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Identification of protein disulfide isomerase as an endothelial hypoxic stress protein

KRISTA K. GRAVEN,[1] CHRISTOPHER MOLVAR,[2] JILL S. RONCARATI,[2]
BRIAN D. KLAHN,[1] SHAWNA LOWREY,[1] AND HARRISON W. FARBER[2]
[1]*Department of Medicine, University of Wisconsin Medical School, Madison, Wisconsin 53706; and*
[2]*The Pulmonary Center, Boston University School of Medicine, Boston, Massachusetts 02118*

Received 9 September 2001; accepted in final form 27 November 2001

Graven, Krista K., Christopher Molvar, Jill S. Roncarati, Brian D. Klahn, Shawna Lowrey, and Harrison W. Farber. Identification of protein disulfide isomerase as an endothelial hypoxic stress protein. *Am J Physiol Lung Cell Mol Physiol* 282: L996–L1003, 2002. First published November 30, 2001; 10.1152/ajplung.00359.2001.—Endothelial cells (EC) exposed to hypoxia upregulate a unique set of five stress proteins. These proteins are upregulated in human and bovine aortic and pulmonary artery EC and are distinct from heat shock or glucose-regulated proteins. We previously identified two of these proteins as the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase and enolase and postulated that the remaining proteins were also glycolytic enzymes. Using SDS-PAGE, tryptic digestion, and NH_2-terminal amino acid sequencing, we report here the identification of the 56-kDa protein as protein disulfide isomerase (PDI). PDI is upregulated by hypoxia at the mRNA level and follows a time course similar to that of the protein, with maximal upregulation detected after exposure to 18 h of 0% O_2. Neither smooth muscle cells nor fibroblasts upregulate PDI to the same extent as EC, which correlates with their decreased hypoxia tolerance. Upregulation of PDI specifically in EC may contribute to their ability to tolerate hypoxia and may occur through PDI's functions as a prolyl hydroxylase subunit, protein folding catalyst, or molecular chaperone.

heat shock protein; glucose-regulated protein; prolyl hydroxylase; hypoxia-inducible factor-α

APPROPRIATE RESPONSES TO DECREASED environmental O_2 are imperative for mammalian survival. These responses govern a wide range of physiological processes, from maintenance of ventilation, cardiac output, and cellular ATP levels to production of various mitogenic, immunological, and vasoactive substances. Numerous studies have shown that many of these responses to hypoxia involve alterations in gene expression. The list of genes regulated by hypoxia continues to grow and includes erythropoietin (14), vascular endothelial growth factor (45), endothelin (26), platelet-derived growth factor-β (25), xanthine oxidase (49), heme oxygenase-1 (31), glucose transporter-1 (3), tyrosine hydroxylase (8), interleukin-6 (51), and several glycolytic enzymes, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (15), enolase (1, 43), aldolase (43, 44), phosphoglycerate kinase 1 (12, 44), and lactate dehydrogenase A (12).

Upregulation of many of these genes is cell specific; however, the mechanisms and significance of this cell specificity remain unclear. Among the numerous cell types in which appropriate sensing of and adaptation to hypoxia are critical are vascular endothelial cells (EC). As a result of their location, EC are frequently exposed to changes in blood P_{O_2} and are important mediators of vascular tone, coagulation, immune response, vascular permeability, and angiogenesis. Thus the ability of EC to synthesize and release various mitogenic, procoagulant, vasoactive, and immunological substances in response to hypoxia and the adaptive strategies these cells have developed to tolerate wide variations in environmental O_2 availability are critical to maintaining homeostasis while, in certain instances, contributing to the pathogenesis of disease.

EC exposed to physiological levels of hypoxia upregulate a distinct set of 34-, 36-, 39-, 47-, and 56-kDa stress proteins (53). This stress response is unlike that of other mammalian cell types, which upregulate heat shock proteins or glucose-regulated proteins on exposure to hypoxia or hypoxia-reoxygenation, respectively (4, 42). These EC proteins are constitutively expressed and are upregulated during exposure to hypoxia, despite a 30–60% decrease in total protein synthesis (53). They have been described in cultured bovine aortic EC (BAEC) and pulmonary artery EC (BPAEC) and human aortic, pulmonary artery, and microvascular EC (16). Upregulation is first detected after 4 h of hypoxia, is maximal at 18 h of hypoxia (3- to 5-fold increase), and remains elevated for ≥24–48 h of hypoxic exposure (53). The pattern of upregulation is similar whether cells are exposed to hypoxia alone or hypoxia followed by reoxygenation. Induction is inversely related to O_2 concentration, with maximal upregulation occurring with exposure to 0% O_2 (25 mmHg P_{O_2} in the medium) (53). EC cultured long term (e.g., 2–3 mo) in 3% O_2 (40 mmHg P_{O_2} in the medium) synthesize these

Address for reprint requests and other correspondence: K. K. Graven, Dept. of Medicine, University of Wisconsin Medical School, 2590 Medical Sciences Center, 1300 University Ave., Madison, WI 53706 (E-mail: kkg@medicine.wisc.edu).

