Hypoxic Induction of Prolyl 4-Hydroxylase α(I) in Cultured Cells*

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Accumulated evidence indicates that hypoxia activates collagen synthesis in tissues. To explore the molecular mechanism of activation, we screened genes that are up-regulated or down-regulated by hypoxia. Fibroblasts isolated from fetal rat lung were cultured under hypoxia. Differential display technique showed that the mRNA level of prolyl 4-hydroxylase (PH) α(I), an active subunit that catalyzes the oxygen-dependent hydroxylation of proline residue in procollagen, increased 2–3-fold after an 8-h exposure to hypoxia. This elevated level was maintained over 40 h and returned to the basal level after reoxygenation. The transcription rate, protein level, and hydroxyproline content (an indicator of the prolyl hydroxylation) were all elevated by hypoxic culture. Analysis of the promoter region of PHα(I) gene indicated that a motif similar to hypoxia-responsive element (HRE) of hypoxia-inducible genes such as erythropoietin, was identified within a 120-base pair sequence upstream of the transcription start site. Luciferase reporter assay and mutational analysis showed that a site similar to the HRE in this motif is functionally essential to hypoxic response. Electrophoretic mobility shift assay revealed that hypoxia-inducible factor-1 was stimulated and bound to the PHα(I) HRE upon hypoxic challenge. Our results indicate that PHα(I), an essential enzyme for collagen synthesis, is a target gene for hypoxia-inducible factor-1.

Restricted oxygen availability is a feature of many physiologic and pathologic conditions, including high altitude residence, fetal development in the uterus, pulmonary fibrosis, wounded tissue, and neoplasm (1). Systemic and cellular responses to reduced oxygen tension (hypoxia) are initiated by activation and/or inactivation of gene expression. Hypoxia-inducible factor-1 (HIF-1), which was originally found to be a critical mediator for the inducible expression of the erythropoietin (Epo) gene by hypoxia (2), is a heterodimer composed of HIF-1α and arylhydrocarbon receptor nuclear translocator (ARNT). HIF-1α and ARNT retain a basic helix-loop-helix domain and a Per-ARNT/aryl hydrocarbon receptor Sim domain in their N termini (2). Hypoxia induces stabilization of HIF-1α (3), heterodimerization of HIF-1α and ARNT (4), and the binding of the heterodimer to the hypoxia-responsive element (HRE) in the regulatory region of the target genes with the transcriptional coactivator p300/CREB-binding protein (5). Although posttranscriptional mechanisms may contribute to the induction of hypoxia-sensitive genes, activation of the HIF-1 complex is an important step leading to hypoxia-mediated induction of glycolytic enzymes (6–9), Epo (2), vascular endothelial growth factor (10), and tyrosine hydroxylase (11).

In the remodeling of the small muscular pulmonary artery observed in hypoxia-induced pulmonary hypertension, type I collagen is actively synthesized and accumulated in the media and the adventitia of the artery (12). Recent studies have revealed that in vivo exposure of rats to hypoxia increases prolyl hydroxylation activity in skeletal muscle (13) and increases the concentration of collagenous proteins in cardiac muscle (14). Moreover, in vitro exposure of rat mesangial cells (15) and dermal fibroblasts (16) to hypoxia results in the enhancement of type IV collagen protein level and type I procollagen mRNA level, respectively. These observations indicate that systemic and cellular hypoxia modulates collagen synthesis in several types of cells. Synthesis of collagen molecules can be regulated at several steps (17). After translation, procollagens are hydroxylated on proline residues at the endoplasmic reticulum and form stable triple-helical trimers. The secreted procollagen trimer is proteolytically cleaved at both N- and C-propeptides in the extracellular space, where collagen trimers polymerize and are deposited nonenzymatically. These sequential steps of collagen synthesis and hypoxic effects on several stages in the expression of multiple types of collagen genes imply that hypoxic regulatory factors may be involved in common reactions during the collagen synthesis process.

To explore the molecular mechanism, we screened hypoxia-responsive genes in isolated fetal rat lung fibroblasts using the differential display method. We subsequently found that prolyl 4-hydroxylase (PH) α(I), an active subunit that catalyzes oxygen-dependent hydroxylation of proline residue in procollagen, is up-regulated by hypoxia via HIF-1 transcription factor complex.

