Erythrocytes Induce Proinflammatory Endothelial Activation in Hypoxia

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Although exposure to ambient hypoxia is known to cause proinflammatory vascular responses, the mechanisms initiating these responses are not understood. We tested the hypothesis that in systemic hypoxia, erythrocyte-derived H₂O₂ induces proinflammatory gene transcription in vascular endothelium. We exposed mice or isolated, perfused murine lungs to 4 hours of hypoxia (8% O₂). Leukocyte counts increased in the bronchoalveolar lavage. The expression of leukocyte adhesion receptors, reactive oxygen species, and protein tyrosine phosphorylation increased in freshly recovered lung endothelial cells (FLECs). These effects were inhibited by extracellular catalase and by the removal of erythrocytes, indicating that the responses were attributable to erythrocyte-derived H₂O₂. Concomitant nuclear translocation of the p65 subunit of NF- κ B and hypoxia-inducible factor-1 α stabilization in FLECs occurred only in the presence of erythrocytes. Hemoglobin binding to the erythrocyte membrane protein, band 3, induced the release of H₂O₂ from erythrocytes and the p65 translocation in FLECs. These data indicate for the first time, to our knowledge, that erythrocytes are responsible for endothelial transcriptional responses in hypoxia.

Keywords: hypoxia; erythrocytes; endothelium; lung; inflammation

Systemic hypoxia, which is characterized by a decrease in the partial pressure of oxygen in blood (P_{O₂}), results from a lack of oxygen in inhaled breath, or from impaired blood oxygenation because of lung disease. The circulatory response to a decrease of P_{O₂} is best characterized by pulmonary arterial vasoconstriction, a protective strategy that redistributes the pulmonary blood flow from hypoxic to well-ventilated regions of the lung. An additional vascular effect may involve a hypoxia-induced innate immune response, characterized by leukocyte activation and tissue injury (1). However, this effect remains controversial. Support for the immune effect derives from evidence in animal models that ambient hypoxia causes lung injury (2–5), systemic inflammation (4, 5), and vascular leakage (2, 6). A 4-hour exposure to 8% O₂ in mice activates NF- κ B in astrocytes and hepatocytes (7), suggesting that hypoxia-induced proinflammatory gene transcription occurs in these cells.

The evidence opposing the hypoxic immune response comes from lung lymph flow studies in adult sheep. According to these studies, ambient hypoxia does not increase lung microvascular permeability to proteins, and it does not cause pulmonary edema

CLINICAL RELEVANCE

Hypoxia is a common outcome of lung disease. However, to what extent hypoxia itself causes lung inflammation, thereby exacerbating lung disease, remains unclear. We show that lung inflammation occurs during hypoxia, and that erythrocytes play a role in the response. This study will lead to new understandings and, possibly, new erythrocyte-based therapy for lung disease.

(8, 9). The clinical evidence for the hypoxic immune effect is mixed, and is based on studies of high-altitude pulmonary edema (HAPE), a form of lung injury that follows exposure to hypoxia at high altitude. Radioactive tracer studies in patients with HAPE confirm the sheep data insofar as lung microvascular permeability does not increase (10). Several studies indicate that the bronchoalveolar lavage (BAL) of patients with HAPE is enriched in leukocytes, proteins, and proinflammatory factors, indicating that HAPE causes lung inflammation (11–13). However, this inflammatory response has not been confirmed by all investigators (14, 15), and when present, it may not be sufficiently severe to account for the pulmonary edema of acute lung injury (12).

To the extent that the innate immune response to hypoxia has been reported in animal models and humans, its underlying mechanisms must be understood. Here, we consider the release of H₂O₂ from erythrocytes as a possible mechanism. In hypoxic erythrocytes, the partial deoxygenation of hemoglobin (Hb) dramatically increases the rate of autoxidation in the oxygenated domains of Hb, resulting in the formation of superoxide and H₂O₂ (16, 17). A fraction of the deoxy-Hb binds the erythrocyte membrane protein, band 3 (18, 19). H₂O₂ formed at this membrane site escapes antioxidants in the erythrocyte cytosol, and is released into the extracellular space (16). The extracellular H₂O₂ can diffuse back into the erythrocyte and undergo hydrolysis by catalase in the erythrocyte cytosol. However, because erythrocytes flow in close contact with the endothelial lining of capillaries, the released H₂O₂ also diffuses to the endothelial cytosol and is detectable as an increase of oxidant-sensitive fluorescence in the microvascular endothelium (20). Consequently, endothelial Ca²⁺ increases and activates the expression of the leukocyte adhesion receptor, P-selectin, resulting in leukocyte recruitment.