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proteins at a continually elevated level compared with normoxic cells of the same cell line and passage number (16, 53). When these cells are exposed acutely to 0% O_2, they upregulate the proteins even further. Two of these proteins have been identified as the glycolytic enzymes GAPDH (15) and enolase-A (1). To determine whether the remaining proteins were glycolytic enzymes, as hypothesized on the basis of their molecular weights, we isolated the 56-kDa protein via subcellular fractionation and SDS-PAGE and identified it via tryptic digestion and limited NH_2-terminal sequencing. We report here that the 56-kDa protein is not a glycolytic enzyme but is protein disulfide isomerase (PDI), a multifunctional protein important in disulfide bond formation and rearrangement and as a critical subunit of prolyl hydroxylases. We also report that PDI is upregulated by hypoxia at the RNA level and is not upregulated by hypoxia in two hypoxia-sensitive cell types.

EXPERIMENTAL PROCEDURES

Materials. O_2 gas mixtures were purchased from Wesco (Billerica, MA). Tissue culture materials were obtained from GIBCO (Grand Island, NY), except for BSA, which was obtained from Hyclone (Logan, UT). Radioactive isotopes were purchased from New England Nuclear (Boston, MA) and nylon membranes (Hybond N^+) from Amersham (Arlington Heights, IL). Reagents used for RNA work were of molecular biology grade and were purchased from Sigma Chemical (St. Louis, MO) or Fisher (Pittsburgh, PA). Random primer labeling of cDNA probes was carried out using a kit from Promega (Madison, WI). PDI monoclonal antibody was obtained from Affinity Bioreageants (Golden, CO) and TO-PRO-3 iodide, Texas red-conjugated goat anti-mouse IgG secondary antibody, 5-dodecanoylaminofluorescein, and ProLong Antifade kit from Molecular Probes (Eugene, OR). All other materials were of standard chemical grade and were purchased from Sigma Chemical or ICN Biomedicals (Irvine, CA).

Cell culture. Bovine EC were isolated from freshly excised calf aortas and pulmonary arteries (BAEC and BPAEC, respectively), as previously described (15). Cultures were maintained from isolation at 37°C in a humidified incubator in 5% CO_2-95% air (21% O_2, "normoxia") or in a humidified sealed chamber (Billups-Rothenburg, Del Mar, CA) gassed with 3% O_2-5% CO_2-92% N_2 (3% O_2, chronic hypoxia). Under normoxic conditions, O_2 levels in the medium surrounding these cells has been measured at 120 mmHg and under chronic hypoxic conditions has been measured at 40 mmHg (10). EC purity was confirmed by typical cobblestone appearance, factor VIII immunofluorescence, and uptake of fluorescent acetylated low-density lipoprotein. All experiments were performed using 80–90% confluent BAEC and BPAEC monolayers at *passages 3–10*, and all cells were fed within 24 h of each experiment. Control and experimental conditions for an individual experiment were performed in parallel on identical cell lines of identical passage number. All experiments were repeated three to five times using different cell lines.

Bovine pulmonary artery smooth muscle cells (SMC) were a kind gift from Dr. Barry Fanburg (Tufts University Medical School) and were cultured in 21% O_2 at 37°C, as previously described (16). Human fetal lung fibroblast (IMR-90) cells were a kind gift from Dr. Ronald Goldstein (Boston University Medical School) and were cultured in 21% O_2 at 37°C, as previously described (16).

Protein induction and labeling. Hypoxic conditions were generated by exposure of cell monolayers to 0% O_2-5% CO_2-95% N_2 (0% O_2, "hypoxia") for the times specified in humidified sealed chambers (Billups-Rothenburg) at 37°C. O_2 levels in the media have been measured at ~25 mmHg during exposure to 0% O_2 (10). After hypoxic exposure, EC were allowed to recover for 2 h in 21% O_2. Some cells were labeled with [^{35}S]methionine (50 μCi/ml) in methionine-free modified Eagle's medium supplemented with 10% bovine calf serum and 1 mM sodium pyruvate during the 2-h recovery period. Cells were then washed three times with PBS, and the nuclear and combined cytoplasmic and subcellular organelle/membrane fractions were isolated as described previously (15). These fractions were solubilized in 25% sample buffer [0.15 M Tris, pH 6.8, 20% (vol/vol) glycerol, 1.5% (wt/vol) SDS, 10% (vol/vol) basal medium Eagle, and 0.025% (wt/vol) bromphenol blue] and boiled for 2 min before electrophoresis. Because the protein of interest was induced only in the cytoplasmic/membrane fraction (Fig. 1), a maximal quantity of this fraction of unlabeled protein was separated in each of four lanes by 10% SDS-PAGE. Two parallel lanes of the gel were loaded with equal radioactive counts of [^{35}S]methionine-labeled protein from normoxic and hypoxic conditions to confirm excision of the desired band. The radioactive lanes were excised, dried, and exposed to Kodak XAR-5 film (Rochester, NY). The remaining proteins were visualized with Coomassie brilliant blue, and the 56-kDa protein was excised from four separate lanes, washed twice with 50% acetonitrile, and frozen at −20°C. Initial sequencing with an