EXPERIMENTAL PROCEDURES

Cell Culture—Fibroblasts were isolated from the fetal rat lung at 19 days of gestation (18). IMR-90 and WI-38, human fetal lung fibroblast cell lines, were obtained from the RIKEN Cell Bank (Tokyo, Japan). These cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, 1× minimum essential medium vitamin, and 15 μM HEPES (pH 7.4). Fetal rat lung fibroblasts between the 20th and 25th passages were used for the experiments. The mouse hepatoma cell lines Hepa-1c1c7 (Hepa-1) and its ARNT-defective derivative, Hepa-c4, were generously supplied by O. Hankinson. Hepa-c4 cells lack ARNT function and also fail to express...
the ARNT protein (19). These cells were cultured in the minimal essential medium-α without nucleosides (Life Technologies, Inc.) supplemented with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 15 mM HEPES (pH 7.4). At subconfluence, cells were exposed to hypoxia or treated with chemicals. Exposures of cells to hypoxia were performed as described previously (9). In some experiments, 200 µM ascorbic acid was added to the medium.

**RNA blotting**—Probes for RNA blot analysis were prepared by reverse transcription-polymerase chain reaction with rat total RNA extracted from the lung as mentioned previously (21). The following sense and antisense primers were used: rat PHα(I), DDBJ/EMBL/GenBank™ accession no. X78949, nucleotides 103–122, and nucleotides 1085–1066. RNA blotting was performed as described previously (22). After analysis of the PHα(I) mRNA level, the probe was stripped with a low salt wash at 90 °C. The membrane was reblotted for 28 S rRNA. The intensity of the mRNA was normalized to the 28 S rRNA signal (23).

**Preparation of Cell Homogenate**—The fibroblast monolayer was washed three times with phosphate-buffered saline (PBS), then scraped and homogenized by centrifugation at 100 × g for 5 min at 4 °C. Cell pellets were washed once with PBS. The cell pellets were suspended in a 10 × volume of 0.2 M NaCl, 0.1 M glycine, 20 mM Tris-HCl (pH 7.5 at 4 °C) containing 0.1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol and kept on ice for 10 min, followed by homogenization in a Potter-Elvehjem homogenizer at 1200 rpm with 10 up-and-down strokes. The homogenate was analyzed for PHα(I) by protein blotting.

**Protein blotting**—Protein blotting was performed as mentioned previously (9) with some modifications. For PHα(I) detection, equivalent amounts of protein from each sample were subjected to 10% SDS-polyacrylamide gel electrophoresis and then electrically transferred overnight at 25 °C from the gel to nitrocellulose membranes. The blots were incubated with mouse anti-rat PHα(I) monoclonal antibody (Fuji Chemical, Toyama, Japan) at 0.3 µg IgG per ml of 0.1% Tween 20-Tris-buffered saline (TTBS) for 1 h at 25 °C, and then rinsed three times in TTBS for 15 min. The blotted membranes were probed with horseradish peroxidase-conjugated goat anti-mouse IgG at a dilution of 1:3000 for 1 h. After four washes with TTBS, antibody-antigen complexes were detected using an enhanced chemiluminescence detection kit (Amer sham, Buckinghamshire, U.K.) according to the manufacturer's manual.

**Quantification of Hydroxy Proline, DNA, and Protein**—To analyze hydroxy proline in the culture medium, fibroblasts at subconfluence were cultured in 10% FCS-DMEM with or without 200 µM ascorbic acid under either 20 or 0% O2 for 24 h. For the next 24 h, cells were maintained in 0.1% FCS-DMEM with or without 200 µM ascorbic acid under either 20 or 0% O2. The culture medium was collected and hydrolyzed in 6 N HCl for 16 h at 110 °C. To analyze the hydroxy proline in the cells and cell-associated matrix, subconfluent fibroblasts were cultured for 8 days. The culture medium was changed every other day. After three washes with PBS, the cell layer was harvested by scraping and hydrolyzed. The 4-hydroxy proline in the hydrolysate was determined by the colorimetric method (24, 25).

The DNA content of the cell homogenate was assayed by the fluorometric method using DNA-binding fluorochrome Hoechst 33258 (26). The protein content was measured by the colorimetric method using the Pierce BCA protein assay kit, according to the manufacturer's manual. BSA was used as the standard.

