Hypoxia-mediated Induction of Endothelial Cell Interleukin-1α

An Autocrine Mechanism Promoting Expression of Leukocyte Adhesion Molecules on the Vessel Surface

R. Shreeniwas, S. Koga, M. Karakurum, D. Pinsky, E. Kaiser, J. Brett, B. A. Wolitzky,*
C. Norton,* J. Plocinski,‡ W. Benjamin,‡ D. K. Burns,* A. Goldstein,§ and D. Stern
*Department of Physiology, Columbia University, College of Physicians and Surgeons, New York 10032; and Departments of *Molecular Genetics, ‡Immunopharmacology, and §Cardiovascular, Hoffmann-LaRoche Inc., Nutley, New Jersey 07110*

Abstract

Tissue injury that accompanies hypoxemia/reoxygenation shares features with the host response in inflammation, suggesting that cytokines, such as IL-1, may act as mediators in this setting. Human endothelial cells (ECs) subjected to hypoxia ($P_{O_2} \approx$ 12–14 Torr) elaborated IL-1 activity into conditioned media in a time-dependent manner; this activity was completely neutralized by an antibody to IL-1α. Production of IL-1 activity by hypoxic ECs was associated with an increase in the level of mRNA for IL-1α, and was followed by induction of endothelial-leukocyte adhesion molecule-1 and enhanced expression of intercellular adhesion molecule-1 (ICAM-1) during reoxygenation. During reoxygenation there was a three- to fivefold increased adherence of leukocytes, partly blocked by antibodies to endothelial-leukocyte adhesion molecule-1 and ICAM-1. Suppressing endothelial-derived IL-1, using either antibodies to IL-1α, specific antisense oligonucleotides or the IL-1 receptor antagonist, decreased leukocyte adherence to reoxygenated ECs, emphasizing the integral role of IL-1 in the adherence phenomenon. Mice subjected to hypoxia ($P_{O_2} \approx$ 30–40 Torr) displayed increased plasma levels of IL-1α, induction of IL-1α mRNA in the lung, and enhanced expression of ICAM-1 in pulmonary tissue compared with normoxic controls. These data suggest that hypoxia is a stimulus which induces EC synthesis and release of IL-1α, resulting in an autocrine enhancement in the expression of adhesion molecules. (*J. Clin. Invest.* 1992. 90:2333–2339.) Key words: hypoxia • endothelium • endothelial-leukocyte adhesion molecule-1 • intercellular adhesion molecule-1 • interleukin-1

Introduction

Recent work has focused attention on the role of leukocytes and other inflammatory cells in the tissue damage which occurs in ischemic syndromes (1–5). Cytokines, such as IL-1 and tumor necrosis factor, play a key role in mediating the host inflammatory response, and are likely candidates to draw leukocytes to loci of hypoxic vascular injury. This response could occur via the increased expression of leukocyte adherence molecules on the surface of endothelial cells (ECs)[1] (6–11), as well as through elaboration of chemoattractant substances (12, 13).

Cultured ECs have been shown to elaborate IL-1, as well as other cytokines, in response to pathophysiologically relevant stimuli such as lipopolysaccharides and thrombin (14–16). This led us to test the hypothesis that hypoxia could induce EC production of IL-1, resulting in autocrine enhancement in the expression of adhesion molecules thereby increasing leukocyte binding to the vessel surface. Our results indicate that hypoxia induces an increase in IL-1 mRNA, and release of active IL-1α by cultured endothelium. IL-1, thus produced, results in induction of endothelial-leukocyte adhesion molecule-1 (ELAM-1) and increased expression of intercellular adhesion molecule-1 (ICAM-1) on ECs. In addition, IL-1α mRNA is induced in the lungs of mice subjected to hypoxia, after which ICAM-1 antigen is expressed at considerably higher levels than in normoxic controls. Taken together, these results support the hypothesis that cytokine production by hypoxic ECs can contribute to the host response in the pathogenesis of ischemic vascular injury.

Methods

Endothelial cell culture and exposure of cells to hypoxia/reoxygenation. Human umbilical vein ECs were prepared from umbilical cords and grown in culture by the method of Jaffe (17) as modified by Thornton (18). Experiments used confluent ECs (passages 1–4) grown in Medium 199 supplemented with fetal bovine serum (10%; Sterile Systems, Logan, UT), recombinant endothelial cell growth factor (8 ng/ml; obtained from Dr. T. Maciag and processed at Hoffmann-La-Roche Inc., Nutley, NJ), heparin (90 μg/ml; Sigma Chemical Co., St. Louis, MO), and antibiotics. For experiments with sense/antisense oligonucleotides for IL-1α (the latter designed to bind nine nucleotides upstream and downstream from the translation initiation codon of IL-1α), as described (19), oligomers were added to EC cultures daily (10 μM, final concentration). When ECs achieved confluence, experiments were performed by placing cultures in an environmental chamber (Coy Laboratory Products, Ann Arbor, MI), which provides a controlled atmosphere containing the indicated amount of oxygen, carbon dioxide (5%), with the balance made up of nitrogen. Use of this chamber for cell culture experiments has been described previously by Ogawa et al. (20). Temperature was maintained at 37°C. Experiments involved exposure of ECs to hypoxia for a maximum of 24 h, during which time oxygen tension in the culture medium was 12–14 Torr and there was no change in medium pH. Reoxygenation was performed by placing ECs in an ambient air atmosphere containing carbon dioxide (5%) at 37°C.

