL3-ALAS2, we doubly digested an 877 bp fragment amplified from human genomic DNA using SalI and BamHI (MBI Fermentas) and inserted the fragment into the BamHI/SalI sites of the pGL3-Promoter Vector (Promega, Madison, WI). Mutations of the putative HRE sequence in the pGL3-ALAS2 plasmid were introduced by polymerase chain reaction-based site-directed mutagenesis. The CTTTC motif replaced the CACGC motif at position 611 to construct pALAS2-M1; the CTTTC motif replaced the CACGC motif at position 621 to construct pALAS2-M2, and the GAAAG motif replaced the GCGTG motif at position 741 to construct pALAS2-M3. We also constructed pALAS2-M12 and pALAS2-M13, which carry dual mutations, and pALAS2-M123, which carries the triple mutations. The following primers were used for amplification and mutation: pALAS2F, 5′-ATGGGATCCGACGCTTCACTGGTAATC-3′; pALAS2R, 5′-AAGGTCGACGTTCTCTGCTCAGTTGTAG-3′; M1F, 5′-CTTGGATTACAGGCTTTCGCCACCACG-3′; M1R, 5′-TGGGCGTGGTGGCGAAAGCCTGTAATC-3′; M2F, 5′-AGGCACGCGCCACCTTTCCCAGTTAAT-3′; M2R, 5′-AAAAATTAACTGGGAAAGGTGGCGCGT-3′; M3F, 5′-GCTGGGATTACAGGAAAGCACCACTGT-3′; M3R, 5′-AGGCACAGTGGTGCTTTCCTGTAATCC-3′; M12F, 5′-AGGCTTTCGCCACCTTTCCCAGTTAAT-3′; M12R, 5′-AAAAATTAACTGGGAAAGGTGGCGAAA-3′. All construct sequences were verified by sequencing.

Cell Transfection with Plasmids or siRNAs. For cell transfection with plasmids, K562 cells were plated in 3.5 cm dishes at 70−80% confluency on the day of transfection with 4 μg of plasmid. Lipofectamine 2000 (Invitrogen) was used as a transfection reagent according to the manufacturer's instructions.

For cell transfection with siRNA, K562 cells were plated in 3.5 cm dishes at 70−80% confluency on the day of transfection. The siRNAs (Dharmacon, Lafayette, CO) were resuspended, and DharmaFECT transfection reagents were used according to the manufacturer's instructions.

RNA Extraction, Reverse Transcription, and Real-Time PCR. Total RNA was extracted from cell samples using TRIzol Reagent (Invitrogen) and quantified using the NanoDrop 2000 spectrophotometer (Thermo Scientific Inc., Bremen, Germany). The first strand of cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's instruction. The expression of targeted mRNAs in cultured K562 cells and CD34+ HPCs was quantified by real-time PCR using the iQ5 Real-Time PCR Detection System (Bio-Rad) with TransScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech). Each PCR was performed in triplex tubes and used β-actin mRNA as an endogenous control to standardize the amount of sample mRNA. The quantification data were analyzed with the iQ5 software (Bio-Rad). The following primers were used for real-time PCR: β-actin-F, 5′-CTGGCACCACACCTTCTACA-3′; β-actin-R, 5′-AGCACAGCCTGGATAGCAAC-3′; ALAS2-F, 5′-AATGACCCTGACCACCTAAAG-3′; ALAS2-R, 5′-GGACACATCACACAACTCCTC-3′; HIF-1α-F, 5′-AGGTGGATATGTCTGGGTTG-3′; HIF-1α-R, 5′-AAGGACACATTCTGTTTGTTG-3′.

Western Blot Analysis. The cell samples were lysed in SDS lysis buffer [50 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), and 10% glycerol]. Total cell extracts were quantified with Synergy 4 (BioTek, Tucson, AZ) using the BCA Protein Assay Kit (VIGOROUS, Beijing, China) and fractionated by electrophoresis

on 10% SDS−polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were blocked with a 5% nonfat dry milk solution and incubated with either anti-HIF1α antibody (Abcam plc, Cambridge, U.K.), anti-ALAS2 antibody (Abnova, Taipei, Taiwan), or anti-ACTB antibody (ProteinTech Group Inc., Chicago, IL) followed by peroxidase-conjugated affinipure goat anti-mouse IgG (H+L) (Zhongshan Goldenbridge, Beijing, China). After being washed with TBS-T buffer, the membrane was treated with Immobilon Western Chemiluminescent HRP Substrate (Millipore) and exposed to Kodak X-omat BT Film.

Flow Cytometric Analysis. Cells were cultured in normoxia or hypoxia for 36 h and then collected and resuspended in PBS. Cells were then fixed with formaldehyde and permeated with methanol. After being rinsed with incubation buffer (PBS with BSA), cells were incubated with unconjugated primary antibody for 1 h at room temperature. After being rinsed with incubation buffer by centrifugation, cells were incubated with fluorochrome-conjugated secondary antibody for 30 min at room temperature. Cells were rinsed, resuspended in PBS, and analyzed using the Accuri C6 flow cytometer system (Accuri Cytometers, Ann Arbor, MI).

Chromatin Immunoprecipitation and PCR (*ChIP−PCR*). K562 cells in four 100 mm culture dishes containing 10 mL of growth medium were cultured under normoxia (21% O_2) or hypoxia (1% O_2) for 24 h and then fixed in 1% formaldehyde (Sigma-Aldrich) at room temperature for 10 min and quenched for 5 min with glycine. Cells were lysed and sonicated to yield 200−1000 bp DNA fragments. Chromatin immunoprecipitation was performed using the EZ-ChIP chromatin immunoprecipitation kit (Millipore) according to the manufacturer's instructions with minor modifications. A ChIP-grade rabbit polyclonal antibody to HIF1α (Abcam plc) was used as the immunoprecipitating antibody, and rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) was used as the control. After reverse cross-linking and DNA purification, the input and immunoprecipitated DNA samples were amplified by PCR, and the products were assayed by agarose gel electrophoresis. The following primers were used for ChIP−PCR with a 386 bp product covering the putative HREs of ALAS2: forward (5′-CAGGATTTACCAGTCCCATTC-3′) and reverse (5′-CAGGATTTACCAGTCCCATTC-3′). We also designed the primers for the positive control [forward (5′-CAGTGACCTGCGATGGTG-3′) and reverse (5′-GCGACAGACCCACTTACG-3′)] with a 371 bp product covering the HRE of *FECH* and the negative control [forward (5′-CTGATAAGACTACACTGGACG-3′) and reverse (5′-CCCAAGATACTACACTACCATC-3′)] with a 486 bp product including no HREs.