**Transient Transfections and Reporter Gene Assays**—The rat genomic library was screened against rat PHα(I) cDNA probe containing a 5'-untranslated region. One clone covering from 1.9 kb 5'-upstream of the transcription starting site to the first exon was selected to analyze responses of PHα(I) gene to hypoxia. A region from −1.9 kb to +68 bases (transcription start site was transiently determined by the 5'-RACE method, Clontech, Palo Alto, CA) was ligated to PhoZip pyruis luciferase reporter vector (pG3l-basic, Promega, Madison, WI) and designated mPHα(I). Deletion constructs were cultured in 60-mm dishes. When the cultures reached subconfluence, cells were transfected by PHα(I)-luciferase chimeric plasmids, the promoter-less luciferase plasmid (a negative control vector, pG3l-basic, Promega, Madison, WI) or the SV40-promoter/enhancer-containing luciferase plasmid (a positive control vector, pGL3-control) concomitantly with 0.1 µg of pRL-SV40, an internal control plasmid containing Renilla reniformis luciferase gene. Test plasmid (0.45 pmol), 5 µl of FuGENE6 transfection reagent (Roche Molecular Biochemicals), and 95 µl of serum-free medium complex were added to the cultured cells. After 24 h incubation, cells were exposed to hypoxic gas for 16 h, and then lysed. Cell lysates were used to determine luciferase activity using Lumat LB 9501 (EG & G Berthold, Bad Wildbad, Germany).

**Preparation of Whole Cell Extracts and Electrophoretic Mobility Shift Assay**—The cell monolayer was washed once with PBS and then scraped. After centrifugation at 100 × g for 5 min at 4 °C, cell pellets were washed once with PBS, quickly frozen in liquid nitrogen for more than 5 min, and thawed on ice for 5–10 min with a 5-fold cell-packed-volume volume of cell lysis buffer (50 mM HEPES-KOH, pH 7.9, 420 mM KCl, 20 mM Tris, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM sodium azide, 0.1% Triton X-100, 1.5 mM glutamine, and 20% glycerol). Cells were lysed at 4 °C by multiple (20) passages through a 26 gauge needle, followed by centrifugation at 12000 × g for 15 min at 4 °C. The supernatants were frozen in liquid nitrogen and stored at –85 °C until use. Mouse Epo 3'-enhancer oligonucleotide (5'-GCC GTA CGT GCC TCG CAT GGC-3') (27), PHα(I) 5'-enhancer oligonucleotide (from –91 to –74, numbered with the transcription start site as +1; 5'-CTG AGC GCA GCT AGC GAG-3') and PHα(I)-M oligonucleotide (5'-CTG AGC GCT CTT AGC GAG-3') were used as probes or competitors. Both strands of oligonucleotide probes were labeled with [γ-32P]ATP (111 TBq/nmol) and T4 polynucleotide kinase and then annealed in 20 mM Tris-HCl, pH 7.5, 10 mM MgCl2, and 50 mM NaCl by heating at 95 °C for 5 min followed by gradual cooling to 25 °C over 60 min. The annealed probes were purified by passing them through their nanoparticle (Amersham Pharmacia Biotech). A typical DNA binding reaction was carried out by mixing 15 µg of cell extract with 20 µl of DNA binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM MgCl2, 0.5 mM EDTA, 5 mM dithiothreitol, 280 ng of sonicated poly(dI-dC), 1× CompleteTM) for 5 min at 25 °C. Probes (20 fmol) and competitors (2 pmol) were added and incubated for a further 10 min at 25 °C. Reaction products were electrophoresed at 4 °C on 5% polyacrylamide in 30 mM Tris/30 mM borax acid, 0.3 mM EDTA (pH 7.3 at 20 °C). For supershift assay, 1 µg of monocular anti-HIF-1α antibody (H1067, Novus Biochemical, Littleton, CO) or anti-Flag-epitope tag antibody as control (M5, Sigma), were incubated with 15 µg of whole cell extract at 4 °C for 90 min.

**Analyses of significant differences between data sets** were performed by the use of Student's t test or Welch's t test after analysis of variance. Values of p < 0.05 were considered statistically significant.