Endothelial cell–leukocyte interaction. The source of leukocytes was either HL60 cells obtained from American Type Culture Collec-

Address correspondence to Dr. R. Shreeniwas or Dr. D. Stern, Department of Physiology, Columbia University, College of Physicians and Surgeons, 630 West 168th Street, New York, NY 10032.

Received for publication 20 April 1992 and in revised form 27 July 1992.

J. Clin. Invest.

0021-9738/92/12/2333/07 $2.00
Volume 90, December 1992, 2333–2339

1. *Abbreviations used in this paper:* EC, endothelial cells; ELAM-1, endothelial-leukocyte adhesion molecule-1; GAPDH, glyceraldehyde-6-phosphate dehydrogenase; ICAM-1, intercellular adhesion molecule-1.

tion, Rockville, MD, and grown in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with fetal calf serum (10%) or peripheral blood leukocytes. For the latter, blood was collected from healthy donors by venipuncture in syringes containing EDTA (5 mM final concentration), diluted 1:1 with NaCl (0.9%), and separated by gradient centrifugation on Ficoll Hypaque (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) as described (21). ECs grown to confluence in 100-mm dishes were either exposed to normoxia, placed in hypoxia ($Po_2 \approx$ 12–14 Torr) or subjected to hypoxia followed by a period of replacement into normoxia (reoxygenation), as indicated. Cultures were then washed once with RPMI 1640 and incubated with either HL60 cells or leukocytes (2×10^6 cells/ml) for 30 min at 25°C in hypoxia or normoxia as indicated. The binding experiment was terminated by washing monolayers twice with cold RPMI 1640 to remove nonadherent cells, and then leukocytes or HL60 cells adherent to ECs were eluted by addition of 1 ml of EDTA (10 mM) for 2 min at 37°C. Under these conditions the EC monolayer remained intact. Detached cells were counted; three separate aliquots per sample, in a hemocytometer, and post hoc comparisons of individual means were performed using the Tukey test for certain binding data. All binding assays were performed in an atmosphere with the indicated oxygen concentration. Where indicated, murine monoclonal antibodies to ELAM-1 (IgG_1, 3B7) and/or ICAM-1 (IgG_1, RR1) (the latter generously provided by Dr. R. Rothlien, Boehringer Ingleheim, CT) were added to EC cultures at a concentration of 10 μg/ml 30 min before reoxygenation. Other agents added to hypoxic/reoxygenated EC cultures included goat anti-IL-1α IgG (2.5 μg/ml), generously provided by Dr. R. Chizzonite (Hoffman-LaRoche Inc.), goat anti–bovine Factor IX IgG (2.5 μg/ml), purified human recombinant IL-1α (generously provided by Dr. P. Lomedico, Hoffmann-LaRoche Inc.), and IL-1RA (200 ng/ml), the latter generously provided by Dr. R. Thompson (Synergen, Boulder, CO).

Western blotting to detect ELAM-1 and ICAM-1 was performed on either hypoxic, normoxic, or reoxygenated ECs (2×10^6 cells) by washing cultures in PBS (Na phosphate, 0.02 M; NaCl, 0.1 M, pH 7.2), scraping cells into suspension, and lysing them in ice cold 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) lysis buffer (CHAPS 0.01 M, NaCl 0.25 M, Tris, 0.05 M, PMSF, 1 mM; final pH 7.2). Samples were prepared for nonreduced SDS-PAGE, subjected to electrophoresis on 5–15% gradient gels (22), and electroblotted onto nitrocellulose membranes (23). Excess sites on the membrane were blocked by incubation overnight in nonfat dry milk (5%), and the migration of ELAM-1 and ICAM-1 was then identified by reacting blots with either anti-ELAM-1 or anti-ICAM-1 antibody (5 μg/ml in each case), and visualizing sites of primary antibody binding with goat anti–mouse IgG coupled to horseradish peroxidase using chemiluminescence (ECL kit; Amersham Corp., Arlington Heights, IL). Positive controls were membrane extracts derived either from COS cells transfected with a full-length cDNA for human ELAM-1 or stably transfected Chinese hamster ovary cells expressing human ICAM-1.