Although these findings indicate that hypoxic erythrocytes are capable of inducing acute proinflammatory effects, it remains unclear whether the erythrocyte effect is sufficiently robust to activate transcriptional responses (such as the expression of E-selectin) that may require a more sustained inflammatory response. Whether these erythrocyte effects are severe enough to cause pulmonary edema also remains unclear. To address these issues, we determined hypoxic responses in freshly recovered lung endothelial cells (FLECs) that reflect endothelial

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responses *in situ* (21). Our findings indicate that erythrocytes are critical for hypoxia-induced NF- κ B activation in lung, providing the first evidence, to our knowledge, that hypoxia-induced paracrine effects between erythrocytes and lung endothelium activate proinflammatory responses.

MATERIALS AND METHODS

Animals

Animal protocols were approved by the Institutional Animal Care and Use Committee of Columbia University, and are described in detail in the online supplement.

Endothelial Cell Isolation

FLECs were isolated on ice (21). Briefly, to clear blood, lungs were perfused with 3 ml of 0.1% BSA-containing PBS. Chilled collagenase and trypsin were infused through the pulmonary artery to obtain a blood-free, endothelial cell-enriched left atrial effluent that was suspended in 0.1% BSA-containing PBS.

Flow Cytometry of FLECs and Assessment of Purity

To correct for background variability, we labeled samples in duplicate, using nonimmune isotype-specific antibodies for one aliquot, and specific antibodies for the other. The nonimmune fluorescence provided a background for the sample. Cells were stained with fluorophore-labeled antibodies (Alexa 633, Alexa 488, and FITC) under permeabilized (0.1% Triton-X), or nonpermeabilized conditions to enable intracellular or surface staining, respectively (21). Flow cytometry (FACScalibur; Beckton Dickinson, San Jose, CA) gating conditions were set against isotype-matched and fluorophore-matched nonimmune IgG. To assess the purity of FLECs, the collected cells were stained with antibodies against the endothelial surface markers von Willebrand factor (vWf), Tie2, and CD31. As shown in the online supplement (Figure E1), vascular cells positive for vWf were also positive for Tie-2 and CD31. Based on this phenotype, endothelial cells represented 70% $\pm$ 2% of the total FLECs in all our experiments. To determine the effect of hypoxia on endothelial reactive oxygen species (ROS) production, we exposed isolated perfused lungs (IPLs) to 8% O₂ for 3 hours. We then added the ROS indicator, dichlorodihydrofluorescein diacetate (H₂DCF-DA), to the perfusion. Because H₂DCF-DA is light-sensitive, we maintained dark conditions. Intracellularly, ROS oxidize nonfluorescent H₂DCF-DA to fluorescent 2',7'-dichlorofluorescein (DCF) (20, 26). FLECs were harvested after 1 hour under chilled conditions.

Treatment of Hypoxic Samples

To block cellular processes, we maintained the lungs under chilled conditions after the experimental hypoxic period. In IPL experiments, we stopped lung perfusion and placed the lungs on ice and infused them with chilled albumin-containing PBS through the pulmonary artery. For whole-animal experiments, we removed the lungs inside the hypoxic chamber, and placed them on ice. Tissue homogenizations were performed on ice.

Erythrocyte Treatments

To load erythrocytes with anti-CDB3 monoclonal antibody (mAb), we applied the osmolysis-resealing approach (22) by exposing erythrocytes for 1 hour to hyperosmolar buffer containing the mAb, followed by dialysis against isosmolar buffer. Because protein A specifically ligates IgG, to confirm mAb loading, we incubated the erythrocyte lysate with protein A-agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). We then eluted the proteins bound to the beads with SDS-PAGE sample buffer, and performed immunoblotting for CDB3. To detect Hb binding to CDB3, we exposed erythrocyte lysates to Concanavalin A-Sepharose (25 μ l/mg protein; GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Glycoproteins bound to concanavalin A were eluted with manno-pyranoside (1 M; Sigma-Aldrich, St. Louis, MO) and then immunoblotted for Hb and CDB3.