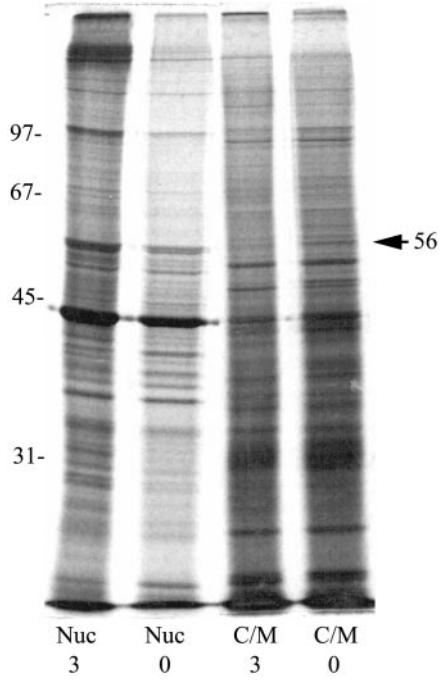

Fig. 1. Subcellular fractionation of bovine aortic endothelial cells (BAEC) after hypoxic exposure. BAEC were maintained in 3% O_2 (3) or exposed to 0% O_2 (0) for 18 h. After hypoxic exposure, cells were returned to 21% O_2 for 2 h and then labeled with [^{35}S]methionine for 2 h. Washed endothelial cells (EC) were centrifuged to provide a nuclear (Nuc) or cytoplasmic/membrane (C/M) fraction, resuspended in Laemmli sample buffer, and separated by 10% SDS-PAGE, with equal counts loaded per lane. Positions of molecular mass markers are shown at *left* in kDa, and the position of the induced 56-kDa protein is shown at *right*. Similar results were obtained from bovine pulmonary artery endothelial cells (BPAEC).

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Applied Biosystems 470A gas phase sequencer equipped with a 120 PTH analyzer in the Biochemistry Facility at Boston University was unsuccessful because of NH_2-terminal blockage. Thus the samples were sent to the Harvard Microchemistry Facility (Cambridge, MA) for sequencing. Samples underwent tryptic digestion, analysis by mass spectrometry to approximate the quantity of peptides present, HPLC separation, and NH_2-terminal amino acid sequencing through repeated rounds of Edman degradation. Two of the four fragments produced high-confidence sequences and were compared with nonredundant GenBank CDS translations, Brookhaven Protein Data Bank, Swiss-Prot, and Protein Identification Resource sequence databases for significant identity using BLAST search protocols (National Center for Biotechnology Information's BLAST world wide web server).

Confocal fluorescence microscopy. EC were plated onto coated glass slides (Nunc Lab-Tek II CC2, Fisher Scientific) and, on reaching 80–90% confluence, were exposed to 0% O_2 for 18 h or maintained in 21% O_2. Cells were then processed using a modification of the technique outlined previously (5). Medium was removed, and the cells were washed twice with PBS and fixed in 4% (wt/vol) paraformaldehyde in MSM-PIPES (5 mM PIPES, pH 6.8, 5 mM $CaCl_2$, 18 mM $MgSO_4$, 24 mM NaCl, and 40 mM KCl) for 30 min, with replacement of the solution once. The slides were washed twice for 1 min each in MSM-PIPES containing nonionic detergents [MSM-PIPES containing 0.5% (vol/vol) Triton X-100 and 0.5% (vol/vol) Nonidet] and incubated in 1 mM sodium cyanoborohydride in PBS for 10 min at 37°C. The slides were then washed twice with MSM-PIPES containing nonionic detergents for a total of 20 min at room temperature with shaking, washed twice with PBS, and incubated in 2% (wt/vol) BSA in PBS for 1.5 h at room temperature. The BSA solution was removed, and the slides were incubated with a 1:1,000 dilution of PDI antibody in 2% BSA (wt/vol) in PBS overnight at 4°C with shaking. On the following day, the slides were washed five times for 10 min each with PBS at room temperature and incubated in a solution containing 1 μM TO-PRO-3 (nucleic acid stain) and a 1:1,000 dilution of Texas red-conjugated anti-mouse IgG (secondary antibody) in PBS for 2 h at room temperature in the dark. At 5 min before the end of this incubation, 5-dodecanoylaminofluorescein (lipid stain) in PBS was added to give a final concentration of 0.1 μM, and the incubation was continued in the dark as described above. Slides were then washed five times with PBS for 10 min each in the dark. The PBS was aspirated, one to two drops of ProLong solution were added, and a coverslip was applied following the manufacturer's protocol. Slides were dried for ≥1 h in the dark at 4°C before imaging. Two separate slides served as negative controls: one was incubated overnight in 2% BSA alone and then processed as described above, and the other was incubated overnight in the primary antibody solution and then incubated in the TO-PRO solution without the addition of secondary antibody. Cells were imaged in the W. M. Keck Laboratory for Biological Imaging at the University of Wisconsin using a laser scanning microscope (model 1024, Bio-Rad, Hercules, CA) equipped with a 15-mW mixed-gas (krypton-argon) laser. Experiments were repeated three times with similar results. Representative images are shown.