Assays of IL-1. IL-1 activity was detected using the D10 assay (24), murine IL-1α antigen was assessed by radioimmunoassay, and changes in expression of IL-1α mRNA were studied by polymerase chain reaction (PCR). For the activity assay, serum-free supernatants that had been centrifuged and filtered (0.2 μm) to remove cellular debris were incubated with the D10(N4)M cell line (24), and mitogenesis was assessed. The limit of sensitivity in this assay was 2 U/ml. IL-1 activity in samples from hypoxic ECs was neutralized by the IgG fraction derived from a monospecific goat anti–human IL-1α. Murine IL-1α antigen was measured in mouse plasma using a radioimmunoassay kit from Cytokine Sciences, Inc. (Boston, MA) according to the manufacturer's instructions (limit of detection 20–30 pg/ml). For PCR, cultured ECs were exposed to normoxia, hypoxia, or hypoxia/reoxygenation, total RNA was extracted using the acid-guanidinium hydrochloride procedure (Stratagene Inc., La Jolla, CA). Random hexanucleotide primed first strand cDNA was prepared and served as template for PCR analysis. IL-1α primers were those described by Maier et al. (19), and glyceraldehyde-6-phosphate dehydrogenase (GAPDH) primers were 5′ CCA TGG AGA AGG CTG GGG 3′ (sense) and 5′ CAA ATG TGT CAT GGA TGA CC 3′ (antisense). For positive controls, 10 ng of plasmid carrying either IL-1α or GAPDH cDNA sequences were used. cDNA was amplified by PCR for 35 cycles, each cycle consisting of incubations at 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. Products were analyzed by agarose gel electrophoresis (2%) and were visualized with ethidium bromide under ultraviolet light, transferred to nylon membranes and Southern hybridization performed using ^{32}P-labeled cDNA encoding full-length human IL-1α. PCR on samples derived from hypoxic mouse lung tissue was performed as described above, except that primers for murine IL-1α were obtained from Clontech Laboratories, Inc. (Palo Alto, CA). Southern hybridization was similarly performed on these samples using ^{32}P-labeled cDNA encoding full-length mouse IL-1α. After washing, the filter was analyzed in a β-scanner to compare bands in hypoxia (8 and 2 h) and normoxia (Fig. 4 *a*, panel *B*). In control studies, the same cDNA (derived from lung) was amplified with the human GAPDH primers described above, except that the primer annealing temperature was dropped to 50°C.

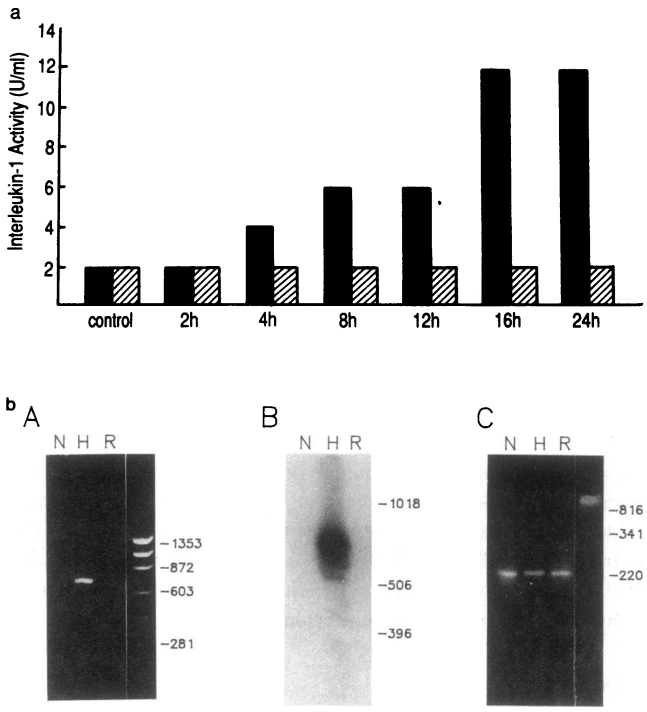

Figure 1. Production of IL-1α by hypoxic ECs. (*a*) IL-1 activity in conditioned media of hypoxic ECs. Confluent ECs were washed in PBS and incubated in Medium 199 in the hypoxic atmosphere ($Po_2 \approx$ 12–14 Torr) for the indicated times. Aliquots of culture supernatant were assayed for IL-1 activity (*shaded bars*) in the D10 assay. The hatched bars indicate culture supernatants prepared identically, but assayed in the presence of anti-IL-1α IgG (2.5 μg/ml). Data shown are the mean of duplicate determinations from a representative experiment. (*b*) PCR analysis of IL-1 transcripts in hypoxic ECs. ECs were maintained either in normoxia (*N*), hypoxia for 16 h (*H*), or hypoxia (16 h) followed by reoxygenation for 4 h (*R*). First-strand cDNA was prepared from total RNA (1 μg) and served as template for PCR in combination with primers specific for IL-1α (panel *A*) and GAPDH (panel *C*). Results shown are ethidium bromide–stained PCR products obtained after 35 cycles for IL-1α and 30 cycles for GAPDH. Amplicons for IL-1α and GAPDH were identical to those obtained on PCR amplification of plasmids bearing full length inserts for these molecules (data not shown). Panel *B* shows Southern hybridization of the PCR products obtained in panel *A* using ^{32}P-labeled cDNA for human IL-1α.

