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Effects of Cell-Free Hemoglobin on Hypoxia-Inducible Factor (HIF-1α) and Heme Oxygenase (HO-1) Expressions in Endothelial Cells Subjected to Hypoxia

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ABSTRACT

We have investigated the impact of diaspirin cross-linked hemoglobin (DBBF-Hb), a blood substitute, on cell signaling pathways that are modulated in part by biological peroxides (*i.e.*, hydrogen peroxide, lipid peroxide, and peroxynitrite). Bovine aortic endothelial cells (BAECs) subjected to hypoxia expressed hypoxia-inducible factor (HIF-1α) in a time course that paralleled the expressions of heme oxygenase (HO-1). Co-incubation of the oxy form ($HbFe^{2+}$) with hypoxic BAECs resulted in an increase in the expression of HIF-1α in a manner that corresponded linearly with the decay of $HbFe^{2+}$ and accumulation of the ferric form ($HbFe^{3+}$). Inclusion of $HbFe^{3+}$ with hypoxic BAECs produced twice as much expression in the HIF-1α and HO-1 proteins as opposed to $HbFe^{2+}$ alone, or $HbFe^{2+}$ plus hypoxia. In addition, higher and more persistent levels of the ferryl form ($HbFe^{4+}$), due to the consumption of endogenous peroxides, were found in the hypoxic media containing hemoglobin. Nitric oxide (NO) released from an NO donor reduced the levels of HIF-1α in the hypoxic cells treated with either $HbFe^{2+}$ or $HbFe^{3+}$, but had little or no effect on the levels of HO-1. DBBF-Hb modulates key cell-signaling pathways by competing with peroxides required for the deactivation of HIF-1α, which may modulate important physiological mediators. *Antioxid. Redox Signal*. 6, 944–953.

INTRODUCTION

BIOLOGICAL PEROXIDES have been implicated as regulators of redox-sensitive cell-signaling pathways (5, 45). For example, reactive species have been implicated in the regulation of hematopoiesis (38). Both *in vitro* and *in vivo* studies indicate that angiogenic response in vascular tissue is triggered by reactive oxygen species (ROS) signaling in a highly coordinated manner (23). Several studies have shown that hydrogen peroxide (H_2O_2) regulates transcriptional and translational events in many cell types (16, 22, 25). The exact targets that H_2O_2 reacts with, to either stimulate or repress a given pathway, are not known, but downstream targets include the mitogen-activated protein kinases, nuclear factor κB, and hypoxia-inducible factor (HIF) (30, 51, 52). These are important components of numerous redox-sensitive signaling pathways that link extracellular stimuli to gene regulation (30). Similarly, lipid peroxides are intermediates in the cascade of reactions forming compounds that mediate an inflammatory response (34, 36).

The vascular endothelium has emerged as the primary target of hemoglobin (Hb) toxicity due to its proximity to the circulating protein (20). Hb-based oxygen (O_2) carriers, unlike red cells, can reach within close proximity to the endothelial barrier lining the vessel wall, where it can potentially scavenge nitric oxide (NO), thus disrupting the physiological balance between NO, superoxide ion ($O_2^{\bullet -}$), peroxynitrite ($ONOO^-$), and H_2O_2 (3). Although the primary event respon-

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sible for the observed microvascular effects of Hb solutions is believed to be the removal of NO by Hb, subsequent oxidative side reactions between Hb and peroxides of the vascular system (*i.e.* H_2O_2 and $ONOO^-$) may potentially cause vascular injury (2, 3).

The effects of reactions between Hb and biologically relevant peroxides in the context of cell signaling have not been explored. The concentrations of cell-free Hb that can be achieved upon administration are high (in the millimolar range in terms of heme) (13) and can potentially compete with endogenous reactions that consume the peroxides mentioned. No systematic studies have been conducted addressing these issues, but reactions between Hb and H_2O_2 generated during reperfusion of hypoxic endothelial cells was demonstrated (24). Therefore, similar to NO, the effects of Hb on cell function may be more subtle than oxidative damage mediated by Hb and may involve perturbation of redox-sensitive signaling pathways (5).

In living organisms, the transcriptional activator HIF- functions as a global regulator of O_2 homeostasis that facilitates both O_2 delivery and adaptation to O_2 deprivation (42). Cells from a wide variety of tissues share this common mechanism of O_2 sensing and signal transduction leading to the activation of HIF. HIF-1 functions as a global regulator of O_2 homeostasis (43). It is a heterodimer composed of an HIF-1α subunit and an HIF-1β subunit, the expression and transcriptional activity of which are precisely regulated by the cellular O_2 concentration (46). HIF-1 binds to DNA in hypoxic cells, but not in normoxic cells. At the mRNA level, both HIF-1α and HIF-1β are constitutively expressed. However, at the protein levels, HIF-1α is found only in hypoxic cells, whereas HIF-1β is constitutively expressed (18). Several mechanisms responsible for HIF activation that include signal transduction via O_2 binding hemoproteins or via the generation of $O_2^{\bullet -}/H_2O_2$, by either an NAD(P)H oxidase or the mitochondrial electron transport chain, have been suggested (42). Both HIF-1 stability and its transcriptional activity are subject to negative regulation by hydroxylation. Under aerobic conditions, HIF is hydroxylated on proline 402 and proline 564. The proline hydroxylations are necessary for binding to von Hippel–Lindau and ubiquitin-mediated degradation by the proteasome (14). Unlike other posttranscriptional modifications such as phosphorylation, hydroxylation is unique in its dependence on cellular O_2 concentration.

Heme oxygenase (HO), an enzyme widely distributed in mammalian tissues, is primarily involved in the degradation of heme to biliverdin, iron, and carbon monoxide. Two distinct isoforms of the protein exist, a constitutive (HO-2) and an inducible (HO-1) form. The latter is regarded as a heat shock protein, and its expression is elicited by many conditions and factors that produce an imbalance in the cellular functions. Oxidative and nitrosative stress (28, 29), polyphenolic compounds, and Hb (27) are factors capable of inducing HO-1 in endothelial cells and other tissues. Overexpression of HO-1 in endothelial cells protected against heme and Hb toxicity has highlighted the importance of HO-1 in vasculature (37).