Dual-Luciferase Reporter Assay. For the transient transfection of plasmids, MCF-7 cells were plated in each well of 24-well plates at 50−60% confluency the day before cotransfection using the PEI [poly(ethyleneimine)] method as described previously (*17*, *18*) with a mixture of 1 μg of pGL3 reporter plasmid and 20 ng of pRL-TK reporter vector encoding Renilla luciferase, which was used to normalize for transfection efficiency. Six hours after transfection, cells were transferred to complete medium and incubated under the specified condition. Cells were lysed using passive lysis buffer, and the dual-luciferase activities were measured with a Modulus Microplate Luminometer (Turner Biosystems, Sunnyvale, CA) using the dual-luciferase reporter assay system (Promega, Milan, Italy) according to the manufacturer's instructions.

Induction of K562 Erythroid Differentiation and Benzidine Staining. To induce K562 erythroid differentiation, we added hemin (Sigma-Aldrich) to the medium at a final concentration of 40 μM. To detect hemoglobin-positive cells, benzidine staining was conducted according to the standard protocol.

Measurement of Cellular Heme Content. The heme level in K562 cells was measured using a modified QuantiChrom Heme Assay (BioAssay Systems). Cells were pelleted and washed with PBS, resuspended in distilled water, and transferred into sample wells. After reagent had been added to the sample wells and the mixture incubated for 5 min at room temperature, the absorbance at 400 nm was measured using a Synergy 4 instrument (BioTek).

Statistics. A Student's *t* test (two-tailed) was performed to analyze our data. *p* values of < 0.05 were considered significant.

RESULTS

Hypoxia-Induced Expression of ALAS2. To examine whether *ALAS2* gene expression is induced under hypoxic conditions, K562 cells and $CD34^+$ HPCs were cultured at normoxia (21% O_2) or hypoxia (1% O_2) for 8−36 h before being harvested. *ALAS2* mRNA levels were determined by real-time PCR. A significantly increased level of *ALAS2* mRNA expression was observed in K562 cells under cobalt chloride-simulated hypoxia or hypoxic conditions (Figure 1A). In K562 and CD34+ erythroid cultures exposed to hypoxic conditions, the level of *ALAS2* mRNA expression gradually increased over time (Figure 1B,C). A Western blot assay was performed to examine ALAS2 protein expression. As expected, obvious increases in HIF1α and ALAS2 protein levels were detected in K562 cells exposed to cobalt chloride-simulated hypoxia or hypoxia (Figure 1D). Increased and sustained ALAS2 protein levels were observed in K562 and CD34+ erythroid cultures exposed to hypoxic conditions (Figure 1E,F). Furthermore, flow cytometric analysis also revealed a notable increase in the level of ALAS2 protein in K562 cells during hypoxia (Figure 1G). These results establish that hypoxia induces expression of ALAS2.

Hypoxia-Induced Expression of ALAS2 Is Regulated by HIF1. To examine whether the hypoxia-induced expression of ALAS2 is regulated by HIF1, we constructed an HIF1α expression plasmid, pcDNA6 V5HisB/HIF1α (pHIF1α), and a plasmid that expresses an HIF1α-specific interference RNA sequence, pSilencer 2.1/U6-HIF1α-RNAi (pSiHIF1α). These plasmids or their corresponding empty vectors were transfected into K562 cells. The mRNA levels of the HIF1α gene (*HIF1A*) and *ALAS2* were determined by real-time PCR. As expected, there was a dramatic increase in the level of *HIF1A* mRNA expression in K562 cells transfected with pHIF1α, and this HIF1α overexpression induced expression of ALAS2 mRNA (Figure 2A). In contrast, there was a sharp decrease in *HIF1A* mRNA levels when K562 cells were transfected with pSiHIF1α, and this was accompanied by a significant decrease in *ALAS2* mRNA levels (Figure 2B). K562 cells were also transfected with siRNA that specifically targeted the HIF1α gene (si HIF1α). Compared to the cells transfected with control siRNA (siCON), RNA interference-mediated knockdown of HIF1 resulted in a decreased level of expression of *ALAS2* mRNA (Figure 2C). Additionally, ALAS2 protein levels were determined by Western blotting. The level of ALAS2 protein expression increased in K562 cells transfected with pHIF1α and decreased in K562 cells transfected with pSiHIF1α, relative to the protein levels observed in cells transfected with the corresponding empty vectors (Figure 2D,E). Significant declines in HIF1α and ALAS2 protein levels were