Statistical Analysis

Experimental data from multiple interventions were compared by ANOVA with the Newman-Keuls correction. Other data were compared according to unpaired *t* test. All data represent the mean $\pm$ SE. Significance was accepted at *P* < 0.05.

RESULTS

Hypoxia Causes Leukocyte Accumulation, but Not Tissue Damage

To determine whether exposure to ambient hypoxia causes leukocyte accumulation and vascular damage, we exposed mice to 8% O₂ for 24 hours. The hypoxic exposure did not increase wet/dry ratios in lungs or other organs (Figure 1A), indicating that no edema, and hence no vascular damage, had occurred. Our findings differ from reports in which similar levels of hypoxic exposure caused pulmonary edema (2, 6). The difference may be attributable to our correction of lung water data to account for the water content in the lung's blood volume (23). This important correction was probably not included in studies that found the opposite result. Despite the absence of pulmonary edema, a marked increase was nonetheless evident in the leukocyte accumulation in the BAL (Figure 1B), confirming that hypoxia induces a proinflammatory response in the lung.

Hypoxia Causes Erythrocyte-Derived H₂O₂ Proinflammatory Effects in the Lung

To determine the role of erythrocytes in the hypoxic proinflammatory response, FLECs were derived from IPLs, because in this preparation, organ perfusion can be established in the presence (ERY⁺) or absence (ERY⁻) of erythrocytes (20). At the end of each lung perfusion, we centrifuged a sample of the perfusate (500 g for 15 min) to confirm the absence of hemolysis. For ERY⁻ perfusions, we removed erythrocytes from murine blood, and then perfused lungs with the erythrocyte-free plasma that we reconstituted in lung perfusion buffer at similar osmotic pressure and osmolarity as for ERY⁺ perfusions. We isolated FLECs (Figure E1) from IPLs exposed to 8% O₂ for 4 hours, and analyzed the FLECs by flow cytometry to detect the leukocyte adhesion molecules, E-selectin and intercellular adhesion molecule-1 (ICAM-1). The dot plots (Figures 2A and 2B) and corresponding histograms (Figures E2C and E2D) revealed increased expressions of E-selectin and ICAM-1 in ERY⁺ perfused

Figure 1. Hypoxia increases alveolar leukocytes. BAL, bronchoalveolar lavage. Swiss Webster mice were exposed to hypoxia (8% O₂ $\times$ 24 hours), as indicated. (A) Extravascular water content of organs. (B) BAL leukocyte counts. Mean $\pm$ SE, *n* = 3 for hypoxia (-), *n* = 4 for hypoxia (+). **P* < 0.05, compared with hypoxia (-).

Figure 2. Hypoxia induces erythrocyte-dependent and superoxide-dependent E-selectin and intercellular adhesion molecule-1 (ICAM-1) expression in freshly recovered lung endothelial cells (FLECs). vWf, von Willebrand factor; ERY, erythrocyte; Hb^{mut}, mutant hemoglobin-expressing mice; N, normoxia; Hy, hypoxia; cat, catalase; WT, wild-type. Isolated blood-perfused lungs (IPLs) of Swiss Webster mice were maintained in 8% O₂ for 4 hours with ERY containing (+) or lacking (-) perfusions. Catalase (17 µg/ml) was added (+) as indicated. FLECs were surface-labeled for the endothelial marker vWf, and either E-selectin or ICAM-1. Flow cytometry plots from single representative experiments indicate (upper right quadrants) cells expressing both the indicated epitopes. The x and y ordinates (gray lines) represent nonimmune, isotype-specific IgG expression. Comparison histograms (corrected for background nonimmune IgG fluorescence) indicate E-selectin and ICAM-1 expression. (A and B) E-selectin. (C and D) ICAM-1. (E) Group data for percent expressions (upper right quadrants). Mean ± SE (n = 4, each bar). *P < 0.05, compared with hypoxia (-). (F) Group data for E-selectin expression in FLEC from isolated perfused lungs (IPLs) of Hb^{mut} mice. Mean ± SE (n = 3, each bar). *P < 0.05, compared with left bar. #P < 0.05, compared with hypoxia, ERY (+).

lungs, but not in lungs receiving ERY⁻ perfusion, or ERY⁺ perfusions containing extracellular catalase (ERY⁺ + CAT). These differences (highlighted for single experiments in the comparative histograms in Figures 2C and 2D) were statistically significant for the group as a whole (Figure 2E). We confirmed that ERY⁻ perfusions contained no erythrocytes (Figure E3). Because increases of E-selectin and ICAM-1 are transcriptionally regulated, and because the expressions of these receptors were blocked by catalase, we conclude that hypoxia induced proinflammatory transcription, and that the effect was attributable to erythrocyte-derived H₂O₂.