**RESULTS**

**Differential Display**—Gene products up-regulated or down-regulated by a 16-h exposure of fetal rat lung fibroblasts to 0% O2 were screened by the differential display technique using 104 combinations of arbitrary primers and anchor primers. Polymerase chain reaction with arbitrary primer 5'-TTTTG-GCTCC-3' and fluorescein isothiocyanate-labeled anchor primer 5'-GT14VA amplified a fragment at base pair 350, where the intensity from hypoxic cells is higher than that from normoxic cells (Fig. 1, left). This difference was confirmed by amplification with the same pair of primers using [35S]dCTP (Fig. 1, right). Cloning of this band combined with a computer search of genomic DNA similarity revealed that this amplified DNA was the 3' fragment of PHα(I) cDNA.

**Kinetics of Increases in PHα(I) mRNA Levels after Hypoxia**—To examine the response of PHα(I) gene expression to low oxygen culture, the steady state level of PHα(I) mRNA was determined by RNA blot analysis. Fibroblasts at subconfluence were cultured for 16 h at various concentrations of O2 from 20

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to 0%. The PHα(I) mRNA level increased in a hypoxic stress-dependent manner (Fig. 2A). The PHα(I) mRNA signal, normalized to 28S rRNA, increased to 1.3-fold the control after 3% O₂ culture. This response was enhanced by hypoxic conditions of 5–0% O₂; inset, lanes 2–7) for 16 h. Following extraction of total RNA, the steady state level of PHα(I) mRNA was determined by the RNA blotting method (inset, top). Radioactivity was estimated as mentioned under "Experimental Procedures" and normalized to the 28S rRNA signal (inset, bottom) and then expressed as a level relative to the control. B, subconfluent fibroblasts were either maintained at 20% O₂ or exposed to 0% O₂ for various lengths of time, as indicated. In certain experiments, after 16 h in 0% O₂, fibroblasts were returned to 20% O₂ for the indicated times (reoxygenation). The steady state level of PHα(I) mRNA was analyzed and expressed as in A.

IMR-90 and WI-38, human fetal lung fibroblast cell lines, also showed increases in the PHα(I) mRNA level by 2.5–3.0-fold the normoxic control in response to 16 h of hypoxic exposure (data not shown).

Run-on Transcription Analysis—The increase of PHα(I) mRNA levels by hypoxic cultures pointed to a transcriptional effect on PHα(I) expression. To assess this aspect, we measured PHα(I) gene transcription in isolated nuclei. The transcriptional rate of PHα(I), normalized to the β-actin signal, significantly increased to 2.2-fold (p < 0.05) and 3.3-fold (p < 0.05) of the normoxic control after 4 and 8 h of exposure to 0% O₂, respectively (Fig. 3). Transcription of β-actin appeared to be unchanged under the same conditions. LDH-A, a positive control gene (6), showed 1.4- and 1.8-fold (p < 0.05) increases in the relative transcription rate after 4 and 8 h of 0% O₂ exposure, respectively. Vector DNA did not show any signal.

Levels of PHα Protein and Hydroxyproline Residue—To determine the protein level of PHα in fibroblasts, we probed the whole cell homogenate by protein blotting for the presence of PHα protein. Anti-rat PHα monoclonal antibody detected a band at 63,000 daltons in the fibroblasts, which co-migrated with authentic PHα(I) (Fig. 4A). After 4 h of exposure to 0% O₂, the protein level of PHαs significantly increased to 1.3-fold (p < 0.05) the control level (Fig. 4B). The protein level reached its peak after 24 h of 0% O₂ exposure, which was 1.9-fold (p < 0.05).
The PH was maintained in 20% O2 or exposed to 0% O2 for 4–32 h. The PH was restored from 20% O2 and from 0% O2 for 4, 8, 16, 24, and 32 h, respectively (right). Levels were determined by the protein blotting method using anti-PH antibody as described under “Experimental Procedures.”

Differences between the control (0 h) and each hypoxic sample (4, 8, 16, 24, and 32 h) was performed, and intensity relative to 20% O2 control was expressed. Means and standard errors from three experiments are shown. Statistically significant differences between normoxia and hypoxia are shown (*, p < 0.05).

