Hypoxic regulation of endothelial glyceraldehyde-3-phosphate dehydrogenase

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Graven, Krista K., Robert J. McDonald, and Harrison W. Farber. Hypoxic regulation of endothelial glyceraldehyde-3-phosphate dehydrogenase. Am. J. Physiol. 274 (Cell Physiol. 43): C347–C355, 1998.—The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is induced by hypoxia in endothelial cells (EC). To define the mechanisms by which GAPDH is regulated by hypoxia, EC were exposed to cobalt, other transition metals, carbon monoxide (CO), deferoxamine, or cycloheximide in the presence or absence of hypoxia for 24 h, and GAPDH protein and mRNA levels were measured. GAPDH was induced in cells by the transition metal cobalt, nickel, and manganese and by deferoxamine, and GAPDH mRNA induction by hypoxia was blocked by cycloheximide. GAPDH induction by hypoxia, unlike that of other hypoxia-regulated genes, was not inhibited by CO or by 4,6-dioxoheptanoic acid, an inhibitor of heme synthesis. GAPDH induction was not altered by mediators of protein phosphorylation, a calcium channel blocker, a calcium ionophore, or alterations in redox state. GAPDH induction by hypoxia or transitional metals was partially blocked by sodium nitroprusside but was not altered by the inhibitor of nitric oxide synthase Nω-nitro-L-arginine. These findings suggest that GAPDH induction by hypoxia in EC occurs via mechanisms other than those involved in other hypoxia-responsive systems.

hypoxia; endothelium; erythropoietin; nitric oxide; cobalt

THE ABILITY OF CELLS AND ORGANISMS TO SENSE AND ADAPT TO ACUTE AND CHRONIC CHANGES IN OXYGEN TENSION IS CRITICAL TO MAINTAINING HOMEOSTASIS. THE MECHANISMS BY WHICH CELLS RESPOND TO DECREASES IN OXYGEN TENSION ARE UNCLEAR BUT CERTAINLY RESULT IN ALTERATIONS IN GENE EXPRESSION VIA CHANGES IN THE RATES OF TRANSCRIPTION AND/OR RNA STABILITY. THE LIST OF GENES REGULATED BY HYPOXIA CONTINUES TO GROW AND INCLUDES ERYTHROPOIETIN (EPO) (13), VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) (30), ENDOTHELIN-1 (ET-1) (18), PLATELET-DERIVED GROWTH FACTOR B (PDGF-B) (17), XANTHINE OXIDASE (31), HEME OXYGENASE 1 (24), GLUCOSE TRANSPORTER-1 (GLUT-1) (3), TYROSINE HYDROXYLASE (7), INTERLEUKIN-6 (IL-6) (35), AND SEVERAL GLYCOLYTIC ENZYMES, INCLUDING GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (GAPDH) (14), ENOLASE (1, 28), ALDOLASE (28, 29), PHOSPHOGLYCEROKINASE (PGK1) (10, 29), AND LACTATE DEHYDROGENASE A (LDHA) (10).