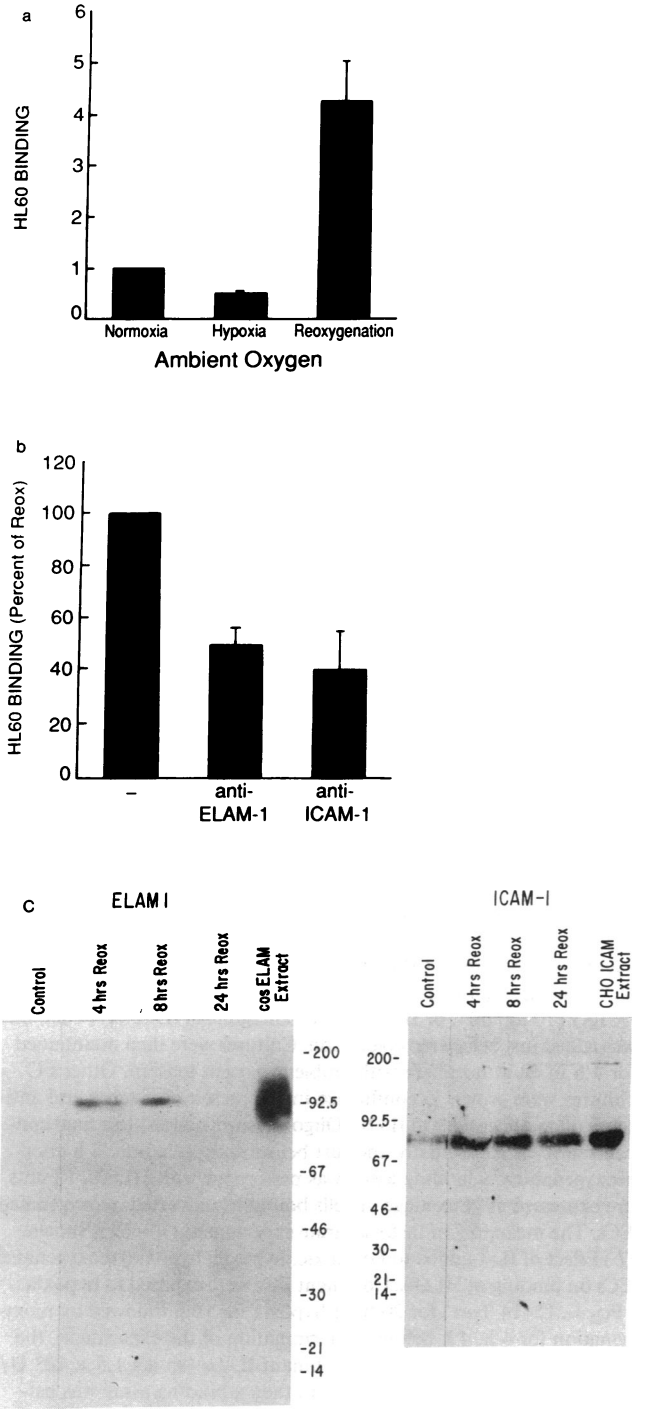

Figure 2. Effect of hypoxia and reoxygenation on the binding of HL60 cells to ECs. (*a*) Binding of HL60s to hypoxic/reoxygenated ECs. Confluent ECs were either placed in hypoxia ($Po_2 \approx$ 12–14 Torr) for 20 h (*Hypoxia*) or in hypoxia for 16 h and then reoxygenated by replacement into an atmosphere with ambient oxygen tension for 4 h (*Reoxygenation*). ECs were then washed in RPMI 1640 and a binding assay with HL60 cells was performed as described in the text. The binding assay was performed in ambient oxygen tension (bars labeled normoxia and reoxygenation), and in an atmosphere with $Po_2 \approx$ 12–14 Torr (bar marked hypoxia). Results shown are the means of three separate experiments (n = 6 for each sample), and are expressed as a ratio of binding of HL60 cells to normoxic endothelium, which was arbitrarily assigned a value of 1. Comparison of HL60 binding to reoxygenated ECs versus normoxic ECs showed a three-to fivefold increase ($P < 0.05$). (*b*) Effect of anti-ELAM-1 and anti-ICAM-1 antibodies on HL60 adherence to hypoxic/reoxygenated ECs. ECs were subjected to hypoxia for 16 h, and 30 min before reoxygenation, blocking antibodies to ELAM-1 (3B7) or ICAM-1 (10 μg/ml in each case) were added. After 4 h of further incubation in an ambient air atmosphere, a binding assay was performed by adding HL60 cells. Results shown are the means of three separate experiments (n = 6). (*c*) Western blot analysis of EC lysates with antibodies to ELAM-1 and ICAM-1. Confluent ECs were subjected to hypoxia ($Po_2 \approx$ 12–14 Torr) for 16 h and then reoxygenated for the indicated times (4, 8, or 24 h). Monolayers were lysed, prepared for nonreduced SDS-PAGE (gradient, 5–15%), and Western blot analysis was performed as described in the text. "Control" designates cultures exposed only to normoxia; extracts of COS-ELAM or Chinese hamster ovary-ICAM were used as positive controls.

Exposure of mice to hypoxia and immunohistology. Mice were subjected to hypoxic conditions and reoxygenated using a controlled environment chamber (access to this chamber was generously provided by Dr. R. Stark, Dept. of Pediatrics, Columbia University). The chamber was equipped with circulating fans, and inflow and outflow valves (Skinner valves; Honeywell Inc., New Britain, CT) connected to a microprocessor and instantaneous on-line oxygen sensor (Horiba Ltd., Kyoto, Japan). This apparatus controlled the flow of the gas mixture and a one way outflow valve was used to maintain the pressure at atmospheric levels. The environment within the chamber was continuously regulated by a custom built interface (K + K Interface Inc., NY) and utilized an environmental control program (K + M) running on an XT clone microprocessor equipped with a Megaboard logic board (Display Telecommunications Corp., Dallas, TX) and an analogue to digital conversion package (LabMaster Scientific Solutions Inc., Solon, OH). The temperature and humidity in the chamber were at room ambient environment, and were also controlled by the automated system above. Mice were placed in the chamber in their usual cages and allowed free access to food and water, and the system parameters were adjusted to a final oxygen concentration of 8–10% with the balance of the gas mixture being made up of nitrogen. Over the first hour, the oxygen content of the atmosphere was reduced from ambient levels to 15%, and over the second hour, it was reduced to 8–10%. Mice were exposed to hypoxia for varying times, plasma samples were obtained, and lung tissue collected for extraction of RNA (see above) and for immunohistology. Tissue was fixed in paraformaldehyde (3.5%) and embedded in paraffin. Sections were stained for ICAM-1 using a specific rat anti-mouse ICAM-1 monoclonal antibody YN.1, obtained from American Type Culture Collection. Sites of primary antibody binding were visualized with a peroxidase-coupled secondary antibody.