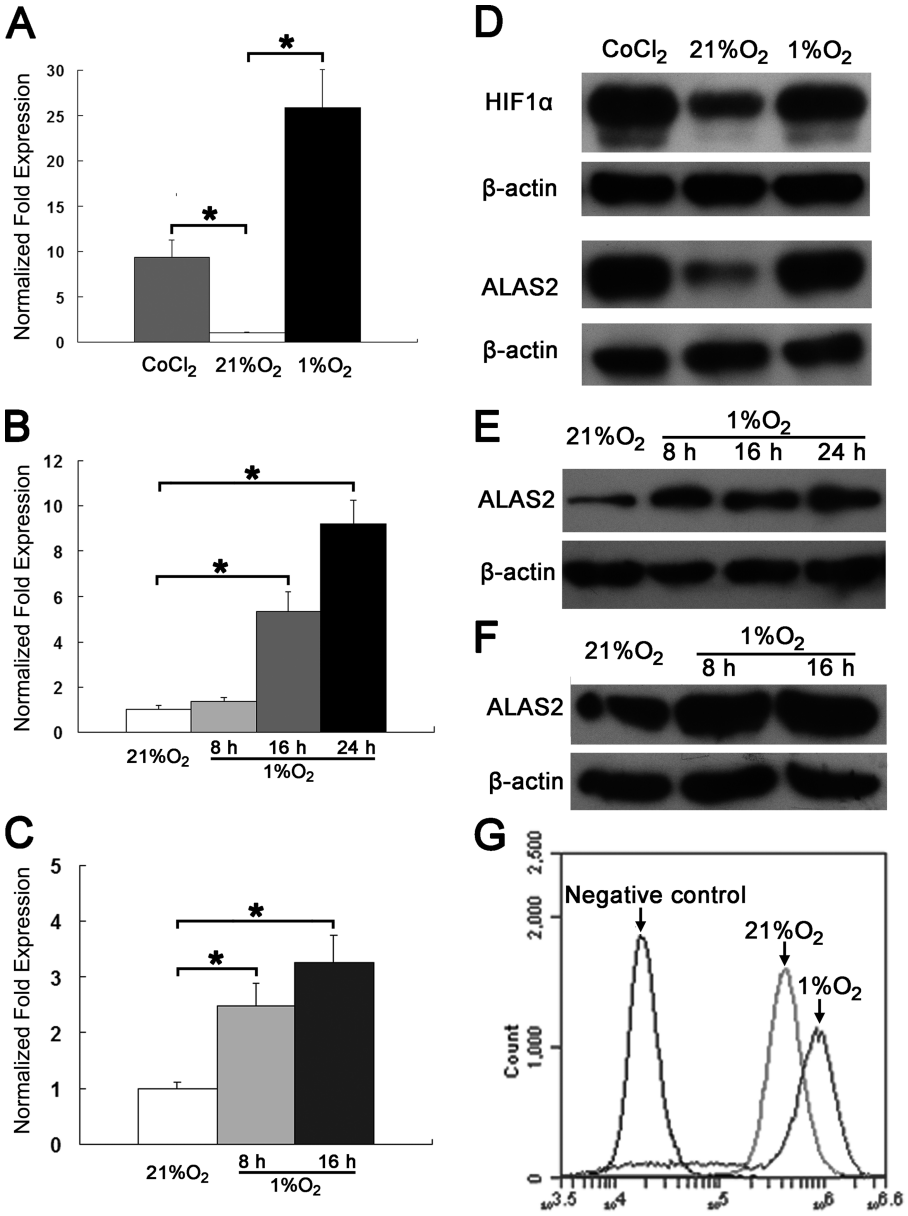

FIGURE 1: Induction of ALAS2 gene expression during hypoxia. (A) Real-time PCR analysis of *ALAS2* mRNA expression [mean ± standard error of the mean (SEM)] in K562 cells that were cultured with 100 μM $CoCl_2$, or under normoxia (21% O_2) or hypoxia (1% O_2) for 24 h. (B) Real-time PCR analysis of *ALAS2* mRNA expression (mean ± SEM) in K562 cells that were cultured under normoxia (21% O_2) or hypoxia (1% O_2) for the indicated hours. (C) Real-time PCR analysis of *ALAS2* mRNA expression (mean ± SEM) in CD34+ erythroid cultures. The asterisk indicates a p value of < 0.01. (D) Western blot assay of HIF1α, ALAS2, and β-actin protein expression in K562 cells cultured in 100 μM $CoCl_2$, or under normoxia (21% O_2) or hypoxia (1% O_2) for 36 h. (E) Western blot assay of ALAS2 protein expression in K562 cells that were cultured under normoxia (21% O_2) or hypoxia (1% O_2) for the indicated number of hours. (F) Western blot assay of ALAS2 and β-actin protein expression in CD34+ erythroid cultures. The UCB-derived CD34+ cells were cultured in erythroid induction culture medium for 4 days and exposed to 21% O_2 (normoxia) for 16 h or 1% O_2 (hypoxia) for the indicated number of hours. (G) Flow cytometry analysis of ALAS2 protein expression in K562 cells that were cultured under normoxia (21% O_2) or hypoxia (1% O_2) for 36 h. After being fixed and permeated, the cells were incubated with unconjugated primary antibody and then with fluorochrome-conjugated secondary antibody. As a negative control, cells were incubated only with secondary antibody.

also observed in the cells transfected with siHIF1α in comparison to those transfected with control siRNA (Figure 2F). These results establish that *ALAS2* gene expression is regulated by HIF1.

Identification of Putative and Functional HREs in the Human ALAS2 Gene. We searched the DNA sequence of the human *ALAS2* gene on NCBI and identified three putative HREs in the 3′-flanking sequence of the gene (Figure 3A). The characteristics of these HREs were extremely similar to those of the consensus HRE described previously (*10*, *19*, *20*). The three HRE core motifs are located within a < 150 bp region, where the CACGC motif is a reverse complementary motif (Figure 3B). The presence of such motifs is necessary but not sufficient for HIF1 recognition and HRE function.

To determine whether each of the three HREs within the 3′-flanking region is actually involved in hypoxia-mediated *ALAS2* gene induction, the DNA fragment containing this region was inserted into the 3′-clone site of a luciferase reporter gene vector (pGL3-promoter), producing pGL3-ALAS2. Specific mutations were individually introduced into the core motif sequences within this construct to generate different constructs. The constructs were transfected into MCF-7 cells, and the cells were cultured under normoxia or hypoxia. The wild-type pGL3-ALAS2 construct showed the hypoxic induction of luciferase activity, and