In this study, we used diaspirin cross-linked Hb (DBBF-Hb), an intramolecularly cross-linked tetramer, which has been extensively studied *in vitro,* in animal models, and in humans as oxygen therapeutic (31, 32). Oxygen binding and the redox chemistry characteristics of DBBF-Hb have been well documented, and thus may provide a useful model for correlating these parameters with the redox-sensitive intracellular pathways. Our aim is to examine the effects of DBBF-Hb and its oxidized forms on the transcriptional and translational processes in endothelial cells subjected to severe hypoxia. We tested the hypothesis that a low O_2 affinity and a highly redox-active Hb, such as DBBF-Hb, will impact the expression and the transcriptional activity of HIF-1α and HO-1 by virtue of its ability to carry oxygen and to consume peroxides under hypoxic conditions. We report here that the expressions of HIF-1α and HO-1 are enhanced in the presence of the ferrous form of Hb ($HbFe^{2+}$), but substantially enhanced in the presence of its nonfunctional ferric form ($HbFe^{3+}$). In the presence of an NO donor, the levels of these proteins were reduced. The physiological relevance of these findings will be discussed.

MATERIALS AND METHODS

Cell culture

Bovine aortic endothelial cells (BAECs) were purchased from Clonetics (San Diego, CA, U.S.A.). Cells were grown in endothelial basal medium (Clonetics), supplemented with10 ng/ml human recombinant epidermal growth factor, 1 μg/ml hydrocortisone, 10 μg/ml bovine brain abstract, 5% fetal bovine serum, 10 μg/ml glutamine, 50 μg/ml gentamicin, and 50 ng/ml amphotericin B. Cells were subcultured twice a week. BAECs were cultured in cell culture dishes (60 mm diameter) at 37°C in a humidified incubator (5% CO_2, 95% air). All experiments were performed with cell passage 3–10 at 90–95% confluence.

Hypoxic experiments

Before exposure to hypoxic conditions, growth medium of confluent BAECs was replaced with phenol red-free basal medium and incubated with cells overnight. To initiate hypoxia, BAECs were placed in a polybicarbonate modular incubator chamber (Billups-Rothenburg, Del Mar, CA, U.S.A.), flushed with hypoxic gas (5% CO_2 and 95% N_2) for 30 min, and clamp-locked, and then the chamber was placed into a humidified incubator at 37°C for the specified period of time. In some experiments, confluent BAECs from a 60-mm dish were incubated with phenol red basal medium containing 50 μ*M* of either $HbFe^{2+}$ or $HbFe^{3+}$ with or without diethylenetriamine nitric oxide adduct (DETA/NO; 100 μ*M*), and then exposed to hypoxic conditions. Cells in medium without Hb and DETA/NO in normoxic conditions served as controls for normalization.

Measurement of oxygen tension

A polarographic Clark-type oxygen electrode (model 125/05, Harvard Apparatus, Holliston, MA, U.S.A.) was placed through a hole on the cover of the hypoxic chamber. The hole was subsequently sealed with silicone sealant (GE RTV 108, Waterford, NY, U.S.A.). The height of the electrode wire was adjusted so as to allow the tip of the electrode to be

fully immersed in cell culture medium. To minimize the error due to electrode drift, the electrode was equilibrated with fresh KCl electrolyte at the start of each experiment. Confluent cells in 60-mm cell dishes without cover were placed inside the hypoxic chamber containing 3 ml of basal medium with or without Hb in different oxidation and ligation states. Mixed gas (95% N_2, 5% CO_2) was flushed through the headspace of the hypoxic chamber. Calibration was conducted using mixed gases (5% CO_2 equilibrated with 95% N_2, 95% air, or 95% O_2). The reading in the amplifier connected to the electrode was later converted to oxygen partial pressure (pO_2) based on calibration curve, which was constructed using known pO_2 values.

Western blot

For western blots, 5×10^6 cells were plated in tissue culture dishes and allowed to reach confluency. BAECs treated with the $HbFe^{2+}$ or $HbFe^{3+}$ (50 μ*M*, in heme) were incubated under normoxia or hypoxia for a 12-h period. At the end of the incubation period, cells were washed with ice-cold phosphate-buffered saline, lysed in cell lysis buffer (145 m*M* NaCl, 0.1 m*M* $MgCl_2$, 15 m*M* HEPES, 10 m*M* EGTA, 1 m*M* Na_3VO_4, 2% leupeptin, 0.5% 4-(2-aminoethyl)benzenesulfonyl fluoride, and 1% Triton X-100, pH 7.2) by scraping, and immediately frozen in dry ice until analysis. Total protein was determined using the bicinchoninic acid protein (BCA) assay kit (Pierce, Rockford, IL, U.S.A.). The protein extract was separated on a 4–20% polyacrylamide gel (Novex, Carlsbad, CA, U.S.A.), and electroblotted on a polyvinylidene difluoride membrane. The membrane was blocked with 2% I-Block in 0.2% Tween phosphate-buffered saline and then probed with either a HIF-1α mouse monoclonal antibody (1:1,000 dilution) or a HO-1 mouse monoclonal antibody (1:1,000) for 1 h at room temperature. The membrane was washed three times in blocking buffer and then incubated with biotin-goat anti-mouse (1:1,000) antibody (Zymed, San Francisco, CA, U.S.A.) for 1 h. After washing for three times with blocking buffer, the membranes were then incubated with streptavidin-alkaline phosphatase conjugate for 30 min. Antigen–antibody complexes were formed after incubation with chemiluminescent substrate phenyl phosphate 1,2-dioxetane (Tropix, Bedford, MA, U.S.A.) followed by exposure to Hyperfilms (Amersham) or by using BCIP/NBT as substrate. The bands on the membrane were scanned by HP ScanJet and analyzed using software Scion Image (version 4.0.2, Scion Corp., Frederick, MD, U.S.A.) provided by the manufacturer. To control for equal loading, we measured protein samples in cell lysates using the BCA method and adjusted the concentration to 15 μg/lane for each sample. Actin was used as an internal control to confirm equal loading and normalization of the proteins.