THE MECHANISMS INVOLVED IN REGULATION OF EPO EXPRESSION BY HYPOXIA ARE THE MOST EXTENSIVELY STUDIED THUS FAR AND SUGGEST A PARADIGM FOR GENETIC REGULATION BY HYPOXIA (5). HYPOXIC INDUCTION OF EPO IS MEDIATED PRIMARILY BY AN INCREASE IN THE RATE OF TRANSCRIPTION. BINDING OF THE TRANSACTIVATING FACTOR HYPOXIA-INDUCIBLE FACTOR-1 (HIF-1) TO A DNA SEQUENCE IN THE 3′ FLANKING REGION OF THE EPO GENE ACCOUNTS FOR MOST BUT NOT ALL OF THIS INCREASE IN TRANSCRIPTION. EPO PRODUCTION AND LIKewise HIF-1 BINDING ARE INDUCED NOT ONLY BY HYPOXIA BUT ALSO BY CERTAIN TRANSITIONAL METALS (COBALT, NICKEL, MANGANESE) AND THE IRON CHELATOR DEFEROXAMINE (DSF), SUGGESTING THAT EPO UPREGULATION BY HYPOXIA INVOLVES A HEME PROTEIN. CONSISTENT WITH THIS HYPOTHESIS, INDUCTION OF EPO BY HYPOXIA IS INHIBITED BY CARBON MONOXIDE (CO) AND 4,6-DIOXOHEPTANOIC ACID (DHA), AN INHIBITOR OF HEMESYNTHESIS. FURTHERMORE, EPO INDUCTION AND HIF-1 INDUCTION REQUIRE NEW PROTEIN SYNTHESIS, SINCE THEY ARE BLOCKED BY CYCLOHEXIMIDE. SUBSEQUENT EXPERIMENTS HAVE SHOWN THAT CERTAIN OTHER HYPOXIA-REGULATED GENES (E.G., VEGF, PGK1, AND GLUT-1) ARE REGULATED SIMILARLY BY TRANSITIONAL METALS, DSF, AND CO AND BY BINDING OF HIF-1, WITH A REQUIREMENT FOR PROTEIN SYNTHESIS, SUGGESTING A UNIVERSAL OXYGEN-SENSING SYSTEM THAT INVOLVES A HEME PROTEIN (5). THIS SYSTEM APPEARS TO BE PRESENT IN MANY DIFFERENT CELL TYPES, SINCE INDUCTION OF HIF-1 ACTIVITY BY HYPOXIA, COBALT, AND DSF HAS BEEN DETECTED IN MANY MAMMALIAN CELL TYPES (23). RECENT EVIDENCE SUGGESTS THAT CERTAIN HYPOXIA-REGULATED GENES (E.G., LDHA, TYROSINE HYDROXYLASE, IL-6) (8, 11, 26, 35) DO NOT FOLLOW THIS PARADIGM PRECISELY. THUS THERE MAY BE MULTIPLE MECHANISMS INVOLVED IN THE CELLULAR SENSING OF HYPOXIA.

VASCULAR ENDOTHELIAL CELLS (EC) UPREGULATE THE GLYCOLYTIC ENZYME GAPDH IN RESPONSE TO HYPOXIA (14). THIS UPREGULATION IS MAXIMAL (4.5-FOLD) AFTER EXPOSURE TO 0% O2 FOR 18–24 h, OCCURS IN BOTH HUMAN AND BOVINE EC FROM VARIOUS VASCULAR BEDS, AND IS NOT REPRODUCED BY EXPOSURE TO GLUCOSE DEPRIVATION, HEAT SHOCK, REDOX STRESS (USING DITHIOHETREIOL OR H2O2), OR EXPOSURE TO CYANIDE, 2-DEOXYGLUCOSE, OR ARSENITE (15). AS DETERMINED BY NUCLEAR RUNOFF STUDIES, UPREGULATION OF GAPDH BY HYPOXIA OCCURS PRIMARILY AT THE LEVEL OF TRANSCRIPTION. FINALLY, GAPDH IS ONE OF FIVE PROTEINS UPREGULATED BY HYPOXIA IN EC (15). THESE PROTEINS, TERMED HYPOXIA-ASSOCIATED PROTEINS, MAY CONTRIBUTE TO THE RELATIVE HYPOXIA TOLERANCE OF EC. ALTERNATIVELY, THEY MAY BE RELATED TO THE SPECIFIC RESPONSES OF EC TO HYPOXIA, SUCH AS THE SYNTHESIS AND RELEASE OF VARIOUS MITOGENIC, PROCOAGULANT, VASOACTIVE, AND IMMUNOLOGIC SUBSTANCES.