RNA isolation and Northern blot analysis. Cells were maintained in 21% or 3% O_2 and exposed to 0% O_2 for the times specified. After hypoxic exposure, total RNA was isolated as described previously (15). Total RNA (15 μg) from each sample was separated by electrophoresis through a 1% agarose-formaldehyde gel and stained with ethidium bromide; RNA was then transferred to a nylon membrane and probed with a human PDI cDNA (American Type Culture Collection, Rockville, MD) probe as previously described (16). To document equal loading, the same blot was stripped using the manufacturer's protocol and probed with a human β-actin cDNA (American Type Culture Collection) or 18S ribosomal RNA oligonucleotide. Hybridization signals were quantitated using a computing densitometer (Molecular Dynamics). PDI mRNA levels were normalized to β-actin or 18S levels and expressed as fold increase above control (21% O_2) mRNA levels. RNA isolation and Northern blot analysis were performed three to four separate times with similar results. Where indicated, densitometric values are expressed as the average ± SE of three or four experiments. Statistical comparisons were made using the ratio paired *t*-test.

RESULTS

Protein purification and sequencing. Previous studies have shown that human and bovine EC exposed to hypoxia induce synthesis of 34-, 36-, 39-, 47-, and 56-kDa proteins (16, 53). The 36- and 47-kDa proteins have been identified as the glycolytic enzymes GAPDH and enolase-A, respectively (1, 15). To determine whether the 56-kDa protein was also a glycolytic enzyme, we purified and partially sequenced this protein in a manner similar to that used for the other two proteins (1, 15). Specifically, we exposed BAEC grown chronically in 3% O_2 to 0% O_2 for 18 h to produce maximal protein induction. After exposure to hypoxia, the combined cytoplasmic-subcellular organelle fraction was isolated, the proteins were separated by SDS-PAGE, and the appropriate band was excised (Fig. 1). Protein sequencing was performed by the Harvard Microchemistry Facility on pooled samples from four identical lanes using tryptic digestion, HPLC separation, and NH_2-terminal amino acid sequencing of several purified fragments. Two of these sequences were deemed high-confidence sequences by the Harvard laboratory: the first fragment produced a 15-amino acid sequence that was 100% identical to bovine and human PDI (Fig. 2), and a second 12-amino acid fragment was 100% and 83% identical to the sequences for bovine and human PDI, respectively (Fig. 2).

PDI expression in hypoxic EC. To evaluate PDI expression and location during hypoxia, confocal microscopy was performed with triple labeling of EC. Cells were plated onto coated glass slides, exposed to 21% or 0% O_2 for 18 h, fixed, and stained with a nucleic acid stain (blue) that labels the nuclei, a lipid stain (green) that labels membranes, and a primary antibody to PDI followed by a secondary antibody conjugated to Texas red. During normoxia, PDI is present in the cytoplasm [presumably in the endoplasmic reticulum (ER)] and increases significantly during hypoxia (Fig. 3). PDI was not detected in the nucleus during normoxia or hypoxia; this observation correlates with findings on subcellular fractionation (Fig. 1). Because of the strong cytoplasmic induction during hypoxia, it was difficult to determine whether a small percentage localized to the cell membrane.

PDI mRNA levels in EC. To determine whether PDI upregulation during hypoxia corresponds to an increase in PDI mRNA levels, a time-course analysis of PDI mRNA levels from BAEC grown in 21% O_2 and

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56kD protein, fragment 1:  L G E T Y K D H E N I V I A K
                           : : : : : : : : : : : : : : :
bovine PDI:                L G E T Y K D H E N I V I A K
human PDI:                 L G E T Y K D H E N I V I A K
                           412                       426