Spectral analysis

After incubation under either normoxic or hypoxic conditions, media of BAECs treated with Hb in different oxidation states were collected and immediately frozen in dry ice. Spectral analysis of Hb oxidation products in the media was performed using a Hewlett Packard HP-8453 rapid scanning diode array spectrophotometer. The oxy (Fe^{2+}), ferric (Fe^{3+}), and ferryl (Fe^{4+}) forms of Hb were identified, and their proportions were calculated as previously described (12). $HbFe^{4+}$ was also determined by measuring the sodium sulfide (Na_2S)-induced formation of sulfHb. Samples were reacted with 2 m*M* Na_2S for 30 min, and the concentration of sulfHb was calculated using an extinction coefficient of 10.5 mM^{-1} at 620 nm (24).

$O_2^{\bullet -}$ determination

Lucigenin, a compound that emits light upon interaction with $O_2^{\bullet -}$, was used to measure intracellular $O_2^{\bullet -}$ production, as previously described (10, 49). In brief, at the end of the hypoxic period, cells were harvested in ice-cold phosphate-buffered saline, and cell pellets were suspended in a balanced salt solution consisting of 130 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 35 m*M* phosphoric acid, and 20 m*M* HEPES, pH 7.4. The viability of the suspended cells was >90%, as determined by trypan blue exclusion. Cell suspensions were added to triplicate wells of 96-well plates containing dark-adapted lucigenin (250 μ*M*) in balanced salt solution at room temperature. The increases in photon emission were measured every 20 s thereafter for 10 min in a Microplate Luminescence Counter (TopCount NXT, Packard Instrument, Meriden, CT, U.S.A.). The net increases in $O_2^{\bullet -}$ generation were calculated by comparison with a standard curve generated using xanthine/xanthine oxidase, and normalized by protein content as determined by the BCA protein assay (Pierce). In another set of experiments, 100 U of superoxide dismutase (SOD) ($O_2^{\bullet -}$ scavenger) was added to the hypoxic and normoxic media, and $O_2^{\bullet -}$ generation was monitored.

Data presentation and statistics

Data are presented as means ± SD for triplicate experiments unless otherwise stated. Statistical analysis of the data was performed by unpaired two-tailed Student's *t* test included in the SAS JMP software (SAS Institute, Cary, NC, U.S.A.; version 3.2). $p < 0.05$ was considered significant.

Materials

Cross-linked human Hb (DBBF-Hb), free of SOD and catalase, was a kind gift from the Walter Reed Army Institute of Research (Washington, DC, U.S.A.). This Hb was purified from outdated human red blood cells and reacted with *bis*(3,5-dibromosalicyl)fumarate (DBBF) to cross-link intramolecularly the α-99 lysine residues. This modification prevents dissociation into αβ dimers and significantly increases the circulation time of DBBF-Hb compared with unmodified Hb (35). $HbFe^{3+}$ was prepared as previously described (6). In brief, $HbFe^{3+}$ was prepared by oxidation of $HbFe^{2+}$ using potassium ferricyanide (Fisher, Fair Lawn, NJ, U.S.A.), followed by purification over a Sephadex G-25 column twice to remove the salts in the buffer. The cyanomet derivative of DBBF-Hb (CNmet-Hb), in which the CN groups are tightly bound to the heme such that the molecule is unable to carry oxygen or participate in redox reactions, was prepared as previously reported (24). The diazeniumdiolate [(*Z*)-1-1[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate] (DETA/NO) was purchased from Alexis (San Diego, CA, U.S.A.). The half-life for NO release at 37°C

is 20–23 h in a buffer solution, pH 7.4 (39). Monoclonal antibodies against HIF-1α were purchased from NOVUS (Littleton, CO, U.S.A.). Anti-HO-1 was purchased from StressGen (Victoria, BC, Canada). All other reagents were obtained from Sigma (St. Louis, MO, U.S.A.).

RESULTS

Oxygen tension in the presence of DBBF-Hb under normoxic and hypoxic conditions

Under normoxic condition, the pO_2 was determined to be ~154 mm Hg for basal media (pH 7.4). No significant changes in the pO_2 measurements were observed when 50 μ*M* Hb was included in the media (Fig. 1). Subsequent *p*O2 measurements were carried out under hypoxic conditions using $HbFe^{2+}$ and its CNmet derivative. The oxygen affinity (P_{50})($P_{50} = pO_2$ at which Hb is half-saturated) for this Hb was previously determined by automatic oxygen equilibrium analyzer to be ~29 mm Hg (12). Under hypoxia and/or in the presence of CNmet-Hb, pO_2 decreased to almost 0 mm Hg within 15–20 min of incubation under hypoxic conditions. In contrast, in the presence of the $HbFe^{2+}$ (>98% of the total heme), the decrease of pO_2 was markedly slower, reaching ~2–5 mm Hg within 60–70 min. The difference in pO_2 measured in the medium may therefore reflect the difference in the oxygen-carrying capabilities of $HbFe^{2+}$ as opposed to its non–oxygen-carrying derivative (CNmet-Hb).

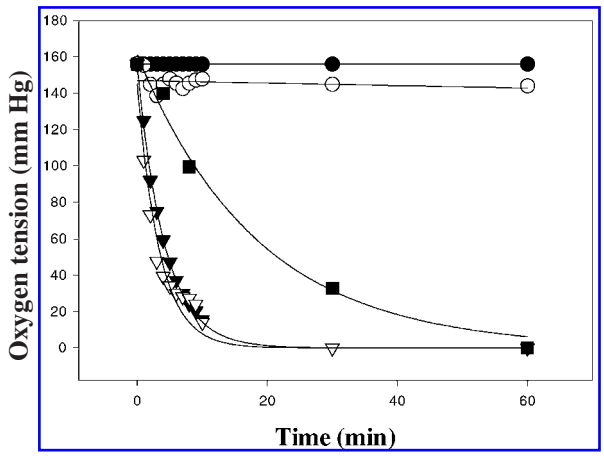

FIG. 1. Time course of oxygen tension measurement during hypoxia/normoxia. Confluent BAEC in 60-mm culture dishes without cover were placed inside the hypoxic chamber containing 3 ml of basal medium with or without Hb. Mixed gas (95% N_2, 5% CO_2) was flushed through the headspace of the hypoxic chamber. Normoxia experiments were conducted when 5% CO_2 and 95% air were fed into the chamber. The reading in the amplifier connected to the electrode was later converted to oxygen partial pressure based on calibration. $HbFe^{+2}$ (50 μ*M*) and CNmet-Hb (50 μ*M*) were used in these experiments (n = 3). Each point represents the mean ± SD of three experiments performed independently. ●, normoxia; ○, normoxia and $HbFe^{2+}$; ▼, hypoxia; ▽, hypoxia and CNmet-Hb; and ■, hypoxia and $HbFe^{2+}$.