TO DETERMINE WHETHER HYPOXIC INDUCTION OF GAPDH IN EC IS SIMILAR TO THAT OF EPO AND OTHER GENES REGULATED BY HYPOXIA, GAPDH mRNA AND PROTEIN LEVELS WERE MEASURED AFTER EXPOSURE OF EC TO COCl2 AND OTHER TRANSITIONAL METALS, DSF, CO, DHA, AND CYCLOHEXIMIDE, IN THE PRESENCE OR ABSENCE OF HYPOXIA. IN ADDITION, BECAUSE RECENT REPORTS SUGGEST THAT EPO AND HIF-1 ACTIVATION BY HYPOXIA ARE REGULATED VIA PROTEIN PHOSPHORYLATION (33), THE REQUIREMENT FOR TYROSINE KINASES OR PROTEIN KINASE C (PKC) IN THE HYPOXIC INDUCTION OF GAPDH WAS EVALUATED USING CERTAIN KINASE INHIBITORS AND STIMULATORS. BECAUSE HYPOXIA AFFECTS CALCIUM HOMEOSTASIS IN EC (2), HYPOXIC UPREGULATION OF...
GAPDH in the presence of a calcium channel blocker or a calcium ionophore was also evaluated. Finally, because it has been hypothesized that the hypoxic induction of EPO and other genes is mediated through the redox state of the cell (5), EC were exposed to glutathione or N-acetyl-L-cysteine in the presence or absence of hypoxia, and GAPDH levels were evaluated (even though previous studies using dithiothreitol or H$_2$O$_2$ have shown that the redox state does not alter GAPDH levels).

Finally, because several studies have demonstrated an effect of nitric oxide (NO) on hypoxic gene regulation (6, 19), the effects of NO on GAPDH induction by hypoxia were evaluated. In the present study, EC were exposed to the NO generator sodium nitroprusside (SNP) or the NO synthase (NOS) inhibitor N$^\text{N}$-nitro-L-arginine (L-NNA) in the presence or absence of hypoxia, and GAPDH mRNA and protein levels were measured.

MATERIALS AND METHODS

Materials. O$_2$ gas mixtures were purchased from Wesco (Billerica, MA). Tissue culture materials were obtained from GIBCO (Grand Island, NY), except for bovine serum albumin, which was obtained from HyClone (Logan, UT). TriReagent-LS was obtained from Molecular Research Center (Cincinnati, OH), radioactive isotopes were purchased from NEN DuPont (Boston, MA), and nylon membranes (Hybond-N$^+$) were obtained 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). Genstein and 12-O-tetradecanoyl-phorbol 13-acetate (TPA) were purchased from LC Laboratories (Woburn, MA), and bisindoylmaleimide was purchased from Calbiochem (La Jolla, CA). All other materials were of standard chemical grade and were purchased from either Sigma or ICN Pharmaceuticals (Irvine, CA).

Cell culture. Bovine aortic EC and bovine pulmonary arterial EC (BAEC and BPAEC, respectively) were isolated from freshly excised calf aortas and pulmonary arteries 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"). Under these conditions, the oxygen level in the medium surrounding these cells has been measured at 140 mmHg. EC purity was confirmed by typical cobblestone appearance, factor VIII immunofluorescence, and assessed by exposure of cell monolayers to 5% CO$_2$-95% N$_2$ (0% O$_2$, "hypoxia") for the times specified in humidified sealed chambers with a gas mixture of 10% CO-5% CO$_2$-balance N$_2$. Stock solutions of verapamil, ionomycin, N-acetyl-L-cysteine, glutathione, bisindoylmaleimide, l-NNA, and cyclohexamide were prepared in distilled water and filtered, and dilutions were performed in standard medium. Genestein was initially dissolved in 95% ethanol and then diluted in standard medium. TPA was initially dissolved in dimethyl sulfoxide, and dilutions were prepared in standard medium. An aqueous stock solution of SNP was prepared immediately before use and kept out of direct light until appropriate dilutions in standard medium were performed. All solutions were prepared immediately before use.

Protein labeling and analysis. After chemical or hypoxic exposure, cells were labeled with 50 µCi/ml of [35S]methionine in methionine-free modified Eagle's medium as previously described (14, 15). After labeling, cells were solubilized in Laemml sample and stored at −20°C until use. [35S]methionine-labeled cell extracts were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography as previously described. The induced protein bands corresponding to GAPDH and the four other hypoxia-associated proteins were quantitated by densitometry (Molecular Dynamics computing densitometer, Sunnyvale, CA), and levels were normalized to the actin bands in each lane.