To assess the in vivo hydroxylation activity, fibroblasts were cultured under either normoxia or hypoxia in the presence or absence of ascorbic acid. We determined the levels of hydroxyproline residue secreted from fibroblasts into the medium over 24 h. With the addition of ascorbic acid, hypoxic culture had no significant effect on the hydroxyproline content of the conditioned medium (Fig. 5A). Under normoxic culture, ascorbic acid depletion decreased the hydroxyproline content of the medium to 7% of the ascorbic acid-sufficient culture. Interestingly, without ascorbic acid, hypoxic culture restored the hydroxyproline level from 7 to 58% of ascorbic acid-supplemented and normoxic cultures. We analyzed the hydroxyproline residue that had accumulated in the cell or cell-associated matrix during the 8-day confluent culture period. Ascorbic acid and hypoxic culture affected the hydroxyproline level in the cell and cell-associated matrix, in the same pattern as was observed in the culture medium (Fig. 5B). These results indicate that hypoxia-induced accumulation of PHα(I) mRNA leads to elevated protein levels, which then contribute to the maintenance of hydroxylation in ascorbic acid-supplemented cultures as well as to elevation of hydroxylation in ascorbic acid-deficient cultures.

Effects of HIF-1 Inducers and Mitochondrial Inhibitors on the PHα(I) mRNA Level—Glucose transporter 1 is up-regulated by depletion of either ATP or oxygen (28). HIF-1 is a transcription factor that regulates hypoxic induction of a variety of genes. CoCl2 and desferroxamine (Des), an iron chelator, can mimic the HIF-1 response to hypoxia (29). To determine whether the HIF-1 related pathway or ATP depletion in mitochondria is involved in the up-regulation of PHα(I) by hypoxia, HIF-1 inducers (CoCl2 and Des) and mitochondrial inhibitors (rotenone, azide, and cyanide) were added to the fibroblast cultures. Total RNA was extracted from cells that were treated with chemicals for 16 h and then the PHα(I) mRNA level was determined by RNA blotting analysis. CoCl2 (50–200 μM) and Des (50–200 μM) increased the PHα(I) mRNA level by 1.8–4.7-fold in a dose-dependent manner (Fig. 6). Rotenone (0.25 and 2.5 μM), NaN3 (1 and 10 μM), and cyanide (3–300 μM) increased the PHα(I) mRNA up to 1.3-fold. This increase was far less than that of hypoxic induction.

FIG. 3. Transcriptional rate of PHα(I) mRNA. Nylon membranes binding plasmid DNA of PHα(I), LDH-A, and β-actin (top) were hybridized with 32P-labeled run-on transcripts from 1 × 107 nuclei isolated from fibroblasts cultured under normoxia or hypoxia for 4 or 8 h. As the control DNA, pBluescript KS bound to nylon membrane was hybridized with nascent 32P-labeled RNA as above. Radioactivities of newly transcribed PHα(I) and LDH-A levels were normalized to β-actin and expressed as increases relative to control levels (bottom). Means and standard errors from three experiments are shown. Statistically significant differences between normoxia and hypoxia are shown (*, p < 0.05).

FIG. 4. Protein level of PHα. Subconfluent fibroblasts were either maintained in 20% O2 or exposed to 0% O2 for 4–32 h. The PHα protein levels were determined by the protein blotting method using anti-PHα antibody as described under “Experimental Procedures.” Lane S represents authentic PHα protein, and lanes 1–6 represent samples harvested from 20% O2 and from 0% O2 for 4, 8, 16, 24, and 32 h, respectively (A). On the right, migration of the simultaneously run molecular mass marker is shown (arrowheads from the top down represent origin and 97.4, 66, 45, 31, and 21.5 kDa, respectively). Densitometric analysis was performed, and intensity relative to 20% O2 control was expressed. Means and standard errors from three experiments are shown (B). Differences between the control (0 h) and each hypoxic sample (4, 8, 16, 24, and 32 h) were statistically significant (*, p < 0.05).
Response of the HIF-1-defective Mutant Cell to Hypoxia—Hepa-c4, the ARNT-defective cell, and Hepa-1, its wild type cell, were exposed to 1, 2, and 20% O₂ for 16 h, and analyzed for PHα(I) response. The level of PHα(I) mRNA in Hepa-1 cells was apparently enhanced (Fig. 7). However, PHα(I) mRNA levels in Hepa-c4 cells did not change under hypoxic stress. This result indicates that ARNT may be an essential transcription factor subunit for hypoxic induction of PHα(I) mRNA in Hepa-1 cells.