Protein expressions in the presence of DBBF-Hb under normoxic and hypoxic conditions

Time-dependent changes in the accumulation of HIF-1α and HO-1 were monitored in BAECs under hypoxic conditions. Figure 2A (top) illustrates the detection by immunoblot analysis of HIF-1α in hypoxic BAECs in the presence and absence of $HbFe^{2+}$ for up to 12 h. Time-dependent changes in the accumulation of HIF-1α are shown in Figure 2A (bottom). The protein levels remained constant at normoxia (pO_2 = 154 mm Hg). Under hypoxic conditions ($pO_2 \approx 2$ mm Hg), on the other hand, there was a time-dependent increase in the

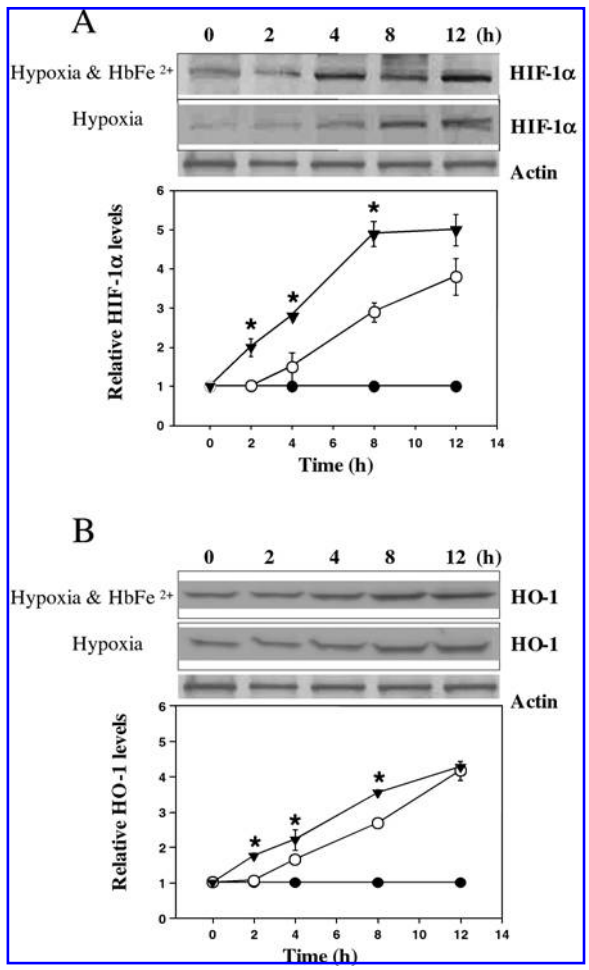

FIG. 2. Time courses of endothelial HIF-1α and HO-1 protein expressions during hypoxia/normoxia in the presence or absence of DBBF-Hb. BAECs were incubated under normoxic or 95% N_2, 5% CO_2 hypoxic conditions in a sealed hypoxic chamber with or without $HbFe^{2+}$ (50 μ*M*) for up to 12 h. After each experiment, cells lysates were analyzed by western blot for **(A)** HIF-1α and **(B)** HO-1 expression **(top)** as described in Materials and Methods. Densitometry was performed to quantify the corresponding bands and plotted as a function of time **(bottom)**. The amount of protein expression is normalized using actin as internal control. Each point represents the mean ± SD of three experiments performed independently. $p < 0.05$, significantly greater than hypoxia alone. ●, normoxia; ○, hypoxia; ▼ hypoxia and $HbFe^{2+}$.

level of HIF-1α, consistent with earlier observations (19, 33). In the presence of $HbFe^{2+}$ (50 μ*M*), however, a two- to three-fold increase in the expression of HIF-1α throughout the first 8-h incubation was seen. HIF-1α expression reached a plateau after 8 h of incubation (Fig. 2A, bottom). Under hypoxic conditions, similar but less significant expressions of HO-1 were seen as compared with hypoxic cells with Hb added (Fig. 2B). Unlike HIF, there was no detectable difference in HO-1 expression at 2 h of hypoxia with or without $HbFe^{2+}$.

Effects of hypoxia on DBBF-Hb oxidation, cellular oxidants, and protein expressions

Monitoring the oxidation status of Hb in the culture media during the 12-h incubation reveals a slight drop in the percent $HbFe^{2+}$ as part of the autoxidation process (Fig. 3A). Under these normoxic conditions, the initial levels of $HbFe^{2+}$ (~95% of the total heme) were reduced to 70%. A corresponding increase in the oxidized forms (Fe^{3+}/Fe^{4+}) in the range of 28–30% of the total heme was seen after the end of the 12-h period. Under hypoxic conditions, however, there was an acceleration in the decay of $HbFe^{2+}$, reaching as low as 2–4% of the total heme. At the end of the experiment, ~80–82% of the total heme was found in the Fe^{3+} and 16–18% in the Fe^{4+} forms of Hb (Fig. 3B). A significant negative correlation ($R = -0.98$; $p < 0.003$) between the levels of HIF-1α and $HbFe^{2+}$ and a positive correlation ($R = 0.97$; $p < 0.0002$) between HIF-1α and $HbFe^{3+}$ clearly establish a relationship between HIF-1α and the oxygenation/oxidation states of Hb (Fig. 4). The following trends were seen between the levels of HO-1 and $HbFe^{2+}$ ($R = -0.94$; $p < 0.005$) and $HbFe^{3+}$ ($R = 0.76$; $p = 0.023$).