Results

IL-1 production by hypoxic ECs. When cultured ECs were exposed to hypoxia, they elaborated IL-1 into the conditioned medium in a time-dependent manner, with levels peaking after 16 h of hypoxia, as measured in the D10 assay. This IL-1 activity was neutralized by an antibody to IL-1α, bringing the activity down to the baseline (Fig. 1 *a*). IL-1 released by hypoxic ECs was not a consequence of cell death, as cellular viability after 16–22 h of exposure to hypoxia remained unaffected, as assessed by several criteria: ECs remained adherent to the surface as a confluent monolayer, excluded trypan blue, and demonstrated only a reversible 20–30% decrease in general protein synthesis (25). Consistent with the specificity of hypoxia as a stimulus for the induction of IL-1, no elaboration of IL-1 by ECs was observed during reoxygenation (data not shown).

In parallel with the increase of IL-1α activity in the culture

supernatant, there was induction of IL-1α mRNA in hypoxic ECs, compared with normoxia, based on PCR, (Fig. 1 *b*, panel *A*). Southern blots of these amplicons, hybridized to a cDNA encoding full-length IL-1α, showed strong induction of IL-1α in the hypoxic samples. (Fig. 1 *b*, panel *B*). Amplification of mRNA for GAPDH demonstrated that levels of transcript for this constitutive enzyme were unaffected by hypoxia (Fig. 1 *b*, panel *B*), confirming selective induction of IL-1α mRNA.

Enhanced leukocyte-EC adherence and increased expression of endothelial cell surface adherence molecules during hypoxia/reoxygenation. Despite the presence of IL-1 in the supernatant of hypoxic ECs, cultures exposed only to hypoxia demonstrated $\approx$ 50% decreased adherence of HL60 cells compared with normoxic controls (Fig. 2 *a*). In contrast, reoxygenated ECs showed a three- to fivefold enhancement in HL60 cell binding compared with normoxia. Increased binding of HL60 cells to reoxygenated ECs could be inhibited by $\approx$ 50% using antibodies to either ELAM-1 or ICAM-1 (Fig. 2 *b*). Addition of exogenous IL-1α to normoxic EC cultures, in amounts similar to that detected in the medium of hypoxic EC, led to increases in HL60 adherence in the range of those detected in Fig. 2 *a*. Experiments with PMNs in place of HL60 cells showed similarly enhanced adherence of reoxygenated ECs (data not shown).

Consistent with a role for ELAM-1 and ICAM-1 in the binding of leukocytes to reoxygenated ECs, Western blot analysis demonstrated upregulation of these cell adhesion molecules when hypoxic cultures were restored to normoxia (Fig. 2 *c*). ELAM-1 antigen was induced by 4 h of reoxygenation, and declined to nondetectable levels 24 h later. ICAM-1, constitutively present in cultured ECs (6, 7), demonstrated an increase in expression by 4 h of reoxygenation which persisted through 24 h. ECs exposed to hypoxia alone did not display increased levels of either ELAM-1 or ICAM-1 (data not shown).

The central contribution of endothelial-derived IL-1 in enhanced adherence of leukocytes to EC cultures subjected to hypoxia/reoxygenation. The data obtained thus far supported the hypothesis that IL-1 produced by ECs during hypoxia was the mediator of enhanced expression of adhesion molecules and increased leukocyte binding during reoxygenation. To test this, the effect of inhibitors of IL-1 translation and IL-1-receptor interaction was assessed (Fig. 3 *a*). Antibody to IL-1α, added to hypoxic EC monolayers before reoxygenation blocked HL60 cell binding during reoxygenation by about 60%, whereas an irrelevant antibody to (coagulation Factor IX) had no effect. Antisense oligonucleotides, used to suppress selectively translation of IL-1α protein (19), similarly suppressed the reoxygenation-associated increase in HL60 adhesion (elaboration of IL-1 activity by endothelial cultures in hypoxia was suppressed by antisense IL-1 oligonucleotides, data not shown). Sense oligonucleotides for IL-1α were without effect. Addition of recombinant IL-1RA to hypoxic ECs resulted in marked depression of HL60 cell adhesion by 70–75% during reoxygenation.