Transcriptional Activity of the PHα(I) Promoter—To assess regulatory elements that delineate hypoxic up-regulation of the PHα(I) gene, we screened the rat genomic library against a rat PHα(I) cDNA probe containing a 5′-untranslated region. A clone covering from 1.9 kb 5′ upstream of the transcription starting site to the first exon was selected to analyze responses of the PHα(I) gene to hypoxia (these DNA sequences are not shown, but they have been deposited in the GenBank™ accession number AF197928). A 1.9-kb fragment (−1.9 kb to +68 bases) of rat PHα(I) promoter region was cloned in front of luciferase, and the resulting construct (prPHLUC19, Fig. 8A) was transiently transfected in fibroblasts. Under normoxia, the transfected fibroblasts exhibited 88 times higher luciferase activity than cells transfected with promoterless Luc vector (pGL3-Basic), but 9 times lower activity than the SV40 promoter vector (pGL3-Cont.) (Fig. 8B). This suggests that the 1.9-kb promoter region has sufficient promoter activity to invest the hypoxic effects on the gene. Exposure of the transfected fibroblasts to hypoxia for 16 h stimulated luciferase expression by 5.5-fold (Fig. 8B). No significant effect was observed after hypoxic culture of cells transfected with promoterless or SV40 vectors. Hepa-1 and its ARNT-defective mutant cell, Hepa-c4, were transfected with prPHLUC19. Hepa-1 showed a 3.7-fold induction of luciferase after 16 h of hypoxic culture (Fig. 8B). In contrast, exposure of Hepa-c4 to hypoxia did not affect luciferase expression (Fig. 8B). These results strongly suggest that the PHα(I) promoter may contain the hypoxia-responsive consensus sequence, which the HIF-1α-only complex targets. Search for potential binding sites in the promoter region revealed one binding site (−79 to −86) matching the consensus for HIF-1α-ARNT/C(G/T)ACGT/GC(G/T) (30) on the antisense strand. A CACAG sequence (31), which seems to be necessary for hypoxic inducibility, was also identified from position −67 to −71 on the antisense strand. To define the promoter region that confers hypoxia responsiveness to PHα(I), we determined the luciferase activity of 5′-deletion constructs (Fig. 9A). The prPHLUC(−117), which included the HRE consensus sequence, showed a response to hypoxic exposure: 5.9-fold induction after 16 h of transfection to fetal lung fibroblasts (Fig. 9B). However, the shorter construct, prPHLUC(−68), which lacked the HRE consensus, did not change luciferase activity after hypoxic culture. This HRE involvement in the PHα(I) response to hypoxia was further supported by a complete loss of hypoxic induction in a mutated chimera luciferase reporter, prPHLUC(−117)mut (Fig. 9B). After 16 h of hypoxia, prPHLUC(−117) showed a 6.0-fold induction in Hepa-1 cells but no change in Hepa-c4 cells (Fig. 9B).

DNA Binding Activity to the PHα(I) HRE—To assess whether the HRE consensus sequence identified in the PHα(I) promoter was the target of HIF-1, whole cell extracts prepared from Hepa-1 cells were analyzed by electrophoretic mobility shift assays (Fig. 10, lanes 1–7). The PHα(I) probe detected hypoxia-specific DNA binding activity (HIF-1) in Hepa-1 cells (Fig. 10, lane 2). This band was absent in normoxic cells (Fig. 10, lane 1). Constitutively expressed DNA binding activity was detected in Hepa-1 cells cultured under both normoxia and hypoxia (Fig. 10, lanes 1 and 2, Con). This hypoxically induced interaction of the PHα(I) probe was also observed in whole cell extracts prepared from fibroblasts (Fig. 10, lane 14), but no hypoxic induction of DNA binding was identified in whole cell extract from ARNT-defective Hepa-c4 cells (lane 12). The specificity of the interaction between the probe and hypoxia-induced factors was tested by competition with nonradioactive oligonucleotides. An excessive amount of cold PHα(I) probe (100× labeled probe) inhibited the binding of the constitutive and inducible complexes (Fig. 10, lane 3). Addition of a mutated PHα(I) oligonucleotide (PH-M) did not compete with labeled PHα(I) probe. Competition with cold oligonucleotides corresponding to the HRE present in the Epo enhancer (Fig. 10, lane 4, EPO) suggested a binding of the HIF-1α-only complex to PHα(I) probe. To further characterize the hypoxia-induced complexes, whole cell extracts were incubated with a monoclonal antibody to HIF-1α before the mobility shift assays. Supershift assays showed that HIF-1α interacts with the HRE sequence of PHα(I) gene (Fig. 10, lane 6). However, Flag monoclonal antibody used as control did not affect the HIF-1 binding to the PHα(I) probe (Fig. 10, lane 7).
Acid-deficient culture.