56kD protein, fragment 2:  Y K P E S D E L T A E K
                           : : : : : : : : : : : :
bovine PDI:                Y K P E S D E L T A E K
human PDI:                 Y K P E S E E L T A E R
                           329                 340
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Fig. 2. Identity of NH_2-terminal sequences for the 56-kDa EC hypoxic stress protein. The cytoplasmic/membrane fraction from hypoxic BAEC (cells grown in 3% O_2 and exposed to 0% O_2 for 18 h) was solubilized, separated by 10% SDS-PAGE, and stained with Coomassie brilliant blue. The 56-kDa band from 4 parallel lanes was then excised and sent to the Harvard Microchemistry Facility for tryptic digestion, HPLC separation, and NH_2-terminal amino acid sequencing. Two fragments produced reliable sequences and were compared with nonredundant GenBank CDS translations, Brookhaven Protein Data Bank, Swiss-Prot, and Protein Identification Resource sequence databases using BLAST search protocols. Identity was found to the sequences for protein disulfide isomerase (PDI) from multiple species. Bovine and human comparisons are shown with amino acid numbering for the known bovine sequences.

exposed to 0% O_2 was performed. This showed that PDI mRNA levels began to rise after 1 h of hypoxia, reached a peak increase of 2.5-fold at 8 h, and remained elevated at 24 h (Fig. 4*A*). These findings correlate with and slightly precede the time course of protein upregulation previously described for this protein (Fig. 4*B*) (16, 53). Hypoxic upregulation of PDI mRNA was also examined in BAEC and BPAEC grown chronically in 21% or 3% O_2 and then exposed acutely to 0% O_2 for 18 h. Figure 5 shows a representative Northern blot of pairs of experiments, with the first lane representing the control and the second lane of each pair showing mRNA from cells exposed to 0% O_2 for 18 h. The first four lanes (from *left* to *right*) show BAEC and BPAEC grown chronically in 3% O_2 and exposed to 0% O_2 for 18 h. BAEC grown in 3% O_2 (1st pair of lanes) upregulated PDI mRNA 1.8-fold when exposed to 0% O_2 for 18 h, whereas BPAEC grown in 3% O_2 (2nd pair of lanes) upregulated PDI mRNA 2.0-fold when exposed to 0% O_2 for 18 h. BAEC grown in 21% O_2 and exposed to 0% O_2 for 18 h (3rd pair of lanes) upregulated PDI mRNA 2.8-fold, and BPAEC grown in 21% O_2 and exposed to 0% O_2 for 18 h upregulated PDI mRNA 1.7-fold. All experiments were repeated three times with similar results.

PDI mRNA levels in other cell types. We previously showed that the 56-kDa hypoxia-inducible protein corresponding to PDI is induced three- to fourfold in various EC types (bovine and human) but not in other cell types, such as rat pulmonary alveolar type II cells, IMR-90, SMC, or mouse renal tubular epithelial cells (16). After identifying the 56-kDa protein as PDI, we evaluated levels of PDI mRNA in SMC and IMR-90 cells exposed to 0% O_2 for increasing time periods. We found that, in each cell type, PDI mRNA was not induced by hypoxia to a significant extent. In IMR-90 cells, PDI is induced a maximum of 1.2-fold after 18 h of hypoxic exposure (Fig. 6). In SMC, PDI is induced a maximum of 1.6-fold after 24 h of hypoxic exposure (Fig. 7). The results of multiple experiments were more variable in SMC than in the other cell types; however, the level of induction did not reach statistical significance at any time point.

DISCUSSION

We previously reported that EC upregulate a unique set of stress proteins in response to hypoxia (53). Two of the proteins were previously identified as the glycolytic enzymes GAPDH and enolase-A (1, 15). A third is reported here to be PDI. We also report that PDI mRNA is upregulated by hypoxia and follows a time course similar to that of PDI protein upregulation. Finally, PDI is not upregulated by hypoxia in two other mammalian cell types (SMC and IMR-90 cells) at the protein and mRNA levels.

PDI is a member of the thioredoxin superfamily (for reviews see Refs. 11 and 13). It is an abundant protein in mammalian cells and resides primarily in the ER. It