RNA isolation and Northern analysis. After chemical or hypoxic exposure, total RNA was isolated from cell monolayers using TriReagent-LS (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Fifteen micrograms of total RNA from each sample were separated by electrophoresis, transferred to a nylon membrane, and probed with a human β-actin cDNA (American Type Culture Collection, Rockville, MD) as previously described (14). To document equal loading, the same blot was stripped using the manufacturer's protocol and probed with a human GAPDH cDNA (Promega, Madison, WI) according to the manufacturer's instructions. Fifteen micrograms of GAPDH cDNA probe was hybridized to a nylon membrane, and probed with a human GAPDH cDNA (American Type Culture Collection, Rockville, MD) as previously described (14). Hybridization signals were quantitated using a Molecular Dynamics computing densitometer. GAPDH mRNA levels were normalized to β-actin levels and expressed as multiples of increase above control mRNA levels.

RESULTS

CoCl$_2$, NiCl$_2$, and MnCl$_2$, but not ZnCl$_2$ or FeCl$_2$, upregulate GAPDH expression. It has previously been shown that EC exposure to hypoxia upregulates GAPDH protein and mRNA levels (14). Maximal induction occurs after exposure to 0% O$_2$ for 18–24 h and ranges from 3.5- to 4.5-fold in human and bovine aortic and pulmonary artery EC. To test whether the mechanism of induction of GAPDH by hypoxia is similar to that of other hypoxia-regulated genes, BAEC and BPAEC were exposed to various concentrations of CoCl$_2$ (50–200 µM), NiCl$_2$ (100–500 µM), and MnCl$_2$ (100–500 µM) for 24 h. The proteins were then labeled with [35S]methionine for 1 h, and total cellular extracts were separated by SDS-PAGE and analyzed by autoradiography. Each metal produced a dose-dependent increase in the 36-kDa protein corresponding to GAPDH. Maximal induction of GAPDH protein followed exposure to 150 µM CoCl$_2$, 400 µM NiCl$_2$, or 400 µM MnCl$_2$ and reached 2.8-fold, 3.0-fold, and 2.7-fold, respectively (Fig. 1A). These levels of GAPDH upregulation were slightly...
lower than the 3.5-fold induction seen after exposure to 0% O₂ for 24 h in parallel experiments. The highest concentrations of each metal resulted in cellular toxicity. When cells were exposed to 150 µM CoCl₂, 400 µM NiCl₂, or 400 µM MnCl₂ in addition to hypoxia for 24 h, there was a slight increase in protein induction above that of either the metal or hypoxia alone (3.8-fold for CoCl₂ plus 0% O₂, 4.1-fold for NiCl₂ plus 0% O₂, and 3.8-fold for MnCl₂ plus 0% O₂); however, the induction was not additive (Fig. 1A). Exposure to ZnCl₂, in concentrations between 50 and 200 µM (Fig. 1A) or to FeCl₂ in concentrations between 100 and 600 µM (data not shown) for 24 h did not result in upregulation of GAPDH and at higher concentrations resulted in cellular toxicity.

To determine whether upregulation of GAPDH protein by these metals is paralleled by an increase in GAPDH message, BAEC and BPAEC were exposed to 150 µM CoCl₂, 400 µM NiCl₂, or 400 µM MnCl₂ for 24 h, total RNA was isolated, and Northern blots were probed with a human GAPDH cDNA. Each metal resulted in upregulation of GAPDH mRNA, although to a slightly lesser extent than hypoxia. Exposure to CoCl₂ resulted in a 2.3-fold increase in GAPDH mRNA (Fig. 1B). Exposure to NiCl₂ or MnCl₂ resulted in 3.0-fold and 3.1-fold increases in GAPDH mRNA, respectively (see Fig. 3B). The amount of induction was slightly less than that seen with 0% O₂ in parallel experiments (3.8-fold induction). Exposure to 150 µM ZnCl₂ or 400 µM FeCl₂ did not alter GAPDH mRNA in the presence or absence of hypoxia (data not shown). Similar to GAPDH protein induction, there was further upregulation of GAPDH mRNA by concomitant 24-h exposure of EC to 0% O₂ and one of the transition metals (3.4-fold for CoCl₂ plus 0% O₂, 4.1-fold for NiCl₂ plus 0% O₂, and 3.9-fold for MnCl₂ plus 0% O₂), but the effects were not additive (see Figs. 1B and 3B).