... cellular protein level, and hydroxy proline level in the ascorbic acid-deficient culture.

In the late 1970s, Levene and Bates (32), and Turto et al. (33) reported that hypoxia increased enzymatic activity of procollagen prolyl hydroxylase. However, the regulatory step of the enzymatic activity remained unclear. In this report, we have shown that exposure of fetal lung fibroblasts to hypoxia activates the PHα(I) gene through the HIF-1 transcription factor complex, the binding of which to the hypoxic responsive element in the 5′-flanking region of the PHα(I) gene leads to an increase in transcription rate, steady state level of mRNA, cellular protein level, and hydroxy proline level in the ascorbic acid-deficient culture.

Several pathways have been proposed for gene induction by hypoxic stress (1). The transcription factors HIF-1α and HIF-2α (34) (also known as HLF or EPAS1) play a major regulatory role in hypoxic gene induction. In addition, nuclear respiratory factor 1 can mediate the hypoxic signal via a decrease in energy production by oxidative-phosphorylation (35). Recently, Disher et al. (36) demonstrated that hypoxia up-regulates β-eno1ase gene expression via destabilization of Sp3, a transcription factor. Unknown factors besides HIF-1 have also been suggested (37). The present results show that the mitochondrial inhibitors azide, rotenone, and cyanide do not affect the PHα(I) mRNA level (Fig. 6). In contrast, both cobaltous ion and Des up-regulate PHα(I) gene expression. CoCl2 and Des mimic HIF-1-mediated hypoxic signals and activate transcription of a variety of genes that are induced by hypoxic stress (29). Moreover, mutant cells incapable of producing ARNT, an essential component of HIF-1 and HIF-2 transcription factor complexes, do not elevate PHα(I) mRNA level and reporter gene activities (Figs. 7 and 8). The importance of HIF-1 transcription factor complex and hypoxia-responsive element in the PHα(I) gene promoter region were confirmed by the reporter gene assay and the electrophoretic mobility shift assays (Figs. 9 and 10). A promoter region covering from 1.9 kb to the transcription start site, which was ligated to luciferase activity (mean ± S.D. of at least three independent experiments). Luciferase activities in hypoxic cells relative to those in normoxic cells are expressed as fold induction, shown at the right.

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DISCUSSION

In the late 1970s, Levene and Bates (32), and Turto et al. (33) reported that hypoxia increased enzymatic activity of procollagen prolyl hydroxylase. However, the regulatory step of the enzymatic activity remained unclear. In this report, we have shown that exposure of fetal lung fibroblasts to hypoxia activates the PHα(I) gene through the HIF-1 transcription factor complex, the binding of which to the hypoxic responsive element in the 5′-flanking region of the PHα(I) gene leads to an increase in transcription rate, steady state level of mRNA, cellular protein level, and hydroxy proline level in the ascorbic acid-deficient culture.

Several pathways have been proposed for gene induction by hypoxic stress (1). The transcription factors HIF-1α and HIF-2α (34) (also known as HLF or EPAS1) play a major regulatory role in hypoxic gene induction. In addition, nuclear respiratory factor 1 can mediate the hypoxic signal via a decrease in energy production by oxidative-phosphorylation (35). Recently, Disher et al. (36) demonstrated that hypoxia up-regulates β-eno1ase gene expression via destabilization of Sp3, a transcription factor. Unknown factors besides HIF-1 have also been suggested (37). The present results show that the mitochondrial inhibitors azide, rotenone, and cyanide do not affect the PHα(I) mRNA level (Fig. 6). In contrast, both cobaltous ion and Des up-regulate PHα(I) gene expression. CoCl2 and Des mimic HIF-1-mediated hypoxic signals and activate transcription of a variety of genes that are induced by hypoxic stress (29). Moreover, mutant cells incapable of producing ARNT, an essential component of HIF-1 and HIF-2 transcription factor complexes, do not elevate PHα(I) mRNA level and reporter gene activities (Figs. 7 and 8). The importance of HIF-1 transcription factor complex and hypoxia-responsive element in the PHα(I) gene promoter region were confirmed by the reporter gene assay and the electrophoretic mobility shift assays (Figs. 9 and 10). A promoter region covering from 1.9 kb to the transcription start site, which was ligated to luciferase activity (mean ± S.D. of at least three independent experiments). Luciferase activities in hypoxic cells relative to those in normoxic cells are expressed as fold induction, shown at the right.
promoter is important to hypoxic up-regulation of the PHα(I) gene.