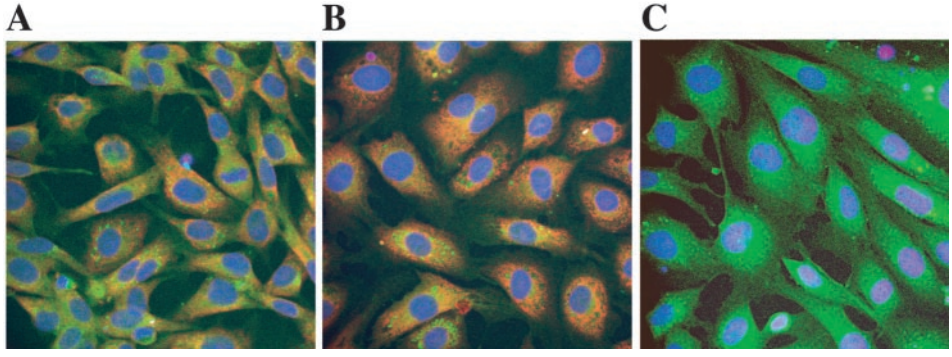

Fig. 3. PDI expression in EC during hypoxia. EC were grown on coated glass slides and maintained in 21% O_2 (*A*) or exposed to 0% O_2 (*B*) for 18 h. Cells were fixed and labeled with TO-PRO-3 (nucleic acid stain, blue), 5-dodecanoylaminofluorescein (lipid stain for membranes, green), and a PDI monoclonal antibody followed by a Texas red-linked anti-mouse secondary antibody (red; *A* and *B*). As a negative control (*C*), cells were exposed to 0% O_2 for 18 h and labeled with TO-PRO-3, 5-dodecanoylaminofluorescein, and secondary antibody alone. Representative results from 3 separate experiments using BPAEC are shown (×60 magnification); similar results were obtained in BAEC.

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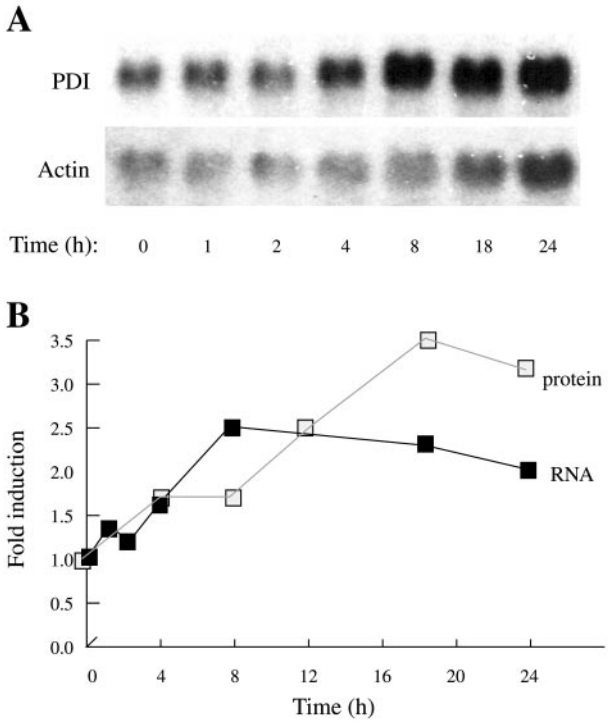

Fig. 4. *A*: time-course analysis of PDI mRNA induction during hypoxia in EC. BAEC were maintained in 21% O_2 (0) or exposed to 0% O_2 for the times indicated. After hypoxic exposure, total RNA was isolated, separated on a 1% agarose-formaldehyde gel, and transferred to a nylon membrane. This was then hybridized with a human PDI cDNA probe (PDI) and then with a β-actin cDNA probe (actin) to control for equal loading. The experiment was repeated 4 times with similar results. Similar results were obtained for BPAEC. *B*: time course of PDI mRNA and 56-kDa protein induction during hypoxia. Relative PDI mRNA and 56-kDa protein levels are plotted against increasing time periods of exposure to 0% O_2. BAEC were exposed to increasing time periods of 0% O_2 as indicated, and total RNA was isolated and analyzed as described in *A*. Densitometric values for PDI mRNA were normalized to the β-actin band and expressed as fold increase above control cells maintained in 21% O_2 (0). Alternatively, BAEC were exposed to 0% O_2 for increasing time periods, total protein was isolated and separated by 10% SDS-PAGE, and induction of the 56-kDa protein was quantitated as previously described (16).

is highly conserved and is usually isolated as a homodimer, each subunit having a molecular mass of 57 kDa. PDI catalyzes the rate-limiting reactions of oxidative formation, reduction, and isomerization of disulfide bonds within the ER. Thus it is critical for protein maturation, and in *Saccharomyces cerevisiae* it is essential for viability (41). Depending on its concentration, PDI has chaperone activity in vitro and in vivo (27, 36), whereas at certain stoichiometric concentrations in vitro, it has been shown to have antichaperone activity (17, 27, 36, 38).

It is unclear why PDI is upregulated specifically in EC during hypoxia. Although primarily an ER resident protein, PDI has been demonstrated on the surface of numerous cell types, including EC (18), platelets (9), lymphocytes (39), hepatocytes (50), pancreatic cells (2), and fibroblasts (7), where it is hypothesized to serve several functions involving disulfide bond formation. PDI has been found on the surface of BAEC, where it has been shown to enhance the adhesion of thrombospondin to the V_3 integrin receptor and support cell spreading (18). In vitro, PDI has been shown to catalyze the formation of thrombospondin-thrombin-antithrombin complexes (9), and it has been proposed that in vivo it functions in this way on the platelet surface (30). Other studies have shown that cell surface PDI is involved in the activation and translocation of diphtheria toxin through the reduction of disulfide-linked toxin on the cell surface (40) and in cell surface events that trigger human immunodeficiency virus entry into lymphocytes (39). Antisense-mediated underexpression of PDI in HT1080 cells caused cells to accumulate in cell islands and never become confluent (21). Finally, antisense inhibition of human erythroleukemia cell surface PDI expression is associated with a significant decrease in cGMP generation after *S*-nitrosothiol exposure, suggesting that the cellular entry of nitric oxide involves a transnitrosation mechanism catalyzed by PDI (52). These findings suggest that cells may be able to manipulate the redox state of cell surface and extracellular thiols/disulfides through targeting PDI to the