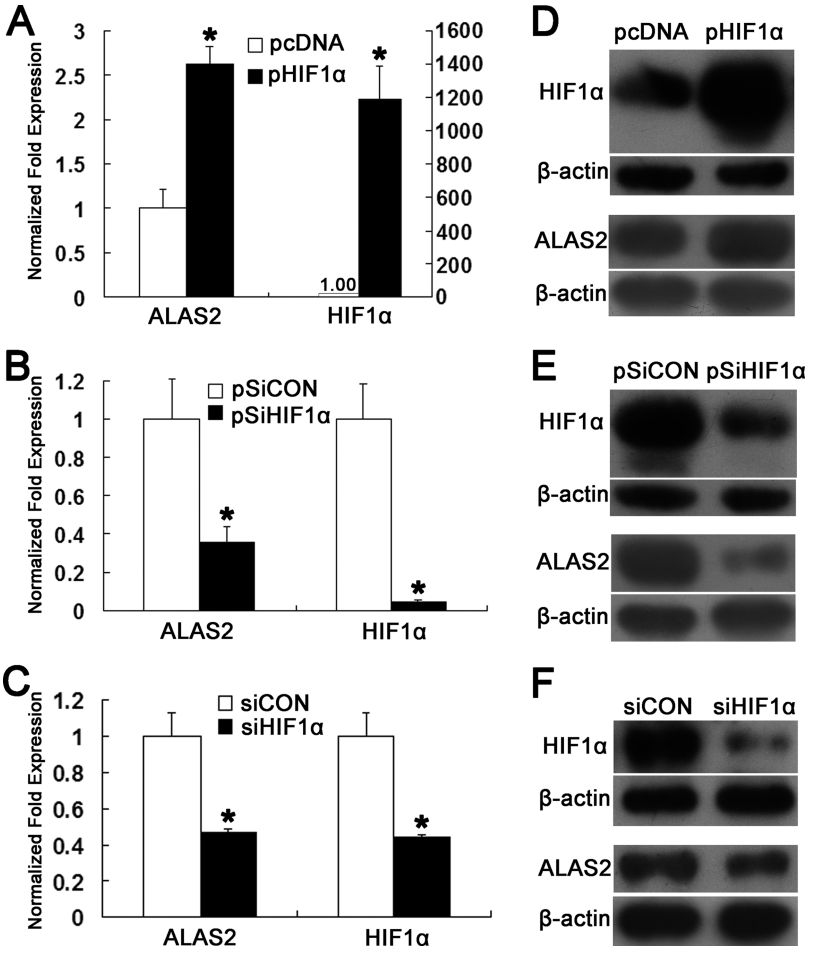

FIGURE 2: Regulation of *ALAS2* gene expression by HIF1. (A) Real-time PCR assay of *HIF1A* and *ALAS2* mRNA levels in K562 cells transfected with the HIF1α expression plasmid (pHIF1α) or empty plasmid (pcDNA) and subsequently incubated under hypoxia for 24 h. (B) Real-time PCR assay of *HIF1A* and *ALAS2* mRNA levels in K562 cells that were transfected with the HIF1α interference vector (pSiHIF1α) or pSilencer control (pSiCON) and subsequently incubated under hypoxia for 24 h. (C) Real-time PCR assay of *HIF1A* and *ALAS2* mRNA levels in K562 cells transfected with siRNA targeted to *HIF1A* (siHIF1α) or siRNA control (siCON). After transfection for 24 h, the transfection medium was replaced with complete medium and the cells were cultured under hypoxia for an additional 24 h. The asterisk indicates a p value of < 0.01. (D) Western blotting assay of HIF1α and ALAS2 protein levels in K562 cells transfected with the HIF1α expression plasmid or corresponding empty vector and subsequently incubated under hypoxia for 48 h. (E) Western blotting assay of HIF1α and ALAS2 protein levels in K562 cells transfected with the HIF1α interference plasmid or the corresponding empty vector and subsequently incubated under hypoxia for 48 h. (F) Western blotting assay of ALAS2 protein levels in K562 cells transfected with siRNA targeting *HIF1A* mRNA or an siRNA control. After transfection for 24 h, the transfection medium was replaced with complete medium and the cells were cultured under hypoxia for an additional 24 h.

this level of activation decreased when the consensus RCGTG sequence was replaced with the RAAAG sequence in the single mutant constructs pALAS2-M1, pALAS2-M2, and pALAS2-M3. Furthermore, dual mutation resulted in a greater loss of hypoxia-induced activity in MCF-7 cells. The triple mutation construct showed the largest decrease in activity (Figure 3C). These results demonstrate that all the three of the putative HREs, found 611, 621, and 741 bp downstream from the human *ALAS2*, gene are responsible for the hypoxic induction of *ALAS2* gene expression.

Validation of HIF1 Binding to the ALAS2 Region Containing the Putative HREs in Vivo. We performed a ChIP assay to examine whether the region of *ALAS2* containing the three HREs was recognized and bound by HIF1 in vivo. DNA was isolated from K562 cells cultured under hypoxic conditions, fragmented, and immunoprecipitated using an anti-HIF1α antibody. When these fragments were amplified by PCR using a primer pair located within the 3′-flanking region of the *ALAS2* gene, an obvious amplification fragment was detected (Figure 4A,B). An obvious PCR product was also observed when the positive control (PC) primers, which amplify a region of *FECH*, a previously identified target of HIF1, were used. However, no obvious PCR product was detected when DNA fragments from cells cultured under nomoxia were subjected to ChIP and PCR. The negative control (NC) primers, which flank a region lacking a HRE motif, were amplified from input DNA, but not from DNA subjected to ChIP, demonstrating that the positive ChIP results were not due to nonspecific binding of HIF1α to a random sequence. The results from the IgG control excluded nonspecific binding of the HIF1α antibody to other proteins. These results indicate that the *ALAS2* fragment containing the putative HREs can be recognized and bound by HIF1 under hypoxic conditions in K562 cells.

Induction of Intracellular Heme and Hemoglobin in Hypoxia. As heme biosynthesis is regulated by ALAS2, an increased level of ALAS2 expression under hypoxic conditions should lead to an increase in the level of heme production. We determined the intracellular heme concentration in K562 cells cultured under normoxia or hypoxia and detected an increase in the level of intracellular heme under hypoxic conditions (Figure 5A). Moreover, an increased level of hemoglobin synthesis under hypoxic