The role of H₂O₂ in E-selectin expression was examined further in the lungs of mice (Hb^{mut}) expressing an Hb mutation that causes Hb instability and decreased oxygen affinity to Hb (24, 25), thereby increasing hypoxia-induced Hb autooxidation and H₂O₂ production (16, 17). Our previous findings indicate that in lungs of Hb^{mut} mice, hypoxia increases erythrocyte-endothelial H₂O₂ transfer as well as Ca²⁺-dependent endothelial P-selectin expression (20). Here, we exposed IPLs of Hb^{mut} mice to hypoxia while perfusing the lungs with Hb^{mut} or wild-type (WT) erythrocytes. In FLECs derived from these lungs, the hypoxia-induced enhancement of E-selectin was greater for Hb^{mut} than for WT erythrocytes (Figure 2F). The removal of Hb^{mut} erythrocytes from the lung perfusion blocked the effect, affirming that erythrocytes were responsible for the E-selectin expression.

To confirm that these responses were capable of generating leukocyte accumulation in lung tissue, we compared BAL responses

in intact mice and in IPLs. To enrich the IPL perfusions with leukocytes and platelets, we isolated these cells from the buffy-coat fraction of whole blood, and added them to the lung perfusions. A 4-hour exposure to 8% O₂ increased BAL leukocyte counts to similar extents in mice (Figure 3A) and IPLs (Figure 3B), affirming that the hypoxic inflammatory response was similar in the intact and isolated lungs. ERY⁻ or ERY⁺ + CAT perfusions completely blocked the IPL inflammatory response (Figure 3B), indicating that leukocyte recruitment was erythrocyte-dependent.

Figure 3. Hypoxia induces erythrocyte-dependent and superoxide-dependent alveolar leukocyte recruitment. BAL, bronchoalveolar lavage. Swiss Webster mice or IPLs were maintained in 8% oxygen (4 hours). Leukocytes were counted in the BAL. (A) Counts from mice. (B) Counts from IPLs. Mean ± SE (n = 4). *P < 0.05, compared with hypoxia (-).

Erythrocyte H₂O₂ Diffuses to Lung Endothelium

To quantify directly the transfer of erythrocyte-derived H₂O₂ to the lung endothelium, we added DCF to IPL perfusions. After 1 hour, we performed flow cytometry on FLECs derived from the IPLs. Figure 4A shows dot plots from single experiments. In a normoxic IPL with ERY⁺ perfusion, DCF fluorescence was relatively low, although it was higher than the autofluorescence, indicating low levels of endothelial ROS production (20). By contrast, in hypoxic IPLs, DCF fluorescence increased in the presence of ERY⁺ but not of ERY⁻ perfusion, indicating that erythrocytes determined the response. The inclusion of catalase in ERY⁺ perfusion blocked the hypoxia-induced increase of DCF fluorescence, indicating that extracellular H₂O₂ was responsible for the effect. The corresponding histograms (Figures E4A ad E4B) and group data (Figure 4B) support these findings. These data affirm our interpretation that 4 hours of hypoxia caused the transfer of erythrocyte-derived H₂O₂ to the lung endothelium.

Erythrocyte H₂O₂ Causes FLEC Tyrosine Phosphorylation

Our next objective was to determine the extent to which H₂O₂ diffusion from erythrocytes caused endothelial activation, as indicated by increased protein tyrosine phosphorylation (TyrP), which is a known effect of H₂O₂ (27). In FLECs derived from mice exposed to hypoxia for up to 24 hours, major increases in TyrP occurred during the first 4 hours, accounting for 70% of the increase seen at 24 hours (Figures 5A and E5A). In FLECs derived from IPLs, 4 hours of hypoxia also increased TyrP (Figures 5B and E5B), confirming that IPL responses were similar to those for mice. The IPL data also showed that the TyrP