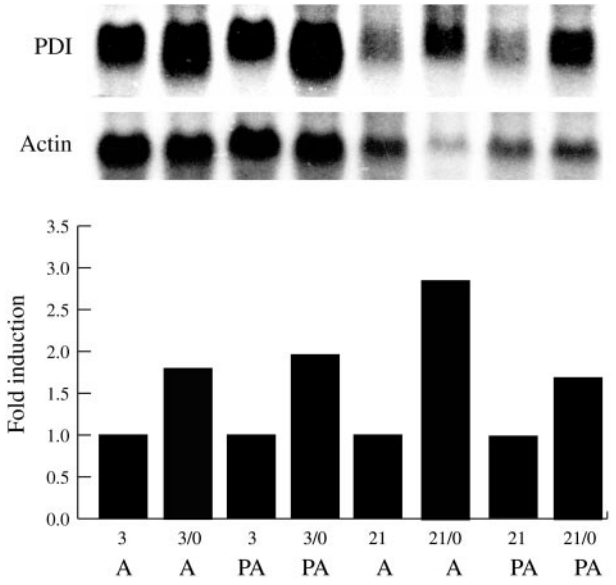

Fig. 5. Northern blot analysis of PDI mRNA levels during hypoxia in different EC types. In pairs of experiments, EC were maintained in 21% or 3% O_2 from the time of isolation and then exposed to 0% O_2 for 18 h. The first of the 2 lanes serves as the control. After hypoxic exposure, total RNA was isolated, separated on a 1% agarose-formaldehyde gel, and transferred to a nylon membrane. This was then hybridized with a human PDI cDNA probe (PDI) and then with a β-actin cDNA probe (actin). Densitometric values for PDI mRNA in each cell type were normalized to actin and expressed as fold increase during acute hypoxia above the corresponding control levels. *Lane 1* (3, A), BAEC grown chronically in 3% O_2; *lane 2* (3/0, A), BAEC grown chronically in 3% O_2 and exposed to 0% O_2 for 18 h; *lane 3* (3, PA), BPAEC grown chronically in 3% O_2; *lane 4* (3/0, PA), BPAEC grown chronically in 3% O_2 and exposed to 0% O_2 for 18 h; *lane 5* (21, A), BAEC grown chronically in 21% O_2; *lane 6* (21/0, A), BAEC grown chronically in 21% O_2 and exposed to 0% O_2 for 18 h; *lane 7* (21, PA), BPAEC grown chronically in 21% O_2; *lane 8* (21/0, PA), BPAEC grown chronically in 21% O_2 and exposed to 0% O_2 for 18 h. The experiment was repeated 3 times with similar results.

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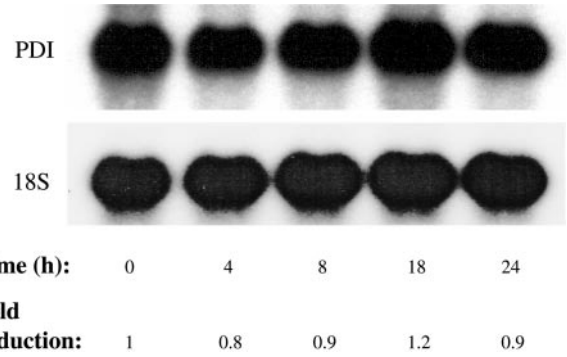

Fig. 6. Time-course analysis of PDI mRNA levels during hypoxia in fibroblasts. Human fetal lung fibroblasts (IMR-90) were maintained in 21% O_2 (0) or exposed to 0% O_2 for the times indicated. After hypoxic exposure, total RNA was isolated, separated on a 1% agarose-formaldehyde gel, and transferred to a nylon membrane. This was then hybridized with a human PDI cDNA probe (PDI) and then with an 18S ribosomal oligonucleotide probe (18S). Densitometric values for PDI mRNA were normalized to the 18S band and expressed as fold increase above control cells (0) maintained in 21% O_2. This experiment was repeated 3 times with similar results.

cell membrane. Our experiments using confocal fluorescence microscopy show that PDI is significantly upregulated within the cytoplasm (and presumably ER) of EC during hypoxia; however, because of the high level of expression, it was difficult to localize PDI specifically to the cell membrane or distinguish differences in membrane localization during normoxia and hypoxia. We cannot exclude the possibility that a fraction of PDI moved to or from the membrane during hypoxia.