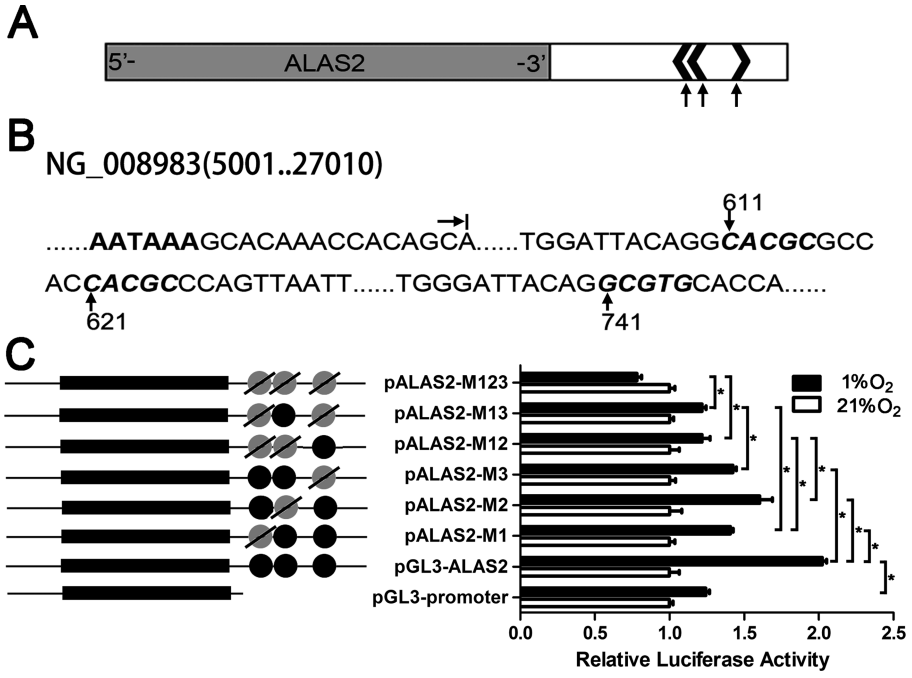

FIGURE 3: Identification of the putative and functional HREs in the human *ALAS2* gene. (A) Location and orientation of the putative HRE core motif 5′-RCGTG-3′ sites within the 3′-flanking region of the human *ALAS2* gene. The coding region of the gene is shaded, and the putative HRE core motifs are indicated by the arrows. (B) Nucleotide sequences matching the consensus HRE core motif are present in the 3′-flanking region within the 1 kb DNA fragment of the human *ALAS2* gene. The nucleotide sequences are numbered in relation to the transcription termination site. The putative HRE motif sequences are shown in bold italics. (C) Identification of the functional HREs. MCF-7 cells were cotransfected with a construct carrying the wild-type 3′-*ALAS2* sequence or a sequence in which one or more of the HREs were mutated, and pRL-TK, which provided an internal control. After transfection, the cells were cultured at 21% or 1% O_2 for 24 h. The mean relative luciferase activity ratio is shown (±SEM; $n = 3$) relative to the activity in cells incubated at 21% O_2. The asterisk indicates a p value of < 0.01.

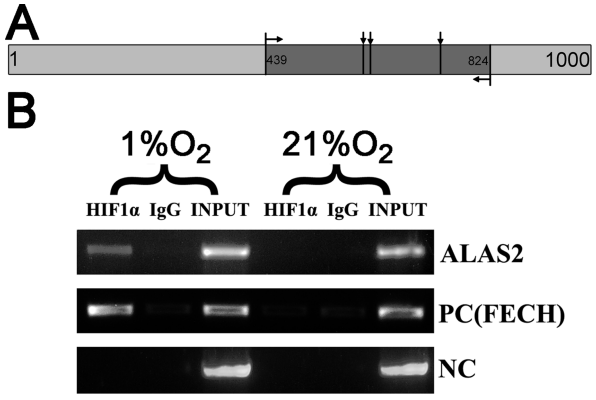

FIGURE 4: ChIP−PCR analysis of binding of HIF1α to the DNA region containing the three HREs. (A) Region of PCR amplification. The three vertical arrows indicate the positions of the three HREs in the 3′-flanking region of the *ALAS2* gene. The two horizontal arrows indicate the locations of the two PCR primers. (B) PCR amplification of immunoprecipitated DNA fragments. K562 cells were cultured under normoxia or hypoxia conditions for 24 h and subjected to a ChIP assay as described in Materials and Methods. Rabbit polyclonal antibody to HIF-1α or IgG was used to precipitate sonicated chromatin. Sonicated cell lysate was used as an input control. NC, negative control; PC, positive control; FECH, ferrochelatase gene, which has been identified as a target gene of HIF1α.

conditions was detected by benzidine staining (Figure 5B). The increase in the level of hemoglobin is likely related to the increased level of heme during hypoxia.

DISCUSSION

In this study, we have demonstrated that under hypoxic conditions the expression of human erythroid-specific ALAS2 is regulated by HIF1. The upregulation of ALAS2 expression contributes significantly to an increased level of heme biosynthesis. We hypothesize that an increased level of ALAS2 expression may contribute to the hemoglobinization of erythroid cells and erythropoiesis under hypoxic conditions, and that ALAS2 may cooperate with EPO to enhance erythropoiesis during hypoxia.

Our data indicate that the HIF1-mediated upregulation of ALAS2 represents an adaptive response that optimizes heme biosynthesis under hypoxic conditions. The expression of ALAS2 is induced by hypoxia, which is consistent with previous findings (*12*, *21*, *22*) that indicated a physiological response to hypoxia. The previous studies indicated that the ALAS2 protein was stabilized by a low level of oxygen and proteasomal inhibition (*22*) and that hypoxia induced ALAS2 expression by transforming growth factor-β signaling (*12*). Previous investigations also examined the possibility that HIF1 regulates ALAS2 by binding to the promoter region of murine *ALAS2*, but the putative HREs were determined to be nonfunctional (*21*). This may reflect the fact that the core motif is necessary but not sufficient for functional HREs. Our study identified functional HREs located downstream of the human *ALAS2* gene in K562 cells, which links the physiological response to hypoxia to the HIF1-mediated regulation network.

On the basis of our results and the previous findings that ferrochelatase and heme oxygenase 1 are regulated directly by HIF1 during hypoxia (*23*, *24*), we hypothesize a new HIF1-mediated heme regulatory pathway under hypoxic conditions (Figure 5C). In this pathway, ALAS2 promotes protoporphyrin IX synthesis in erythroid cells, and ferrochelatase controls the incorporation of iron into protoporphyrin IX to form heme. However, heme oxygenase 1 facilitates the degradation of heme to reduce redundant heme toxicity and achieve the recycling of iron. This "easy come, easy go" pathway would balance the physiological demand