Upregulation of GAPDH by DSF and lack of inhibition of the hypoxic response by CO and DHA. It has been proposed that the regulation of other genes by hypoxia (e.g., EPO and VEGF) involves a heme-binding protein, since they are induced by transitional metals and DSF and the effects of hypoxia are abrogated by CO or inhibitors of heme-synthesis (5, 13). To determine whether a similar system is involved in the regulation of GAPDH by hypoxia, BAEC and BPAEC were preincubated with either the iron chelator DSF (130 µM) or the heme synthesis inhibitor DHA (2 mM) for 24 h and then exposed to 21% O₂, 0% O₂, or one of the transitional metals for another 24 h in the continued presence of DSF or DHA (fresh solutions of each chemical were added after the preincubation period). Exposure to DSF alone resulted in a twofold increase in GAPDH protein levels and a threefold increase in GAPDH mRNA levels compared with normoxic controls (Fig. 2, A and C). At the protein level, concomitant exposure to DSF and 0% O₂ or CoCl₂ resulted in greater increases (2.6-fold and 4-fold, respectively, above normoxic levels), although these effects were not additive. At the mRNA level, concomitant exposure to DSF and 0% O₂ resulted in
upregulation of GAPDH mRNA 5.3-fold above normoxic controls. Exposure to DHA did not inhibit the upregulation of GAPDH protein (Fig. 2A) or mRNA (data not shown) by 0% O2 or CoCl2. Exposure of EC to 0% O2 in the presence of 10% CO for 24 h did not block the hypoxic upregulation of GAPDH at either the protein or mRNA level (Fig. 2B and C).

Modulators of NO production alter hypoxic upregulation of GAPDH. Because it has been shown that NO is involved in modulation of certain hypoxic responses in EC (24), we treated cells with the NO donor SNP at a concentration of 1 mM or the NOS inhibitor L-NNA at a concentration of 2.5 mM for 24 h in the presence or absence of hypoxia, CoCl2, NiCl2, or MnCl2. Exposure of BAEC to SNP alone for 24 h had no effect on GAPDH protein or mRNA levels (Fig. 3); however, concomitant exposure of BAEC to SNP and 0% O2, CoCl2, NiCl2, or MnCl2 blocked GAPDH protein and mRNA upregulation (Fig. 3). Exposure to L-NNA alone for 24 h did not alter GAPDH protein or mRNA levels (Fig. 4). Concomitant exposure to L-NNA and 0% O2, CoCl2, NiCl2, or MnCl2 for 24 h did not alter GAPDH expression at the protein level (Fig. 4A, manganese not shown). Likewise, concomitant exposure to L-NNA and 0% O2 for 24 h did not alter GAPDH expression at the mRNA level (Fig. 4B).
Inhibition of hypoxic induction of GAPDH mRNA by cyclohexamide at early time points only. It has previously been shown that GAPDH mRNA upregulation by hypoxia is, at least in part, due to an increase in the rate of transcription (14). To investigate the need for newly synthesized protein in this response, BAEC were pretreated for 2 h with 100 µM cyclohexamide and then exposed to increasing periods of 0% O₂. GAPDH mRNA levels were compared with levels in parallel cultures of EC exposed to 0% O₂ in the absence of cyclohexamide. At time points up to 8 h, cyclohexamide inhibited GAPDH mRNA induction by hypoxia (Fig. 5). After 18 h of 0% O₂ plus cyclohexamide, GAPDH mRNA levels were not changed significantly from those induced by
hypoxia alone, and after 24 h GAPDH mRNA levels were superinduced above those seen with hypoxia alone.