Figure 4. Erythrocytes increase lung endothelial superoxide production in hypoxia. H₂DCFDA, dichlorodihydrofluorescein diacetate, DCF, 2',7'-dichlorofluorescein; vWf, von Willebrand factor; ERY, erythrocytes; RBC, erythrocytes. IPLs of Swiss Webster mice were maintained in 8% O₂ (3 hours), using ERY (+) or (-) perfusions. The light-sensitive reactive oxygen species (ROS) indicator, H₂DCFDA (20 μ M), was added to the blood reservoir in darkness, and hypoxia was maintained for another hour. Catalase (17 μ g/ml) was added (+), as indicated. FLECs were surface-labeled for the endothelial marker vWf. Flow cytometry plots are based on single representative experiments. The x ordinates (gray lines) indicate labeling with nonimmune IgG, isotype-specific for anti-vWf antibody. The y ordinates indicate autofluorescence at DCF settings in FLECs from H₂DCFDA-untreated IPLs. (A) vWf and DCF. (B) Group data for mean intensity of DCF fluorescence above autofluorescence in vWf (+) FLECs (upper right quadrant of plots). Mean $\pm$ SE ($n = 4$). * $P < 0.05$, compared with bar on left.

response began within 15 minutes of hypoxic exposure. Moreover, in the IPL studies, ERY⁻ or ERY⁺ + CAT perfusion eliminated the TyrP response (Figures 5C and E5C), indicating that erythrocytes released sufficient amounts of H₂O₂ in hypoxia to induce endothelial TyrP. Taking the IPL and murine data together, we interpret that TyrP developed rapidly and was sustained for 24 hours of hypoxia.

Erythrocytes Activate Transcription Factors

Because the up-regulation of E-selectin and ICAM-1 indicated that proinflammatory genes were induced, we considered the role of NF- κ B in these responses. In FLECs derived from IPLs, hypoxic exposure induced the nuclear translocation of p65, confirming NF- κ B activation (Figure 6A). This effect was blocked by ERY⁻ or ERY⁺ + CAT perfusion (Figure 6A), indicating that erythrocytes and H₂O₂ were responsible for the effect. The well-reported hypoxia-induced stabilization of hypoxia inducible factor-1 α (HIF1 α) in nuclear fractions (29) was also evident here for ERY⁺ perfusion, but blocked for ERY⁻ or ERY⁺ + CAT perfusion (Figure 6B), indicating for the first time that erythrocyte-derived H₂O₂ contributes to the hypoxic HIF1 α response.

To determine whether these IPL responses could be replicated *in vivo*, we exposed mice to 8% oxygen for 4 hours. In FLECs from WT mice, hypoxia caused the nuclear translocation of p65 (Figure 6C, lanes 5 and 6), confirming that the p65 responses *in vivo* and in the IPL were similar. The hypoxia-induced p65 translocation was inhibited in mice overexpressing the H₂O₂ inhibitor catalase (28) (Figure 6C, lanes 2 and 4), indicating that H₂O₂ played a critical role in the response. In mice expressing endothelial-specific heterozygous mutations on Ser³² and Ser³⁶ of the negative regulator of NF- κ B ($I\kappa B\alpha$ ^{+/-}) that block NF- κ B activation (30), hypoxia did not cause nuclear p65 translocation (Figure 6C, lanes 7 and 8), affirming that NF- κ B was activated in hypoxia. Further, in FLECs derived from $I\kappa B\alpha$ ^{+/-} mice, HIF1 α stabilization in hypoxia was absent (Figure 6D), and the hypoxia-induced increase of E-selectin expression was markedly blunted (Figures 6E, 6F, E6A, and E6B). Taking these results together, we conclude that 4 hours of hypoxia generated sufficient erythrocyte-derived H₂O₂ to activate endothelial NF- κ B.