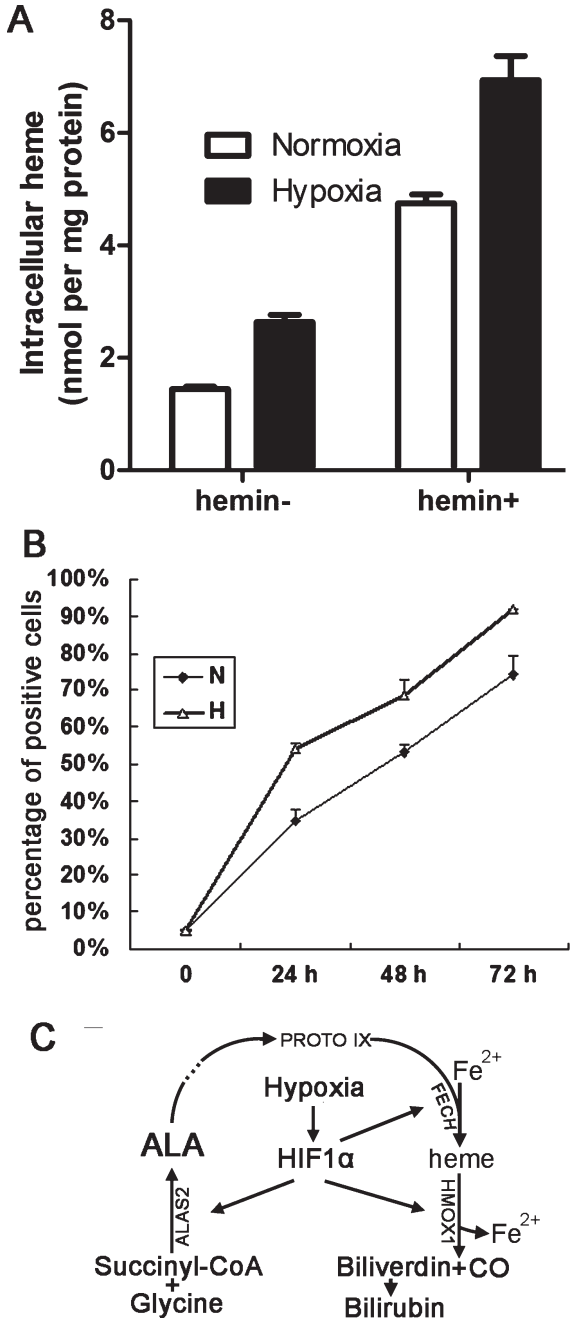

FIGURE 5: Regulation of heme and hemoglobin biosynthesis under hypoxia. (A) Quantification of intracellular heme concentration. K562 cells were cultured under normoxia or hypoxia, and the heme concentration was determined with the QuantiChrom Heme Assay. The amount of heme in each sample is expressed as the nanomoles of heme per milligram of total protein. (B) Benzidine staining of K562 cells. K562 cells were induced with 40 μM hemin. After 12 h, the cells were cultured under normoxia or hypoxia for the indicated number of hours. (C) Hypothetical scheme of heme biosynthesis induced by hypoxia. ALA, δ-aminolevulinate; PROTO IX, protoporphyrin IX; HMOX1, heme oxygenase 1.

for heme under hypoxia with the toxicity of redundant heme. ALAS2 plays a critical role in the cycling of heme.

The expression of *ALAS2* is regulated at the trancriptional and translational levels. Transcriptional regulation is mediated by erthroid-specific factors interacting with promoter and intronic enhancer sites (*25*−*27*). Translational regulation is controlled by iron levels through a 5′-iron regulatory element (IRE), which interacts with an IRE binding protein (IRP), maintaining a balance between protoporphyrin synthesis and iron availability (*28*, *29*). Iron depletion inhibits *ALAS2* translation (*30*). However, the addition of an iron−sulfer cluster (Fe/S), which is generated and exported by the mitochondria, prevents IRP from binding to the IRE and permits translation (*31*−*33*).

Meanwhile, HIF also regulates key genes involved in iron metabolism, such as *TF*, *TFR1*, ceruloplasmin, *DMT1*, *DCYTB*, and *HMOX1* (*34*−*36*). These genes cooperate with *FECH* and *ALAS2* to contribute to the maintainance of iron and heme homeostasis under hypoxia. Iron depletion is one of the functions of the HIF pathway (*2*, *37*, *38*), but the cellular uptake of iron occurs as a consequence. In response to this dilemma, as our data indicate, ALAS2 attempts to keep pace with the consequences instead of aggravating the causes. In other words, it is precisely because iron depletion inhibits *ALAS2* mRNA translation and activates the HIF pathway that more *ALAS2* mRNA is required to meet the physiological need and to maintain heme homeostasis.

HIF takes part in the coordinated regulation of erythropoietin and ALAS2. EPO functions through its receptor on the surface of erythroid progenitor cells and stimulates proliferation, differentiation, and cell survival (*39*). EPO is essential for normal erythropoiesis, and hypoxia is the primary physiological stimulus for its production. EPO is induced by HIF, and serum EPO levels are increased up to several hundred-fold depending on the severity of the hypoxic condition, promoting erythropoiesis. During the latter stages of erythropoiesis, large quantities of globin chains and heme molecules are synthesized for assembly into hemoglobin tetramers. The regulation of heme synthesis in erythroid cells is mediated by erythroid-specific transcription factors and the availability of iron as Fe/S and in nonerythroid cells by heme-mediated feedback inhibition (*13*, *40*). In animal cells, heme production is controlled by the first and rate-limiting mitochondrial enzyme δ-aminolevulinate synthase (ALAS) (*41*−*43*). HIF1-mediated upregulation of ALAS2 expression in human erythroid cells could participate in the hemoglobinization of erythroid cells and erythropoiesis under hypoxia. In fact, erythroid-specific ALAS2 is expressed during erythroid cell differentiation to accommodate the unique requirement of heme synthesis to support hemoglobin production (*40*, *44*). Furthermore, heme itself is required for globin expression and the suvival of erythroid precursors (*45*−*48*). Deficient heme and globin synthesis were observed in embryonic stem cells that lack an intact erythroid-specific δ-aminolevulinate synthase gene (*47*). Heme deficiency in the erythroid lineage causes an arrest in differentiation and cytoplasmic iron overload (*49*). The biological role of heme in hematopoiesis has been reviewed (*50*). Moreover, because ALAS2 deficiency causes X-linked sideroblastic anemia (*44*, *51*−*53*), it is evident that erythropoiesis is dependent on the level of ALAS2 activity. HIF-mediated induction of ALAS2 and EPO could coordinate to jointly contribute to erythropoiesis under hypoxic conditions.

In summary, we have demonstrated that hypoxic induction of the human erythroid-specific *ALAS2* gene is directly mediated by HIF-1. This discovery points toward a possible mechanism for enhanced erythropoisis under hypoxic conditions.