Modulators of intracellular calcium levels, protein kinases, and redox potentials have no effect on GAPDH levels. Other studies have implicated calcium, tyrosine kinases, PKC, and oxidation/reduction states as important to the hypoxic response (4, 9, 20–22, 33). To investigate involvement of these mechanisms in the hypoxic upregulation of GAPDH, EC were exposed for 24 h, in the presence or absence of hypoxia, to the calcium channel blocker verapamil (10 µM), the calcium ionophore ionomycin (5 µM), the tyrosine kinase inhibitor genistein (100 µM), the PKC activator TPA (10–50 ng/µl), the PKC inhibitor bisindoylmaleimide (5 µM), or to alterations in the redox state with (10–50 ng/µl), the PKC inhibitor bisindoylmaleimide (5 µM), or glutathione (50 µM), and protein upregulation was evaluated by SDS-PAGE. None of these agents alone altered GAPDH protein levels, nor did they alter GAPDH upregulation either by 0% O2 or by CoCl2, NiCl2, or MnCl2 (Table 1).

Effects of transition metals, DSF, CO, DHA, SNP, and cyclohexamide on other EC hypoxic stress proteins. It has previously been shown that hypoxia upregulates, although to a lesser extent, four other proteins with relative molecular masses of 34, 39, 47, and 56 kDa in EC in a time-dependent manner similar to that of GAPDH (the 39-kDa protein being variably induced) (37). On the same SDS-PAGE gel used in the evaluation of GAPDH levels, the effects of CoCl2, NiCl2, MnCl2, CO, DSF, and DHA on these proteins were noted. CoCl2, NiCl2, and MnCl2 upregulated the 34-, 47-, and 56-kDa proteins in a manner similar to GAPDH, although to a lesser extent, ~1.5- to 1.8-fold (Fig. 1A). DSF alone resulted in slight induction of the same three proteins (~1.7-fold) and further increased protein levels when combined with hypoxia (~2.0-fold). DHA and CO had no effect on hypoxic upregulation of these proteins (Fig. 2A and B). Modulators of NO production affected hypoxic and transitional metal upregulation of these three proteins similarly to that of GAPDH, such that SNP blocked the ~2.5-fold induction of each of these proteins by either hypoxia, CoCl2, NiCl2, or MnCl2 and L-NNA had no effect (Figs. 3A and 4A). Effects of all modulators on hypoxic induction of the 39-kDa protein were difficult to assess, since induction of this protein is small and variable.

**Table 1. Effects of various mediators on endothelial cell GAPDH levels**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Effect on GAPDH</th>
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<tr>
<td></td>
<td>Normoxia</td>
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<tr>
<td>Deferoxamine</td>
<td>Induction</td>
</tr>
<tr>
<td>4,6-Dioxoheptanoic acid</td>
<td>None</td>
</tr>
<tr>
<td>CO</td>
<td>None</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>None</td>
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<tr>
<td>Sodium nitroprusside</td>
<td>None</td>
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<tr>
<td>N’-nitro-L-arginine</td>
<td>None</td>
</tr>
<tr>
<td>N-acetyl-L-cysteine</td>
<td>None</td>
</tr>
<tr>
<td>Glutathione</td>
<td>None</td>
</tr>
<tr>
<td>Genistein</td>
<td>None</td>
</tr>
<tr>
<td>Bisindoylmalimide</td>
<td>None</td>
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<tr>
<td>Verapamil</td>
<td>None</td>
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<td>Ionomycin</td>
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**DISCUSSION**

It has been proposed that a universal oxygen-sensing system exists in mammalian cells (5, 23). This proposal was based on two general lines of evidence: 1) many genes regulated by hypoxia share the same properties first defined for the EPO gene, and 2) hypoxia upregulates EPO-enhancer-directed reporter genes in non-EPO-producing cell types. In support of the latter finding, the transcription factor found to regulate EPO induction by hypoxia, HIF-1, is induced by hypoxia in many mammalian cell lines (34). The properties shared by some, but not all, hypoxia-regulated genes include 1) induction not only by hypoxia but also by CoCl2 (and NiCl2 and MnCl2, when evaluated) and DSF, 2) abrogation of hypoxic induction by CO and cycloheximide, and 3) presence of a HIF-1 binding region in the inducible gene. These findings support the hypothesis that decreased O2 is sensed by a heme protein and that this cellular response requires ongoing protein synthesis. Originally it was proposed that in such a model CoCl2 and certain other transition metals would substi-
of the heme moiety. It remains unclear whether this model is an accurate one, and permutations of this model have been proposed on the basis of more recent findings.