Hb Binding to Erythrocyte Band 3 Causes Inflammatory Responses

Despite the presence of cytosolic antioxidants, hypoxic erythrocytes have been thought to release H₂O₂ as a consequence of H₂O₂ formation at the erythrocyte membrane where deoxy-Hb binds band 3 (18, 19). To affirm the role of erythrocyte band 3 in these responses, we applied the osmotic lysis-resealing approach to load erythrocytes with the mAb that recognizes the cytoplasmic domain of band 3 (CDB3), thereby specifically blocking ligand interactions with band 3 (22). Immunoblots of CDB3, using the protein A approach to pull down bound mAb, confirmed that we were successful in loading erythrocytes with the anti-CDB3 mAb (Figure 7A). We perfused IPLs with untreated or mAb-loaded erythrocytes. We then immunoblotted concanavalin A-bound membrane fractions of erythrocytes recovered from the lung perfusion buffer. As expected, hypoxia induced membrane Hb binding in untreated erythrocytes (Figure 7B). By contrast, no membrane Hb binding was evident in erythrocytes containing anti-CDB3 mAb (Figure 7B), indicating that the mAb blocked the binding. Further, in FLECs from hypoxic lungs perfused with mAb-loaded erythrocytes, the nuclear translocation of p65 was blocked (Figure 7C). These findings

Figure 5. Hypoxia induces erythrocyte-mediated lung endothelial tyrosine phosphorylation (TyrP). vWf, von Willebrand factor. Swiss Webster mice or IPLs were maintained in 8% oxygen (4 hours). FLECs were fixed, permeabilized, and fluorescently labeled for the endothelial marker vWf and for TyrP. Flow cytometry plots are based on single representative experiments. The *x* and *y* ordinates (gray lines) indicate nonimmune, isotype-specific IgG labeling. Group data (bars) represent means of fluorescence intensities above background in the *top right quadrants* of dot plots (TyrP⁺/vWf⁺ cells). (A) Responses in FLECs from mice exposed to hypoxia for 4 hours or 24 hours. (B) Responses in FLECs from IPLs in hypoxia for 15 minutes and 4 hours. (C) Responses in FLECs from IPLs in hypoxia for 4 hours under the indicated conditions. Mean $\pm$ SE ($n = 4$). * $P < 0.05$, compared with hypoxia (–).

indicate that deoxy-Hb binding to erythrocyte band 3 was critical for hypoxia-induced endothelial NF- κ B activation.

DISCUSSION

Our findings indicate that the exposure of lung tissue to 4 hours of hypoxia induced the nuclear translocation of the p65 subunit of NF- κ B and the up-regulation of the endothelial leukocyte adhesion receptors E-selectin and ICAM-1. These results confirm previous findings that hypoxia activates NF- κ B (7). However, to the extent that all of these effects were blocked in the absence of erythrocytes, we believe this is the first evidence that erythrocyte-derived mechanisms were responsible for the proinflammatory transcriptional responses.

A mechanistic understanding of the erythrocyte effect was developed through a consideration of the role of Hb binding to erythrocyte membrane protein, band 3 (32). Although band 3 has multiple erythrocyte functions, including bicarbonate-chloride exchange and cytoskeletal stability (33), the N-terminal

cytoplasmic region of band 3 is the site for the competitive binding of deoxy-Hb and several glycolytic enzymes (22, 31, 34). In hypoxia, band 3 was shown to sequester a pool of partly deoxygenated Hb at the erythrocyte membrane (18). Because Hb autooxidation releases H₂O₂ from deoxy-Hb (18), the membrane-sequestered deoxy-Hb in hypoxia is a potential source of the erythrocyte-derived H₂O₂ that is released into the extracellular space.

To test this hypothesis, we needed to detect the transfer of erythrocyte-derived H₂O₂ to the endothelium. Previously, we achieved this by fluorescence microscopy of single microvessels (20). Here, we took an approach involving FLECs to determine the effect at a whole-organ level (21). In this approach, because endothelial cells are freshly recovered under cooled conditions, *in situ* responses are preserved. Using our new flow cytometry-based DCF approach, we found that hypoxia, but not normoxia, caused an erythrocyte-dependent, catalase-inhibitable increase of DCF fluorescence in FLECs. These findings indicate that in hypoxia, H₂O₂ was transferred from erythrocytes to the endothelium.