REFERENCES

1. Semenza, G. L., and Wang, G. L. (1992) A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol. Cell. Biol. 12*, 5447–5454.
2. Wang, G. L., Jiang, B. H., Rue, E. A., and Semenza, G. L. (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. *Proc. Natl. Acad. Sci. U.S.A. 92*, 5510–5514.

3. Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S., and Kaelin, W. G., Jr. (2001) HIFα targeted for VHL-mediated destruction by proline hydroxylation: Implications for O_2 sensing. *Science 292*, 464–468.
4. Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., Kriegsheim, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J., Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. (2001) Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. *Science 292*, 468–472.
5. Yu, F., White, S. B., Zhao, Q., and Lee, F. S. (2001) HIF-1α binding to VHL is regulated by stimulus-sensitive proline hydroxylation. *Proc. Natl. Acad. Sci. U.S.A. 98*, 9630–9635.
6. Lando, D., Peet, D. J., Whelan, D. A., Gorman, J. J., and Whitelaw, M. L. (2002) Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. *Science 295*, 858–861.
7. Lando, D., Peet, D. J., Gorman, J. J., Whelan, D. A., Whitelaw, M. L., and Bruick, R. K. (2002) FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes Dev. 16*, 1466–1471.
8. Masson, N., and Ratcliffe, P. J. (2003) HIF prolyl and asparaginyl hydroxylases in the biological response to intracellular O_2 levels. *J. Cell Sci. 116*, 3041–3049.
9. Schofield, C. J., and Ratcliffe, P. J. (2004) Oxygen sensing by HIF hydroxylases. *Nat. Rev. Mol. Cell Biol. 5*, 343–354.
10. Wenger, R. H., Stiehl, D. P., and Camenisch, G. (2005) Integration of oxygen signaling at the consensus HRE. *Sci STKE 2005*, re12.
11. Tsiftsoglou, A. S., Vizirianakis, I. S., and Strouboulis, J. (2009) Erythropoiesis: Model systems, molecular regulators, and developmental programs. *IUBMB Life 61*, 800–830.
12. Kaneko, K., Furuyama, K., Aburatani, H., and Shibahara, S. (2009) Hypoxia induces erythroid-specific 5-aminolevulinate synthase expression in human erythroid cells through transforming growth factor-β signaling. *FEBS J. 276*, 1370–1382.
13. Ajioka, R. S., Phillips, J. D., and Kushner, J. P. (2006) Biosynthesis of heme in mammals. *Biochim. Biophys. Acta 1763*, 723–736.
14. Outten, F. W., and Theil, E. C. (2009) Iron-based redox switches in biology. *Antioxid. Redox Signaling 11*, 1029–1046.
15. Jeon, H., Kim, H., Choi, D., Kim, D., Park, S. Y., Kim, Y. J., Kim, Y. M., and Jung, Y. (2007) Quercetin activates an angiogenic pathway, hypoxia inducible factor (HIF)-1-vascular endothelial growth factor, by inhibiting HIF-prolyl hydroxylase: A structural analysis of quercetin for inhibiting HIF-prolyl hydroxylase. *Mol. Pharmacol. 71*, 1676–1684.
16. Berchner-Pfannschmidt, U., Petrat, F., Doege, K., Trinidad, B., Freitag, P., Metzen, E., de Groot, H., and Fandrey, J. (2004) Chelation of cellular calcium modulates hypoxia-inducible gene expression through activation of hypoxia-inducible factor-1α. *J. Biol. Chem. 279*, 44976–44986.
17. Boussif, O., Lezoualc'h, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B., and Behr, J. P. (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethylenimine. *Proc. Natl. Acad. Sci. U.S.A. 92*, 7297–7301.
18. Akinc, A., Thomas, M., Klibanov, A. M., and Langer, R. (2005) Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. *J. Gene Med. 7*, 657–663.
19. Semenza, G. L. (1998) Hypoxia-inducible factor 1 and the molecular physiology of oxygen homeostasis. *J. Lab. Clin. Med. 131*, 207–214.
20. Zagorska, A., and Dulak, J. (2004) HIF-1: The knowns and unknowns of hypoxia sensing. *Acta Biochim. Pol. 51*, 563–585.
21. Hofer, T., Wenger, R. H., Kramer, M. F., Ferreira, G. C., and Gassmann, M. (2003) Hypoxic up-regulation of erythroid 5-aminolevulinate synthase. *Blood 101*, 348–350.
22. Abu-Farha, M., Niles, J., and Willmore, W. G. (2005) Erythroid-specific 5-aminolevulinate synthase protein is stabilized by low oxygen and proteasomal inhibition. *Biochem. Cell Biol. 83*, 620–630.
23. Liu, Y. L., Ang, S. O., Weigent, D. A., Prchal, J. T., and Bloomer, J. R. (2004) Regulation of ferrochelatase gene expression by hypoxia. *Life Sci. 75*, 2035–2043.
24. Lee, P. J., Jiang, B. H., Chin, B. Y., Iyer, N. V., Alam, J., Semenza, G. L., and Choi, A. M. (1997) Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia. *J. Biol. Chem. 272*, 5375–5381.
25. Srivastava, G., Borthwick, I. A., Maguire, D. J., Elferink, C. J., Bawden, M. J., Mercer, J. F., and May, B. K. (1988) Regulation of 5-aminolevulinate synthase mRNA in different rat tissues. *J. Biol. Chem. 263*, 5202–5209.
26. Surinya, K. H., Cox, T. C., and May, B. K. (1997) Transcriptional regulation of the human erythroid 5-aminolevulinate synthase gene. Identification of promoter elements and role of regulatory proteins. *J. Biol. Chem. 272*, 26585–26594.
27. Surinya, K. H., Cox, T. C., and May, B. K. (1998) Identification and characterization of a conserved erythroid-specific enhancer located in intron 8 of the human 5-aminolevulinate synthase 2 gene. *J. Biol. Chem. 273*, 16798–16809.