Regulation of EC GAPDH by hypoxia has similarities to and differences from the regulation found in the EPO system (and others such as VEGF and PGK1). The ability of cobalt, nickel, manganese, and DSF to mimic hypoxic induction of GAPDH is similar to effects of these mediators on EPO, VEGF, and PGK1 expression, and the finding that the effects of concomitant exposure to hypoxia and these metals are not additive is also similar to results in those gene systems. However, for GAPDH, unlike EPO and VEGF, reversal of hypoxic induction by CO or the heme synthesis inhibitor DHA was not seen. This suggests that either 1) the affinity of the hypothesized heme-binding protein for O\textsubscript{2} vs. CO in the EPO and VEGF systems is different from that in the GAPDH system, 2) the hypoxia-sensing system in EC is functionally different from that in other cell types used in EPO and VEGF systems (e.g., hepatoma or epithelial cell lines), or 3) the hypothesized heme-binding protein does not exist in the hypoxia-sensing pathway for GAPDH. Like upregulation of EPO, VEGF, and PGK1, upregulation of GAPDH mRNA by hypoxia is inhibited by cycloheximide, at least at time points up to 12 h (10, 13, 30). However, unlike most of these genes, after 24 h of hypoxia plus cycloheximide, GAPDH mRNA is superinduced. This has been described for VEGF in hypoxic EC (25) and cyclooxygenase-2 in hypoxic lung homogenates (6), although the mechanism remains unclear.

Additional mechanisms of hypoxic induction of proteins have been suggested, some of which may involve a heme protein pathway and others of which may not. For example, in the EPO system in Hep 3B cells, 2-aminopurine, an inhibitor of serine/threonine kinases, or NaF, an inhibitor of serine/threonine phosphatases, partially or completely inhibits HIF-1 activity induced by hypoxia, suggesting that serine/threonine phosphorylation is involved in step(s) in hypoxia sensing (34). In these particular studies, compounds that affected PKC activity had no effect on HIF-1 induction by hypoxia, suggesting that this particular serine/threonine kinase is not involved in hypoxic signal transduction. In addition, genistein, an inhibitor of tyrosine kinases, interfered with the expression and activity of HIF-1, whereas sodium orthovanadate, an inhibitor of tyrosine phosphatase, increased the basal level of HIF-1 activity, suggesting that tyrosine phosphorylation is also involved in hypoxia signal transduction (32). In support of these findings, staurosporine, a nonspecific kinase inhibitor, and genistein decreased EPO mRNA in rat hepatocytes (35). However, in another study using Hep 3B cells, phorbol ester, in doses thought to stimulate PKC activity, 1-oleoyl-2-acetylglycerol, a synthetic analog of diacylglycerol, and the calcium ionophore A-23187 inhibited hypoxic induction of EPO, suggesting that PKC is indeed involved in hypoxia signaling (9). In the VEGF system, genistein has been shown to block hypoxia-induced mRNA levels in rat hepatocytes (12) as well as hypoxia-induced stabilization of the VEGF 3′-untranslated region transcripts and hypoxia-induced protein binding to this untranslated region (20), suggesting that tyrosine phosphorylation is also involved in this signal transduction pathway. However, in cardiac myocytes, inhibition of PKC by H-7, which can also inhibit protein kinase A (PKA), inhibited the hypoxic induction of VEGF mRNA, whereas inhibition of PKA (using KT-5720), calcium influx (using ethylene glycol-bis(\(\beta\)-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, EDTA, verapamil, or diltiazem), and calcium/calmodulin-dependent protein kinase II (using W-7) had no effect on the hypoxic induction of VEGF mRNA (21). Although somewhat contradictory, these studies suggest a role for serine/threonine and/or tyrosine phosphorylation in hypoxic signaling pathways of VEGF and EPO. In contrast, induction of ET-1 by hypoxia was not affected by inhibitors of PKC, PKA, calcium/calmodulin-dependent protein kinase II, or guanosine 3′,5′-cyclic monophosphate (cGMP)-dependent kinase (4). In our system, genistein, as well as stimulation or inhibition of PKC, had no effect on upregulation of GAPDH by hypoxia or metals, suggesting that tyrosine phosphorylation and PKC-mediated serine/threonine dephosphorylation are not involved in this hypoxia-sensing pathway.