Figure 6. Hypoxia induces superoxide-dependent NF-κB nuclear translocation, HIF1α stabilization, and E-selectin expression. IB, immunoblot; IP, immunoprecipitate; cat, catalase-overexpressing mice; *lkbα^{+/-}*, mice heterozygous for endothelial-specific Ser³² and Ser³⁶ mutations of IκBα; wt, wild-type mice; vWf, von Willebrand factor; HIF1α, hypoxia inducible factor-1. IPLs of Swiss Webster mice or transgenic and WT control mice were maintained in 4% oxygen (4 hours). (A) IB of p65 in nuclear fractions of FLECs from IPLs (*n* = 4). Jurkat cell lysates served as positive controls. Histone deacetylase 1 (HDAC1) bands served as loading controls. (B) IP and IB for HIF1α in nuclear fractions of FLECs (*n* = 2). (C) IB of p65 in nuclear fractions of FLECs from cat, the negative regulator of NF-κBα (*lkbα^{+/-}*), and wt control mice in two from a total of three experiments. (D) IP and IB of HIF1α in nuclear fractions of FLECs from *lkbα^{+/-}* mice and strain-matched WT mice (*n* = 3). (E) Flow cytometry plots from single representative experiments show endothelial marker vWf and E-selectin in surface-labeled FLECs from *lkbα^{+/-}* mice and their wt controls. The x and y ordinates (gray lines) indicate nonimmune, isotype-specific IgG labels. (F) Group data (bars) represent endothelial expressions (upper right quadrants of plots). Mean ± SE (*n* = 4, each bar). **P* < 0.05, compared with wt.

We emphasize that this H₂O₂ transfer probably occurs only under conditions in which close apposition occurs between substantial parts of the erythrocyte surface and the adjoining capillary endothelium. In lung capillaries, erythrocyte-endothelial interactions are extensive. These interactions, in which up to 50% of the erythrocyte surface makes endothelial contact at any given time, could be critical for erythrocyte-endothelial H₂O₂ transfer. Because such intimate interactions may be absent in vessels larger than capillaries, the transfer of erythrocyte H₂O₂ in hypoxia may not occur in large vessels.

To detect erythrocyte-mediated endothelial inflammatory responses, we took advantage of the fact that H₂O₂, through its phosphatase-inhibiting effect, increases protein TyrP (27). Hence we interpreted the extent of TyrP in FLECs as an indicator of the effect of H₂O₂. To the extent that FLEC recovery included nonendothelial cells, we cannot rule out the possibility that endothelial responses may have been underestimated. Nevertheless, hypoxia enhanced FLEC TyrP. Further, this

effect was blocked in the absence of erythrocyte perfusion, as well as by catalase infusion. These findings affirm that FLEC TyrP resulted from the paracrine effects of erythrocyte-derived H₂O₂.

To determine the role of band 3, we loaded erythrocytes with the mAb against CDB3 to block the binding of Hb to band 3 at the deoxy-Hb binding site (22). Confirmation of mAb binding was achieved in immunoblots of band 3, which we immunoprecipitated by the concanavalin A approach to avoid interference with the mAb binding site. Our findings indicate that in hypoxia, perfusion with erythrocytes loaded with the anti-CDB3 mAb blocked Hb binding to the erythrocyte membrane, and inhibited p65 translocation in FLECs. These findings, together with the H₂O₂ release data already discussed, lead us to conclude that endothelial NF-κB activation in hypoxia is initiated by erythrocyte H₂O₂ release. Our findings implicate for the first time, to the best of our knowledge, Hb binding to erythrocyte band 3 as a mechanistic event in the activation of an endothelial transcription factor.

Figure 7. Hypoxia-induced hemoglobin band 3 binding to erythrocyte membranes induces the nuclear translocation of lung endothelial NF- κ B. ERY, erythrocyte; CDB3, cytoplasmic domain of band 3, IB, immunoblot; Hb, hemoglobin; osmo, osmosealed. Isolated murine (SW) lungs exposed to 8% O₂ (4 hours) were perfused with ERY loaded with or lacking anti-CDB3 antibody. HDAC1 bands served as loading controls. (A) IB of CDB3 in protein A bound ERY membranes. (B) IBs of Hb and CDB3 on concavalin A (Con A) bound proteins in lysates of lung perfusate ERYs. (C) IB of p65 in nuclear fractions of FLECs. All immunoblots were replicated three times.