28. Melefors, O., Goossen, B., Johansson, H. E., Stripecke, R., Gray, N. K., and Hentze, M. W. (1993) Translational control of 5-aminolevulinate synthase mRNA by iron-responsive elements in erythroid cells. *J. Biol. Chem. 268*, 5974–5978.
29. Bhasker, C. R., Burgiel, G., Neupert, B., Emery-Goodman, A., Kuhn, L. C., and May, B. K. (1993) The putative iron-responsive element in the human erythroid 5-aminolevulinate synthase mRNA mediates translational control. *J. Biol. Chem. 268*, 12699–12705.
30. Gray, N. K., and Hentze, M. W. (1994) Iron regulatory protein prevents binding of the 43S translation pre-initiation complex to ferritin and eALAS mRNAs. *EMBO J. 13*, 3882–3891.
31. Wingert, R. A., Galloway, J. L., Barut, B., Foott, H., Fraenkel, P., Axe, J. L., Weber, G. J., Dooley, K., Davidson, A. J., Schmid, B., Paw, B. H., Shaw, G. C., Kingsley, P., Palis, J., Schubert, H., Chen, O., Kaplan, J., and Zon, L. I. (2005) Deficiency of glutaredoxin 5 reveals Fe-S clusters are required for vertebrate haem synthesis. *Nature 436*, 1035–1039.
32. Napier, I., Ponka, P., and Richardson, D. R. (2005) Iron trafficking in the mitochondrion: Novel pathways revealed by disease. *Blood 105*, 1867–1874.
33. Rouault, T. A., and Tong, W. H. (2005) Iron-sulphur cluster biogenesis and mitochondrial iron homeostasis. *Nat. Rev. Mol. Cell Biol. 6*, 345–351.
34. Rolfs, A., Kvietikova, I., Gassmann, M., and Wenger, R. H. (1997) Oxygen-regulated transferrin expression is mediated by hypoxia-inducible factor-1. *J. Biol. Chem. 272*, 20055–20062.
35. Tacchini, L., Bianchi, L., Bernelli-Zazzera, A., and Cairo, G. (1999) Transferrin receptor induction by hypoxia. HIF-1-mediated transcriptional activation and cell-specific post-transcriptional regulation. *J. Biol. Chem. 274*, 24142–24146.
36. Haase, V. H. (2010) Hypoxic regulation of erythropoiesis and iron metabolism. *Am. J. Physiol. 299*, F1–F13.
37. Jiang, B. H., Zheng, J. Z., Leung, S. W., Roe, R., and Semenza, G. L. (1997) Transactivation and inhibitory domains of hypoxia-inducible factor 1α. Modulation of transcriptional activity by oxygen tension. *J. Biol. Chem. 272*, 19253–19260.
38. Kallio, P. J., Wilson, W. J., O'Brien, S., Makino, Y., and Poellinger, L. (1999) Regulation of the hypoxia-inducible transcription factor 1α by the ubiquitin-proteasome pathway. *J. Biol. Chem. 274*, 6519–6525.
39. Klingmuller, U. (1997) The role of tyrosine phosphorylation in proliferation and maturation of erythroid progenitor cells: Signals emanating from the erythropoietin receptor. *Eur. J. Biochem. 249*, 637–647.
40. Ponka, P. (1997) Tissue-specific regulation of iron metabolism and heme synthesis: Distinct control mechanisms in erythroid cells. *Blood 89*, 1–25.
41. May, B. K., Dogra, S. C., Sadlon, T. J., Bhasker, C. R., Cox, T. C., and Bottomley, S. S. (1995) Molecular regulation of heme biosynthesis in higher vertebrates. *Prog. Nucleic Acid Res. Mol. Biol. 51*, 1–51.
42. May, B. K., Bhasker, C. R., Bawden, M. J., and Cox, T. C. (1990) Molecular regulation of 5-aminolevulinate synthase. Diseases related to heme biosynthesis. *Mol. Biol. Med. 7*, 405–421.
43. Ferreira, G. C., and Gong, J. (1995) 5-Aminolevulinate synthase and the first step of heme biosynthesis. *J. Bioenerg. Biomembr. 27*, 151–159.
44. Sadlon, T. J., Dell'Oso, T., Surinya, K. H., and May, B. K. (1999) Regulation of erythroid 5-aminolevulinate synthase expression during erythropoiesis. *Int. J. Biochem. Cell Biol. 31*, 1153–1167.
45. Ranu, R. S., and London, I. M. (1976) Regulation of protein synthesis in rabbit reticulocyte lysates: Purification and initial characterization of the cyclic 3′:5′-AMP independent protein kinase of the heme-regulated translational inhibitor. *Proc. Natl. Acad. Sci. U.S.A. 73*, 4349–4353.
46. Han, A. P., Yu, C., Lu, L., Fujiwara, Y., Browne, C., Chin, G., Fleming, M., Leboulch, P., Orkin, S. H., and Chen, J. J. (2001) Heme-regulated eIF2α kinase (HRI) is required for translational regulation and survival of erythroid precursors in iron deficiency. *EMBO J. 20*, 6909–6918.
47. Harigae, H., Suwabe, N., Weinstock, P. H., Nagai, M., Fujita, H., Yamamoto, M., and Sassa, S. (1998) Deficient heme and globin synthesis in embryonic stem cells lacking the erythroid-specific δ-aminolevulinate synthase gene. *Blood 91*, 798–805.
48. Muta, K., and Krantz, S. B. (1995) Inhibition of heme synthesis induces apoptosis in human erythroid progenitor cells. *J. Cell. Physiol. 163*, 38–50.

49. Nakajima, O., Takahashi, S., Harigae, H., Furuyama, K., Hayashi, N., Sassa, S., and Yamamoto, M. (1999) Heme deficiency in erythroid lineage causes differentiation arrest and cytoplasmic iron overload. *EMBO J. 18*, 6282–6289.
50. Abraham, N. G. (1991) Molecular regulation: Biological role of heme in hematopoiesis. *Blood Rev. 5*, 19–28.
51. Bottomley, S. S., May, B. K., Cox, T. C., Cotter, P. D., and Bishop, D. F. (1995) Molecular defects of erythroid 5-aminolevulinate synthase in X-linked sideroblastic anemia. *J. Bioenerg. Biomembr. 27*, 161–168.
52. May, A., and Bishop, D. F. (1998) The molecular biology and pyridoxine responsiveness of X-linked sideroblastic anaemia. *Haematologica 83*, 56–70.
53. Furuyama, K., and Sassa, S. (2002) Multiple mechanisms for hereditary sideroblastic anemia. *Cell. Mol. Biol.* (*Paris, Fr., Print*) *48*, 5–10.