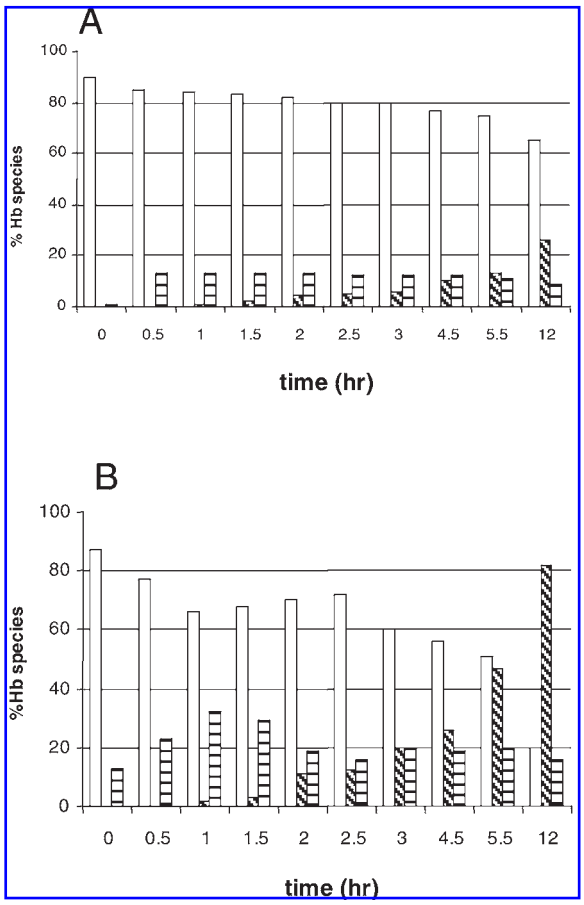

FIG. 3. Time courses of DBBF-Hb oxidation in media of BAECs under normoxic and hypoxic conditions. In each experiment, normoxic or hypoxic BAECs were incubated with 50 μ*M* $HbFe^{2+}$ for 12 h, and spectra were recorded at specified time intervals using a PerkinElmer spectrophotometer. **(A)** Autoxidation of Hb under normoxic conditions. **(B)** Redox transition of Hb in cell cultures subjected to hypoxic conditions. Columns represent $HbFe^{2+}$ (empty), $HbFe^{3+}$ (slanted), and $HbFe^{4+}$ (barred).

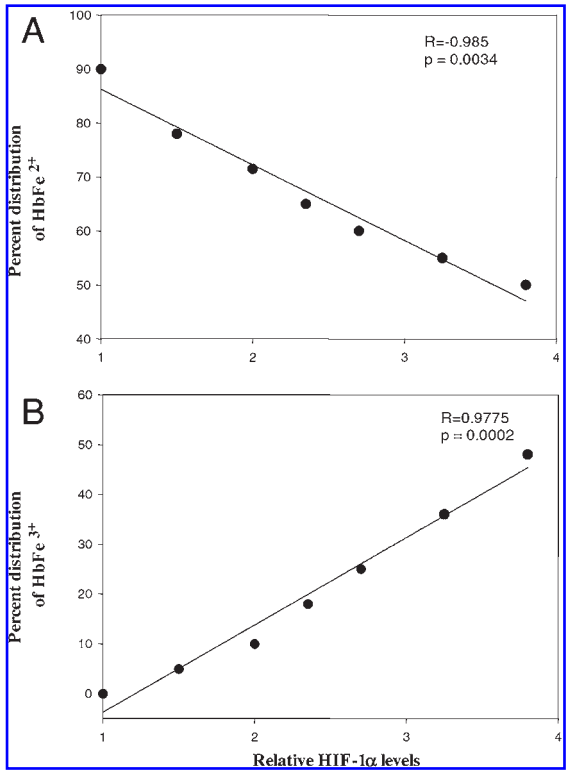

FIG. 4. Correlation between Hb oxygenation and oxidation states and relative values of HIF-1α expressed in BAECs subjected to hypoxia. (A) Correlation between the disappearance of $HbFe^{2+}$ with the relative levels of HIF-1α in BAECs. **(B)** Correlation between formation of $HbFe^{3+}$ and the levels of HIF-1α in BAECs. The data represent a replot of the combined data in Figs. 2A and 3B.

Figure 5A shows the levels of $O_2^{\bullet -}$ produced by these BAECs as detected by the luminescence method under normoxic and hypoxic conditions. The levels of $O_2^{\bullet -}$ reached a maximum at 1–2 h after introduction of hypoxia. This, interestingly, parallels the increases seen with the ferryl species ($HbFe^{4+}$), which also reached a maximum in the medium of these hypoxic cells in approximately the same time frame (Fig. 3B). Adding the enzyme SOD (scavenger of $O_2^{\bullet -}$) to the hypoxic cells reduced the $O_2^{\bullet -}$ levels to that found under normoxic conditions (Fig. 5A).