In the classic pathway of NF- κ B activation, inhibitor κ kinase (IKK) causes serine phosphorylation on I κ B, inducing the nuclear translocation of NF- κ B components p65 and p50 (35). The role of H₂O₂ in NF- κ B activation remains less clear, although H₂O₂-induced activation is implicated in a lymphoblast-like cell line, in Jurkat cells, and in HeLa cells (36–38). Because hypoxic NF κ B activation in our experiments was blocked in the *I κ B α ^{+/-}* mouse, which carries a serine mutation, we conclude that the H₂O₂ effect was attributable to serine phosphorylation on I κ B. The mechanisms underlying serine phosphorylation remain unclear. Among the possibilities, H₂O₂ inactivates the serine phosphatase, Src homology 2-containing inositol-5-phosphatase-1, directly causing serine phosphorylation on I κ B (36), or else H₂O₂ activates IKK through the phosphorylation of protein kinase D (37, 38). Both possibilities are potentially applicable here.

Among our new findings, erythrocyte-derived H₂O₂ enhanced hypoxia-induced HIF1 α stabilization in FLECs. HIF1 α is rapidly degraded under normoxia by prolyl hydroxylases (PHDs) (29). Recent evidence suggests the presence of regulatory interactions between the HIF1 α and NF- κ B pathways. The small interfering RNA knockdown of PHDs in HeLa cells activates IKK, and hence probably NF- κ B (39). NF- κ B transcriptionally activates HIF1 α in liver, brain, and macrophages (7). In our present experiments, the hypoxic stabilization of HIF1 α was blocked in *I κ B α ^{+/-}* mice, expressing dysfunctional NF- κ B in endothelium, and supporting the role of the NF- κ B pathway in hypoxia-induced HIF1 α activation. In the absence of erythrocytes, the HIF1 α -stabilizing effect of hypoxia was present as expected. However, as shown in Figure 6D, the presence of erythrocytes enhanced stabilization, indicating that a previously unreported erythrocyte effect augments HIF1 α stabilization in hypoxic lung capillaries.

Although our findings provide definitive evidence for proinflammatory transcriptional and leukocyte recruitment responses in the lung, the clinical and physiologic implications of our findings must be approached with the caveat that these responses were not accompanied by evidence of lung injury. To this extent, our data support previous findings that lung leukocyte accumulation can occur in the absence of overt lung injury (40). Nevertheless, our findings bear relevance to an issue that has received increasing consideration, namely that tissue injury

may be the consequence of multiple proinflammatory factors acting together. We suggest that hypoxia may be one of these factors.

The paracrine effectiveness of erythrocyte-derived H₂O₂ is likely to depend on the extent of contact between erythrocytes and the endothelium. In the absence of intimate contact between the membranes of these cells, H₂O₂ released from the erythrocyte membrane could rapidly diffuse back to the catalase-rich erythrocyte cytosol without inducing paracrine effects on the endothelium. Thus, during studies in which we placed erythrocytes on cultured endothelial monolayers, we failed to detect evidence of hypoxia-induced H₂O₂ transfer to endothelial cells, using dichlorofluorescein or Amplex Red as detectors (our unpublished observations). This lack of transfer probably occurred because contact interactions between erythrocytes and the endothelial monolayer do not replicate the extensive erythrocyte–endothelial membrane interactions occurring *in vivo*. In lung capillaries, more than 50% of the erythrocyte surface makes endothelial contact at any time. Hence, in lung capillaries, sufficient intercellular contact exists to enable H₂O₂ transfer from erythrocytes to the endothelium, providing the basis for the paracrine effects of erythrocytes in lung tissue.

Our findings have some bearing on the increasing understanding that erythrocytes modify vascular function. Erythrocytes have been identified as key players in vasoactivity, acting as O₂-responsive transducers (41), partly by modulating the availability of nitric oxide (42–46). Erythrocyte–blood vessel interactions are implicated in transfusion-related lung inflammation (47–49). Our evidence that erythrocytes play a determining role in the activation of vascular NF- κ B and HIF1 α reveals a relatively unappreciated proinflammatory mechanism that may be more widely prevalent, particularly under conditions that promote deoxy-Hb binding, and hence H₂O₂ formation at the erythrocyte membrane. Therefore, we need to consider the role of erythrocytes as inducers of hypoxic inflammation, and perhaps the roles of other unrecognized conditions that increase erythrocyte-derived H₂O₂ production. Acting alone, or together with other predisposing factors, these mechanisms may underlie the life-threatening pulmonary edema that occurs in humans exposed to high-altitude hypoxia (50).

Author disclosures are available with the text of this article at www.atsjournals.org.

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