To explain the apparent paradox that IL-1 was produced in hypoxia, while the EC response to IL-1 occurred during reoxygenation, we considered whether hypoxic endothelial cultures were capable of responding to exogenous IL-1 (Fig. 3 *b*). Addition of exogenous IL-1α (55 pg/ml or 83 U/ml, and 110 pg/ml or 166 U/ml) to normoxic and reoxygenated EC cultures demonstrated a comparable enhancement of the adhesion of HL60 cells. The same amounts of IL-1α, in contrast, had a diminished effect on the hypoxic ECs. In view of the blunted response of hypoxic ECs to IL-1, these results lead us to speculate that the effects of the low amounts of IL-1 (12 U/ml) made by hypoxic ECs were not evident until the period of reoxygenation.

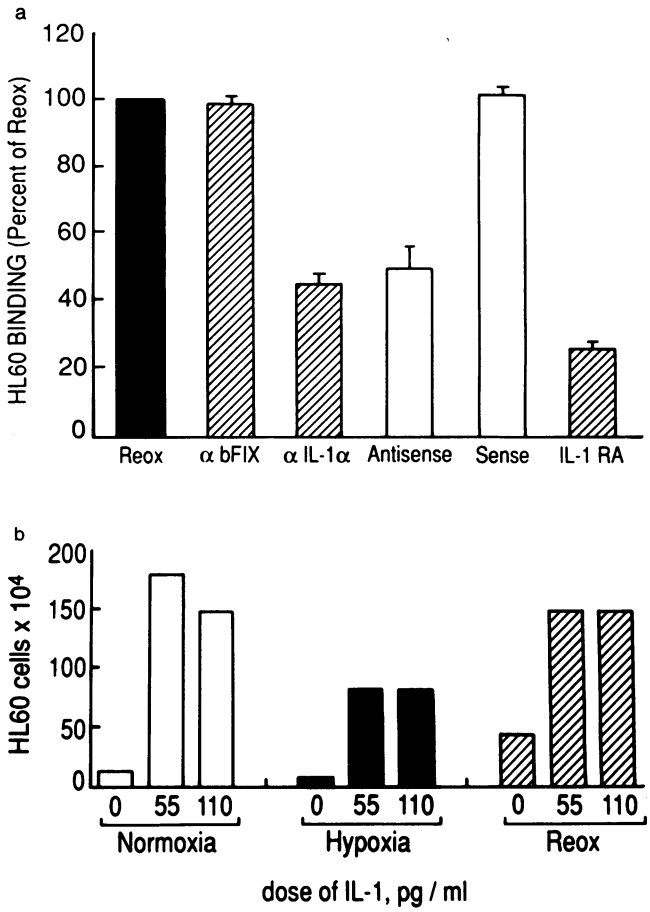

Figure 3. Role of IL-1 in the binding of HL60s to normoxic, hypoxic, and hypoxic/reoxygenated ECs. (*a*) Effect of blocking IL-1. Confluent ECs were subjected to hypoxia ($Po_2 \approx$ 12–14 Torr; 16 h), and then either goat anti-IL-1α IgG (10 μg/ml), goat anti–bovine Factor IX IgG (10 μg/ml), or IL-1 receptor antagonist (IL-1RA) (1 μg/ml) was added just before reoxygenation. Cultures were then maintained for 4 h in an atmosphere with ambient oxygen tension. Other EC cultures were grown to confluence in the presence of sense and antisense oligonucleotides to IL-1α. Oligomers were added to a final concentration of 10 μM daily and just before reoxygenation. 4 h after reoxygenation, a binding assay was performed with HL60s. Results are expressed as percentage of cells bound to untreated, reoxygenated ECs. The mean±SE of three separate experiments ($n = 6$) is shown. (*b*) Effect of IL-1 added to normoxic, hypoxic, hypoxic/reoxygenated ECs on binding of HL60s. Confluent ECs were exposed to hypoxia ($Po_2 \approx$ 12–14 Torr) for 20 h, or hypoxia for 16 h followed by reoxygenation for 4 h. 4 h before the termination of the experiment, the indicated concentration of recombinant IL-1α (sp act 1.5×10^9 U/mg) was added to the medium, and then a binding assay was performed with HL60 cells. Results are expressed as the total number of HL60 cells bound per well (9.6-cm^2 growth area) $\times 10^4$, and are the means of four independent experiments. At each dose of exogenously added IL-1, the number of HL60 cells bound to hypoxic EC monolayers was less than that bound to normoxic cultures ($P < 0.05$) based on a paired *t* test. In contrast, there was no difference in the response of reoxygenated ECs to exogenously added IL-1, as indicated by binding of HL60 cells ($P > 0.05$).