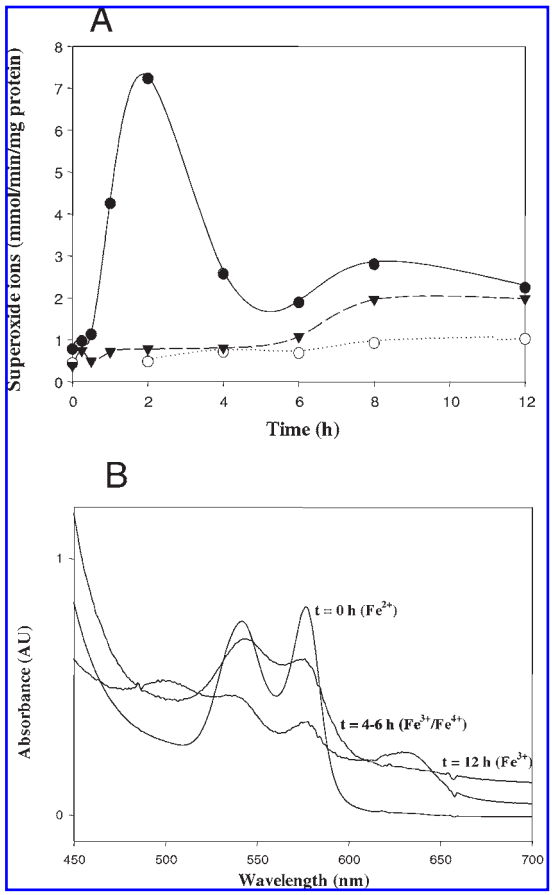

FIG. 5. Time courses of intracellular $O_2^{\cdot -}$ formation and detection of $HbFe^{4+}$ intermediate in BAECs subjected to hypoxia. (A) Time course of intracellular $O_2^{\cdot -}$ generation under normoxic and hypoxic conditions and in the presence of SOD (100 U). Cells were exposed under hypoxic conditions for a given time period, washed, and harvested in ice cold phophate-buffered saline. After centrifugation, cells were resuspended in superoxide incubation buffer. $O_2^{\cdot -}$ was measured in a TopCount Luminescence counter (see Materials and Methods). **(B)** Time-dependent changes in the oxidative states of Hb were monitored spectrophotometrically. Shown as deconvulated spectra of ferrous Hb at $t = 0$ h (normoxia), characterized by absorption bands at 577and 541 nm, and of the ferric/ferryl Hb intermediate ($t = 4$–6 h), which is characterized by absorption bands at 544 and 581 nm, with a flattened region between 600 and 700 nm. The final spectrum is that of the ferric form of Hb. ●, hypoxia; ○, hypoxia and SOD; ▼, normoxia.

Figure 5B shows typical time-dependent changes in the spectral profile of $HbFe^{2+}$ in the cell medium during the 12-h incubation time under hypoxia. Hypoxia induced spectral changes indicative of the transformation of $HbFe^{2+}$ ($t = 0$, normoxia) to the Fe^{4+}/Fe^{3+} oxidation states. The loss of absorption at the 577- and 541-nm bands typical of $HbFe^{2+}$ began immediately, yielding a final spectrum (12 h) characteristic of $HbFe^{3+}$, as witnessed by the appearance of a characteristic absorption peak at 630 nm. Subsequent spectral analysis revealed the presence of spectral intermediates characteristic of both $HbFe^{4+}$ and $HbFe^{3+}$ species at approximately the midpoint (4–6 h) of the incubation time. The presence of $HbFe^{4+}$ was further confirmed via its derivatization to sulfHb using Na_2S (24). Detection of sulfHb formation via reaction of ferryl Hb was measured at 620 nm (data not shown).

In subsequent experiments, 12 h of hypoxia was chosen to investigate the effects of Hb and its oxygenation and/or oxidation states on BAECs in the presence and absence of NO.

HIF-1α accumulation in the presence of DBBF-Hb and NO

We examined the effects of the Fe^{2+} and the Fe^{3+} forms of Hb on HIF-1α expression in cells under normoxic and hypoxic conditions. To study the effects of NO on the HIF-1α accumulation, we used DETA/NO, a slow NO releaser with a half-life of >20 h at 37°C. Figure 6 shows the relative HIF-1α levels expressed in cells after 12 h of hypoxia in the presence or absence of NO using either $HbFe^{2+}$ or $HbFe^{3+}$. In the presence of hypoxia, there was an approximately four-fold increase in levels of HIF-1α as compared with control samples ($p < 0.01$), consistent with previous observations (33). Incubation of either $HbFe^{2+}$ or $HbFe^{3+}$ with BAECs for 12 h induced smaller, but significant, changes in HIF-1α expression compared with its respective controls under normoxic conditions ($p < 0.01$). Under these conditions, HIF-1α levels remained approximately the same, regardless of the oxidation state of Hb. Hypoxia induced a slight increase in the expression of HIF-1α in the presence $HbFe^{2+}$. However, there was a significant increase in the expression of HIF-1α in cells subjected to hypoxia in the presence of $HbFe^{3+}$ when compared with normoxia ($p < 0.01$) and hypoxia in the presence of $HbFe^{2+}$ ($p < 0.05$). Interestingly, the HIF-1α expression in

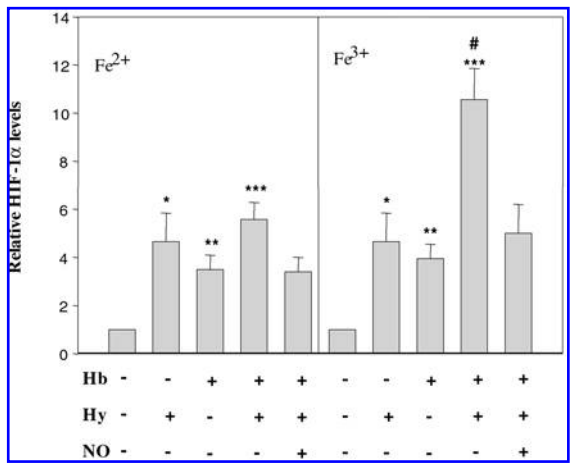

FIG. 6. Effects of ferrous or ferric forms of DBBF-Hb and the NO donor (DETA/NO) on hypoxia-induced expression of HIF-1α. BAECs were exposed to either normoxia (control) or 12 h of hypoxia (Hy) in the presence or absence of 50 μ*M* $HbFe^{2+}$ **(left panel)** or 50 μ*M* $HbFe^{3+}$ **(right panel)** with or without 100 μ*M* DETA/NO. HIF-1α expression was determined by western blot and quantitated as described in Materials and Methods. Each column represents the mean ± SD of three to five experiments performed independently. *$p <$ 0.01 versus control; **$p < 0.01$ versus control; ***$p < 0.01$ versus control; #$p < 0.05$ versus hypoxia with either ferrous or ferric Hb.

BAECs decreased by 1.5-fold and twofold in $HbFe^{2+}$ and $HbFe^{3+}$ solutions, respectively, in the presence of 100 μ*M* NO donor (DETA/NO) under hypoxic conditions. As a control for these experiments, we incubated DETA/NO with hypoxic and normoxic BAECs in the absence of Hb solutions and observed a 30% reduction in the HIF-1α expression under hypoxic conditions, but little or no change under normoxia, consistent with previous reports (33) (data not shown).