Hypoxia-mediated PHα(I) gene expression could be also modulated at the posttranscriptional level. An increase in the steady state-level of PHα(I) mRNA became evident after 8 h of hypoxic exposure (Fig. 2), but the protein level of PHα was elevated by 4 h hypoxic exposure (Fig. 4). The fact that the protein response is more rapid than that of the mRNA, may be ascribed to the stabilization of the PHα protein under low oxygen conditions. Exposure of C6 glioma cells and PC12 cells to hypoxia increases mRNA levels of vascular endothelial growth factor (39) and tyrosine hydroxylase (40), respectively. Increases in both stability and transcription rate of these mRNAs after hypoxic exposure contribute to their elevated steady state levels. However, the way in which these modifications in the posttranscriptional steps contribute to the hypoxically activated expression of the PHα(I) gene remains unclear.

In collagen synthesis, PH is a key enzyme, which catalyzes the formation of 4-hydroxyproline, an essential residue for the folding of the procollagen polypeptide chains into triple helical molecules (41). The active enzyme has two kinds of heterotetramers, each having two pairs of subunits, (αI/β2)/2 and (αII/β2)/2. The α subunits contain the major portion of the catalytic site (42). The β subunit is identical to the enzyme protein disulfide-isomerase and is produced in excess of the α subunits (42). Thus, the abundance of the α subunits restricts enzyme activity. For the catalytic reaction of PH activity, Fe3⁺, 2-oxoglutarate, O2, and ascorbic acid are required. The 2-oxoglutarate is the hydroxylated, 5-deoxyglucarate residue, with one atom of the O2 molecule being incorporated into the succinate, whereas the other is incorporated into the hydroxyl group formed on the proline residue (41, 42). Although O2 is a requirement for enzymatic activity, the cell culture study done by Levene and Bates (32) showed that exposure of fibroblasts to anoxia (less than 0.1% O2) enhanced hydroxylation of proline in newly synthesized collagen in the cells. The present study has shown that the hydroxy proline content is not affected by hypoxic conditions under ascorbic acid sufficiency (Fig. 5). In the ascorbic acid-deficient culture, hypoxic exposure increased the hydroxy proline content in comparison with the normoxic culture. Reducing reagents such as glutathione and cysteine can be substituted for ascorbic acid to maintain PH catalytic activity in vitro, even though ascorbic acid is the most efficient reducing reagent for PH enzymatic reaction both in vitro and in vivo system (43). In normoxic conditions in the absence of ascorbate, the oxidizing environment would convert Fe2⁺ to Fe3⁺, which would not support normal hydroxylation. However, under low oxygen, the relative increase in hydroxylation would be due to the increase in prolyl hydroxylation, as well as a preservation of Fe2⁺. Taken together, these results suggest that in a cell culture system, hypoxic culture may not be the rate-limiting factor in procollagen hydroxylation and may reduce the requirement for ascorbic acid in prolyl hydroxylation.

Hydroxyproline residue is critical in stabilizing triple-helical collagen chains. In addition to collagen, more than 10 proteins, including CIq of complement, acetylcholinesterase, pulmonary surfactant proteins A and D, and conglutin, have collagen-like domains and are potential substrates for prolyl hydroxylase (41). Secretion of surfactant protein D is inhibited by 2,2'-dipyridyl, an inhibitor of prolyl and lysyl hydroxylase (44). In late pregnancy, pulmonary arterial pO2 in the fetus is less than 3% O2 (45), although synthesis of collagen and surfactant proteins A and D is active in the lung parenchima (46–48). Certainly, this active synthesis is essential for the start of ventilation after the birth. HIF-1-mediated activation of PHα(I) may play an important role in maintenance of prolyl hydroxylation of procollagen and collagenous protein under low oxygen conditions in the fetal lung.

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