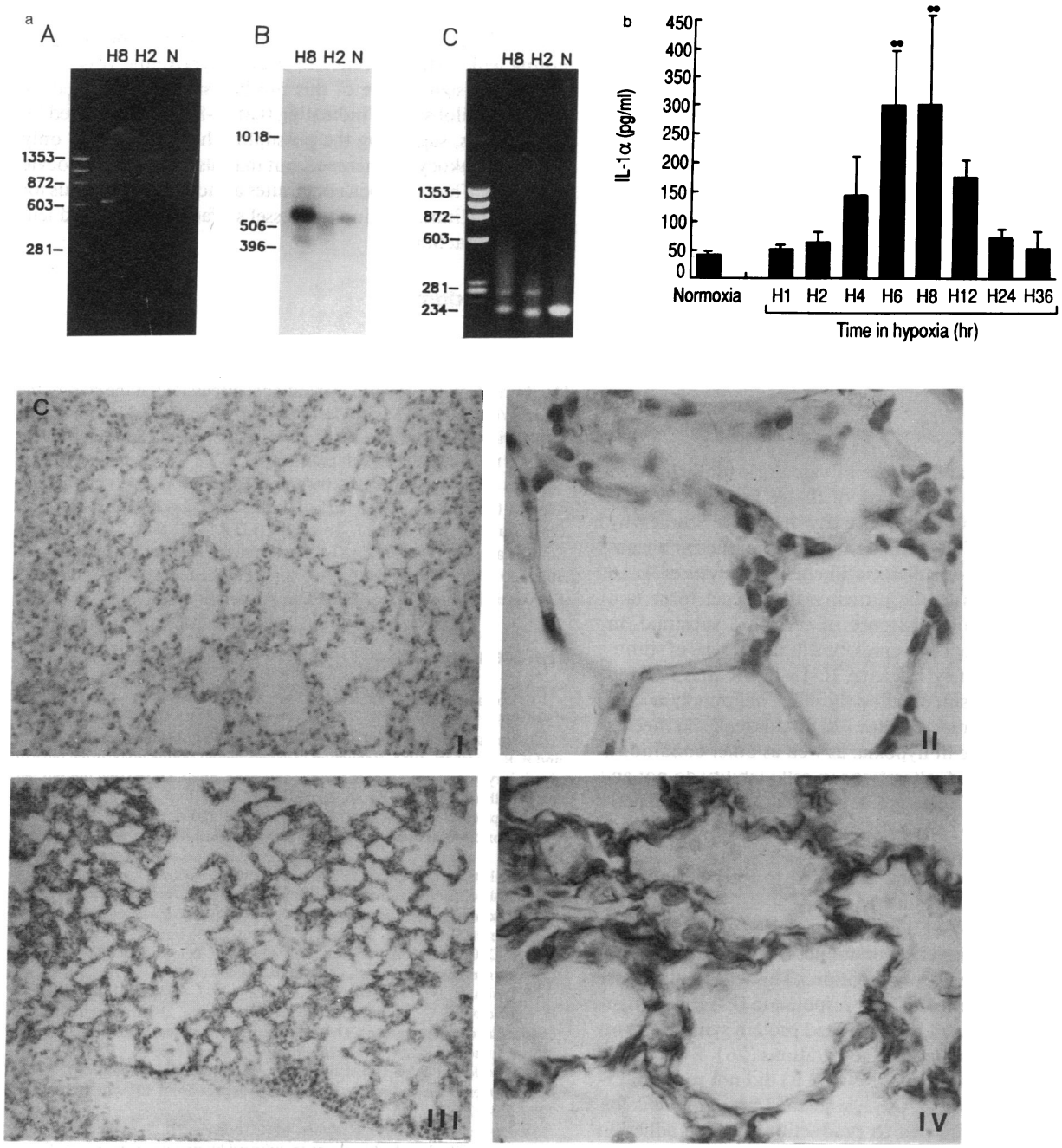

Figure 4. Expression of IL-1 mRNA and ICAM antigen in lungs of hypoxic mice, and induction of IL-1 in hypoxic mouse plasma. (*a*) Amplification of mRNA from lungs of hypoxic mice by PCR. Mice were exposed to an ambient air atmosphere (normoxia, $n = 3$ in each case) or hypoxia ($Po_2 \approx 30$–40 Torr; for either 2 or 8 h, $n = 3$), and PCR was performed as described in the text. Panel *A* shows an ethidium bromide–stained PCR product taken after 35 cycles and demonstrates a band for IL-1α present at 8 h of hypoxia (*H8*), but not in normoxia (*N*) or after 2 h of hypoxia (*H2*). Panel *B* shows results of Southern hybridization of the same PCR products with ^{32}P-labeled cDNA for murine IL-1α. Panel *C* shows that GAPDH transcript is present in all three samples. (*b*) IL-1 antigen in plasma from hypoxic mice. Mice were exposed to hypoxia as above, and at the indicated times plasma samples were obtained for the IL-1 radioimmunoassay. At each time point, results were from three animals (each sample was assayed in duplicate), and ·· indicates $P < 0.01$. The mean±SD is shown. (*c*) Expression of ICAM antigen in lungs of hypoxic mice. Mice were exposed to normoxia (panels *I* and *III*) or hypoxia (panels *II* and *IV*) for 16 h, and lung tissue was harvested and stained for ICAM. *I* and *III*, ×100; *II* and *IV*, ×500.