HO-1 accumulation in the presence of DBBF-Hb and NO

The relative HO-1 levels expressed in BAECs after 12 h of hypoxia in the presence or absence of DETA/NO using either $HbFe^{2+}$ or $HbFe^{3+}$ are shown in Fig. 7. Introducing hypoxia led to three- to fourfold increases in the levels of HO-1 in BAECs, consistent with earlier observations under similar experimental conditions (28). Incubation of the Fe^{2+} or Fe^{3+} forms of Hb with BAECs for 12 h induced an approximately twofold increase in HO-1 expression compared with their respective controls under normoxic conditions ($p < 0.05$) (28). Combining hypoxia with $HbFe^{2+}$ produced a twofold increase in HO-1 in contrast to a fivefold increase in HO-1 when $HbFe^{3+}$ was used ($p < 0.01$). Unlike HIF responses, however, the HO-1 expressions in BAECs did not respond significantly to the addition of NO (Fig. 7).

Both the expression of HIF-1α and its transcriptional activity are modulated by the cellular O_2 concentration. In both cases, there is evidence that the O_2 signal is converted to a redox signal, although the details remain far from clear. A variety of mechanisms have been proposed involving either increased or decreased ROS generation and either direct ROS effects on HIF-1α or ROS-initiated signal transduction pathway leading to altered phosphorylation of HIF-1α (40). Regardless of the mechanisms, ROS seem to be key players: either they inhibit the HIF activation pathway or they activate it (41).

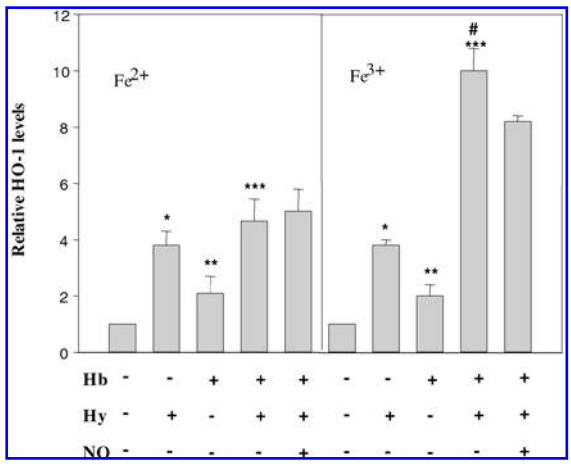

FIG. 7. Effects of ferrous or ferric forms of DBBF-Hb and the NO donor (DETA/NO) on hypoxia-induced expression of HO-1. BAECs were exposed to either normoxia (control) or 12 h of hypoxia (Hy) in the presence or absence of 50 μ*M* $HbFe^{2+}$ **(left panel)** or 50 μ*M* $HbFe^{3+}$ **(right panel)** with or without 100 μ*M* DETA/NO. HO-1 expression was determined by western blot and quantitated as described in Materials and Methods. Each column represents the mean ± SD of three to five experiments performed independently. *$p < 0.01$ versus control; **$p < 0.05$ versus control; ***$p < 0.01$ versus control; #$p < 0.05$ versus hypoxia with either ferrous or ferric Hb. $p < 0.05$ versus control is considered significant.

DISCUSSION

Under hypoxic conditions (1–3% O_2), the pO_2 was reported to be between 7 and 21 mm Hg (8). In the present study, the pO_2 measured in the media by an oxygen electrode under our experimental conditions was ~2 mm Hg. Little or no change was observed throughout the 12-h incubation time. This value is close to previously reported values using the same incubator chamber (26). Oxygen transport by extracellular Hbs takes place through diffusion of oxygen chemically bound as oxyHb ($HbFe^{2+}$). This mechanism is known to augment oxygen transport due to simple diffusion of dissolved oxygen (11). To confirm the role of the ferrous iron in oxygen transport, we incubated $HbFe^{2+}$ and its nonfunctional derivative, CNmet-Hb, with endothelial cells subjected to hypoxia. Data reported in Fig. 1 show that $HbFe^{2+}$ with a low oxygen affinity (large P_{50}) clearly contributes to tissue oxygen content under these hypoxic conditions. This is in keeping with recent experimental and theoretical calculations of oxygen transport by extracellular Hb solutions (11, 44). At oxygen tensions lower than 30 mm Hg, cell-free Hb was shown to exhibit higher effective oxygen diffusion coefficients than blood (11).

Our results show time-dependent increases in the expression of HIF-1α and in the stress protein, HO-1, in BAECs under hypoxia. Hypoxic induction of all of these diverse genes appears to depend on a common mode of oxygen sensing and signal transduction, triggering the activation of a critical transcription factor, HIF-1α.

Coincubating $HbFe^{2+}$ (98%, in heme) with BAECs subjected to hypoxia induced time-dependent increases in the level of HIF-1α expression. Under these conditions, the rate of Hb oxidation (*i.e.*, $HbFe^{2+}$ decay) was three times faster than that seen under normoxic conditions (0.064 h^{-1} versus 0.017 h^{-1}). The rate of $HbFe^{3+}$ accumulation was also three times faster under hypoxic conditions (0.073 h^{-1} versus 0.022 h^{-1}) (Fig. 3). This rapid buildup of nonfunctional $HbFe^{3+}$ under hypoxia reached almost 50% of the total functional heme in the first 5–6 h. Combining the data reported in Figs. 2 and 3 as represented in Fig. 4 reveals that time-dependent increases in HIF-1α under hypoxia are correlated linearly with the oxygenation/oxidation status of DBBF-Hb. HIF-1α expression in these cells was found to be inversely proportional to Fe^{2+} disappearance ($R = -0.98$; $p < 0.003$), but was correlated positively with the appearance of $HbFe^{3+}$ ($R = 0.97$; $p < 0.002$) (Fig. 4). Co-incubation of $HbFe^{3+}$ with BAECs under hypoxia led to substantial increases in the expression of HIF-1α compared with that of $HbFe^{2+}$ ($p < 0.05$). This difference in the levels of expression may well be attributed to differences in the O_2 content of the $HbFe^{2+}$ as opposed to the non–O_2-carrying $HbFe^{3+}$, and thus establishes the relationship between the cellular expressions of HIF-1α and the

O_2-carrying and redox status of DBBF-Hb under our experimental conditions.