The potential role of protein phosphorylation also suggests that calcium fluxes within the cell have a role in hypoxic gene regulation. Hypoxia has been shown to increase calcium flux in EC (2). This influx can be partially blocked by verapamil. Interestingly, cobalt, nickel, and manganese ions have been shown to block calcium influx through receptor- and voltage-activated channels in hepatocytes (16). Although we did not look at calcium flux, we did not see any effect of the calcium channel blocker verapamil or the calcium ionophore ionomycin on the induction of GAPDH synthesis during hypoxia.

Several studies have demonstrated an effect of NO on hypoxic gene regulation (6, 19). NO is produced by several different cell types including EC and has many functions within the cell and organism (27). It has been shown to 1) activate guanylate cyclase, presumably by binding to its heme moiety, resulting in increased cGMP levels, 2) inhibit other heme proteins such as cytochrome-c oxidase and cytochrome P-450, 3) increase nonheme iron within the cell, affecting both heme and nonheme iron homeostasis, and 4) scavenge and inactivate superoxide radicals while generating other active free radicals. Inhibitors of NO synthesis have been shown to inhibit hypoxia-induced EPO production in the isolated perfused rat kidney (36). The opposite effect was seen with hypoxic induction of ET-1 and PDGF-B in EC (19). Generation of NO with SNP inhibited the hypoxic induction of these two genes, whereas inhibition of NOS with L-NNA in the presence of hypoxia induced ET-1 and PDGF-B synthesis above
that seen with hypoxia alone. NO may be acting through a heme protein in these systems, although the mechanism of this inhibition remains uncertain. Similar to the ET-1 and PDGF-B systems, generation of NO by SNP in the experiments reported here blocked GAPDH upregulation by hypoxia and transition metals; however, unlike the ET-1 and PDGF-B systems, inhibition of NOS by L-NNA had no effect on hypoxic induction of GAPDH. These differences may be related to the types of EC used in each study, since human umbilical vein EC were used in the former studies and BAEC were used here. Alternatively, L-NNA may not have been physiologically active in our experiments, although the concentration and time of exposure used here were the same as those used by others (19).

It has previously been shown that hypoxia upregulates four other proteins with relative molecular masses of 34, 39, 47, and 56 kDa in EC in a time-dependent manner similar to that of GAPDH (the 39-kDa protein being variably induced). All of the mediators studied here had a similar effect on expression of these proteins, suggesting that GAPDH and the four other proteins are regulated in a similar manner. The 47-kDa protein has recently been identified as nonneuronal enolase (1), and investigations are needed to characterize its expression more fully in EC, particularly in comparison to GAPDH expression, during hypoxia and exposure to transition metals, DSF, CO, DHA, SNP, and L-NNA. Investigations are also needed to identify the other three proteins so that more extensive studies of expression can be performed at both the protein and RNA levels.

Overall, the studies presented here suggest that the mechanism of GAPDH induction by hypoxia is completely or partially different from that of other hypoxia-regulated genes. This mechanism is less likely to involve a heme protein on the basis of the data presented here and may be unique to EC. If a heme protein is involved in GAPDH regulation by hypoxia, it is functionally or structurally different from that hypothesized to exist in the EPO system, since regulation by CO, DHA, and modulators of NO production result in different alterations in each system. In addition, it appears that the specific steps governing hypoxia gene regulation that involve protein phosphorylation and the cellular redox state are different for various other hypoxia-regulated genes. Whether this is related to the particular experimental methods used or is truly related to distinct mechanisms of the hypoxic response will require further investigation.

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