PDI may also affect cellular processes through regulation of transcription factors. PDI has been shown to regulate dimerization and DNA binding of E2A helix-loop-helix proteins through formation of disulfide bonds (29). Because this dimerization event is critical for the formation of B cell lines, PDI may play an important role in B lymphopoiesis. PDI may also play a role in the redox control of nuclear factor-κB and activator protein-1 transcription factors, since these proteins have decreased affinity for DNA under nonreducing conditions (6). In relation to this, PDI has been found in the nuclei of spermatids, where it may play a role in the redox-dependent condensation of spermatid chromatin (33, 34). Furthermore, it has been shown to be important in the redox regulation of factor binding to enhancers of interferon-induced genes (22). Our experiments using confocal fluorescence microscopy show that PDI is not present in the nucleus of EC during normoxia or hypoxia. This confirms previous findings from use of subcellular fractionation (15). Changes within the cell membrane were difficult to assess because of strong induction of PDI within the cytoplasm/ER during hypoxia. Of particular interest is PDI's function as the β-subunit of both known types of vertebrate prolyl 4-hydroxylase (P4H). The type I and type II enzymes are $[\alpha(II)]_2\beta_2$ and $[\alpha(I)]_2\beta_2$ tetramers, respectively, and catalyze the formation of 4-hydroxyproline in collagens and other proteins with collagen-like sequences (24). Two recent studies identified proline hydroxylation as a key step in regulation of the transcription factor hypoxia-inducible factor-α (HIF-α) (19, 20). Under normoxic conditions, HIF-α is present at very low levels because of protein degradation by an E3 ubiquitin ligase containing the von Hippel-Lindau tumor suppressor protein (23, 32, 46). During hypoxia, degradation is inhibited and the protein is upregulated. Recent studies show that HIF-α is targeted for ubiquitin-mediated destruction by proline hydroxylation. The authors hypothesize that this is catalyzed by a yet-unidentified proline hydroxylase that resides primarily within the cytoplasm (in contrast to P4H, which resides primarily within the ER) and may play a role in O_2 sensing, since proline hydroxylation requires O_2 and Fe^{2+}. The role of PDI upregulation in this hypoxia-sensing pathway is unclear. In P4H the PDI subunit is synthesized in a large excess over the α-subunits; thus regulation of active P4H occurs mainly through the latter. P4H has been shown to be active in hypoxia (28), and prolyl hydroxylase Iα was recently shown to be upregulated by hypoxia in fibroblasts (47).

Through the regulation of HIF-α expression, regulation of other transcription factors, or regulation of disulfide bond formation in response to hypoxia, PDI may contribute to the ability of EC to tolerate prolonged hypoxia (16). Upregulation of PDI has been demonstrated in neuroblastoma cells in response to hypoxia, where it is hypothesized to serve a protective role (48). Overexpression of PDI in these cells resulted in attenuation of cell viability in response to hypoxia and protected hippocampal CA1 cells from apoptotic cell death in response to brain ischemia (48). Studies are underway to determine whether PDI plays a protective role in EC as well.

This work was supported by National Heart, Lung, and Blood Institute Grants HL-45537 (H. W. Farber) and HL-63171-02 (K. K. Graven) and the American Lung Association through a Research

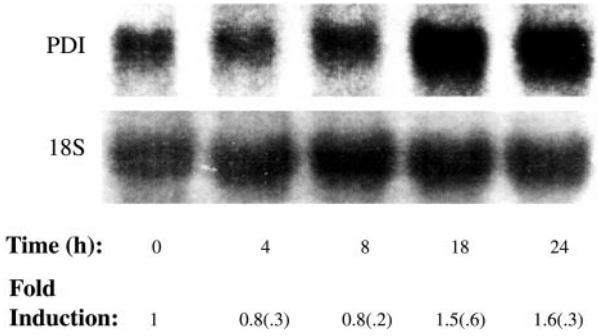

Fig. 7. Time-course analysis of PDI mRNA levels during hypoxia in pulmonary artery smooth muscle cells. Bovine pulmonary artery smooth muscle cells were maintained in 21% O_2 (0) or exposed to 0% O_2 for the times indicated. After hypoxic exposure, total RNA was isolated, separated on a 1% agarose-formaldehyde gel, and transferred to a nylon membrane. This was then hybridized with a human PDI cDNA probe (PDI) and then with a human 18S ribosomal oligonucleotide probe (18S). Densitometric values for PDI mRNA were normalized to the 18S band and expressed as fold increase above control cells (0) maintained in 21% O_2. Because of increased variability of PDI mRNA expression in these cells, means of 3 separate experiments are shown; SE are in parentheses.

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Grant to K. K. Graven and a Career Investigator Award to H. W. Farber.

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