We have previously reported that under similar hypoxic conditions, coincubation of DBBF-Hb and endothelial cells causes transient oxidation of the protein to its higher oxidation state ($HbFe^{4+}$) in a time frame (~2 h) that corresponds closely with H_2O_2 production (~10 μ*M*) and lipid peroxidation by these cells (24). It is quite feasible that intracellular reactive oxygen intermediates (*i.e.*, $O_2^{\bullet -}$ and H_2O_2) required for the oxidative modification of HIF-1α and its subsequent degradation by proteasome have been scavenged by DBBF-Hb. This hypothesis is supported by the following: (A) The time-dependent changes in the oxidative state of Hb are rapid as seen in Fig. 5B. The oxidation of DBBF-Hb, presumably by endogenous endothelial ROS sources, began immediately with a burst in the Fe^{4+} levels that peaked at ~30% of the total heme in the first 1–2 h, consistent with our earlier observations (24). These increases in the Hb hypervalent iron corresponded with increases in intracellular $O_2^{\bullet -}$, which can be dismutated readily to H_2O_2. (B) Hypoxia-induced $O_2^{\bullet -}$ production was inhibited by pretreating these cells with SOD, an $O_2^{\bullet -}$ radical scavenger that does not cross intact endothelial cell membranes or intercellular junctions (49). In contrast, SOD treatment of controls did not significantly affect their intracellular ROS levels.

$O_2^{\bullet -}$, as well as other ROS, in particular, is considered a potential signaling molecule. This is based on the fact that it can be produced by a cell when stimulated to do so, have an action in a cell, either the cell that produces it or a nearby cell, and be removed in order to turn off, or reverse, the signal (8, 17). Clearly $O_2^{\bullet -}$ detected under our hypoxic conditions fulfills these criteria. However, the contribution of other oxidants (*i.e.*, lipid peroxide, and $ONOO^-$) to the oxidation of Hb and/or cell signaling pathways has yet to be determined.

Stimulation of HO-1 expression by most, if not all, inducers is controlled primarily at the level of gene transcription (21). HIF-mediated transcriptional activation of the HO-1 gene in response to hypoxia was demonstrated in a number of model systems (15, 21, 47). In this study, we observed as expected an activation of HO-1 protein in BAECs in response to hypoxia. In the presence of hypoxia, co-incubation of $HbFe^{3+}$, but not DBBF-$HbFe^{2+}$, caused a substantial increase in the expression of HO-1 in cells. This confirms earlier observations that $HbFe^{3+}$ is more susceptible to the loss of its heme than $HbFe^{2+}$ (7). Intriguingly, unlike HIF-1α, the incubation of DETA/NO with the Fe^{2+} or the Fe^{3+} forms of DBBF-Hb produces little or no change in the expression level of HO-1. Again the rapid redox transformation by NO of heme centers in Hb to ferric or ferric-NO complex compounds may have contributed to the pool of Fe^{3+} heme in the medium (2).

HIF-1α activation can be modulated by various factors, including NO (40, 41). NO has been shown to induce HIF-1α under nonhypoxic conditions, but inhibits hypoxia-induced HIF-1α expression (43). NO released from short- and long-lived NO donors induced variable degrees of HIF expression in proximal tubular LLC-PK1 cells (50) and HO-1 expression in BAECs (28). DETA/NO was shown to evoke a delayed HIF response that lasted 12–14 h (33). Recent studies showed that DETA-NO caused a marked 52 and 70–74% complex 1 inhibition, suggesting that NO suppresses the electron mitochondrial transport in mammalian cells (1). In our experiments, coincubation of DETA/NO with the cultured media containing either the Fe^{2+} or the Fe^{3+} forms of DBBF-Hb under hypoxia produced ~ 50% reduction in the levels of HIF. In addition, both $HbFe^{2+}$ and $HbFe^{3+}$ are known to react with NO. NO also reacts with the Fe^{4+} form of Hb, reducing it back to Fe^{3+} heme. When $HbFe^{3+}$ (~100%) was incubated with these hypoxic cells, a nearly 50:50 (Fe^{3+}/ Fe^{4+}) heme ratio existed in the solution after 12 h of incubation. This ratio was altered in the presence of DETA/NO to 83:17 (Fe^{3+}/Fe^{4+}) heme ratio, confirming the antiferryl activity of NO. The interaction of NO with $O_2^{\bullet -}$ resulting in the formation of $ONOO^-$ (9) is another possible mechanism by which NO could modulate cellular responses to hypoxia.

In summary, we observed increases in HO-1 expression in BAECs exposed to hypoxia, consistent with the notion that hypoxic induction of this protein is dependent on HIF-1α, a key component of an "oxygen sensing" mechanism (42). During hypoxic stress, HIF-1α transcription factor, which regulates the metabolic adaptation of oxygen, is activated and phosphorylated. Introduction of cell-free Hb and hypoxia alters the balance between ROS and reactive nitrogen species. Hb, in particular DBBF-Hb, reacts avidly with these species, leading subsequently to the loss of its function and its transformation into various oxidation states, including the ferryl form. DBBF-Hb, as we demonstrated here, induces changes in the levels of these proteins, the extent of which is dependent more strongly on the redox rather than oxygen-carrying state of Hb. Various modified Hbs, developed as oxygen-carrying blood substitutes with diverse oxygen-carrying and redox activities, may differentially modulate cell-signaling pathways (48). HIF expression may therefore be used to assess the oxygen-carrying and redox properties of these Hbs in a simple endothelial cell model system (4).

ABBREVIATIONS

BAECs, bovine aortic endothelial cells; BCA, bicinchoninic acid; CNmet-Hb, the cyanomet form of DBBF-Hb; DBBF-Hb, diaspirin cross-linked hemoglobin; DETA/NO, diethylenetriamine nitric oxide adduct; Hb, hemoglobin; $HbFe^{2+}$, oxy/ferrous form of DBBF-Hb; $HbFe^{3+}$, the ferric form of DBBF-Hb; $HbFe^{4+}$, the ferryl form of DBBF-Hb; HIF-1α, hypoxia-inducible factor–1α; HO-1, heme oxygenase –1; H_2O_2, hydrogen peroxide; NO, nitric oxide; $O_2^{\bullet -}$, superoxide ion; $ONOO^-$, peroxynitrite; P_{50}, O_2 affinity of hemoglobin; pO_2, oxygen partial pressure; ROS, reactive oxygen species; SOD, superoxide dismutase.

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