Induction of IL-1 and increased expression of ICAM-1 in a mouse model of hypoxia. To extrapolate these observations in tissue culture to the in vivo setting, mice were exposed to hypoxia ($Po_2 \approx 30$–40 Torr) and production of IL-1 was assessed. PCR analysis of RNA from hypoxic mouse lungs demonstrated time-dependent increase in IL-1α mRNA evident by 8 h (Fig. 4 *a*, panel *A*). Southern hybridization of these PCR products with a cDNA encoding full-length murine IL-1α demonstrated an increase in the level of IL-1α mRNA in hypoxia, compared with the low levels detectable in normoxic controls

(Fig. 4 *a*, panel *B*). β-scanning demonstrated an approximately seven-fold increase in the signal in the hypoxic lane (Fig. 4 *a*, panel *B*, H8). The same samples showed equal levels of GAPDH transcripts (Fig. 4 *a*, panel *C*). Consistent with these results, IL-1α antigen levels in mouse plasma reached a peak 8–12 h after exposure to hypoxia (Fig. 4 *b*). Probable evidence of the action of IL-1 in vivo was seen in the positive staining for ICAM-1 on pulmonary endothelium of small veins, venules, and lymphatics, as well as the structures of the alveolar wall (Fig. 4 *c*, panels *I–IV*), which occurred in hypoxic animals 4 h after peak plasma levels of the cytokine, consistent with the patterns of staining in injured lungs as previously reported (26). An example of intense staining of alveolar elements for ICAM-1 in the hypoxic lung is seen at high resolution in Fig. 4 *c*, panel *IV* (compare with normoxic lung, Fig. 4 *c*, panel *II*).

Discussion

These studies show that exposure of vascular endothelium to hypoxia results in the production of IL-1α: both the level of mRNA for IL-1α and IL-1α activity increase. Studies by Schindler et al. (27) of IL-1 production in mononuclear phagocytes have demonstrated a dissociation between levels of IL-1β mRNA and synthesis of the protein with respect to certain stimuli such as C5a or adherence of cells to a substrate. In contrast, hypoxia appears to represent a different type of stimulus for ECs, coordinately inducing IL-1α at the message and protein levels. This result parallels the effect of lipopolysaccharide on IL-1β in the macrophage (28). Although the mechanisms of IL-1α release in hypoxia, as well as other conditions, remain to be elucidated, alterations in cell viability do not appear to be involved. The period of hypoxia used in our experiments did not lead to cell death; rather, cells remained intact and were reversibly stimulated to produce IL-1. After reoxygenation, EC IL-1 production decreased to the baseline, and cells could be further subcultured.

IL-1α produced by ECs during the period of hypoxia appeared to have its major effect on induction of leukocyte adhesion molecules during reoxygenation. This effect was most likely caused by a blunted cellular response to IL-1 during hypoxia, perhaps consequent to attenuated protein synthesis in an atmosphere with low oxygen concentrations (26). Even longer incubations of ECs in hypoxia (20–24 h) did not result in expression of ELAM-1 or ICAM-1, whereas 16 h of hypoxia followed by reoxygenation led to production of these adhesion molecules within 4 h. Thus, a period of hypoxia was necessary, but not sufficient, for enhanced expression of ELAM-1 and ICAM-1 by cultured ECs. Our observations are consistent with the results of Yoshida et al. (29), who demonstrated increased CD11/18-mediated leukocyte adhesion to cultured ECs exposed to anoxia during the reoxygenation period. In contrast to these results in cell culture, increased expression of ICAM-1 was seen in the lungs of hypoxic mice, suggesting that higher local concentrations of IL-1 or other factors in the in vivo milieu facilitated endothelial production of adhesion molecules. These data support observations in other studies (1, 2) which show that blocking leukocyte–endothelial interactions decreases vascular and tissue injury in ischemia reperfusion.

Although multiple issues remain to be clarified with respect to the production of IL-1α by hypoxic ECs, such as the relative contribution of membrane-associated IL-1 versus IL-1 elaborated into the culture supernatant, this phenomenon represents a potentially important autocrine mechanism through which vascular ECs can alter their adhesive properties, thereby promoting leukocyte adherence to sites of hypoxemic vessel wall injury. The significance of this mechanism is emphasized by our recent pilot studies indicating that IL-8 is also produced by hypoxic ECs, suggesting the possibility that hypoxia not only alters EC-leukocyte adherence, but may also increase leukocyte migration. Thus, hypoxia constitutes an activating stimulus for the endothelium, priming the vessel surface for enhanced leukocyte interactions.

Acknowledgments

Dr. Gabriel Godman provided invaluable suggestions throughout the course of this work, and Dr. Peter Caldwell reviewed the manuscript. Dr. Ramzi Cotran provided inspirational discussions at the start of the work. We thank Dr. Susan Garfunkel for suggestions, Ms. Sonia Lyn for providing secretarial assistance, and Ms. Virginia Schwinge for assistance with endothelial cell cultures.

This work was supported by grants from the U. S. Public Health Service (HL-08284, HL-34625, HL-42833, HL-42507, HL-21006), the Council for Tobacco Research (CTR1971 and 2101R1), the Schultz and Stony-Wold Foundations, and the New York Heart Association. D. Stern completed this work during the tenure of a Genentech-EI Award from the American Heart Association.

References

1. Horgan, M. J., S. D. Wright, and A. B. Malik. 1990. *Am. J. Physiol.* 259:L315–319.

2. Simpson, P. J., R. F. Todd, J. C. Fantone, J. K. Michelson, J. D. Griffin, and B. R. Lucchesi. 1988. Reduction of experimental canine myocardial reperfusion injury by a monoclonal antibody (anti-Mo1, anti-CD11b) that inhibits leukocyte adhesion. *J. Clin. Invest.* 81:624–629.

3. Repine, J., J. Cheronis, T. Rodell, S. Linas, and A. Patt. 1987. Pulmonary oxygen toxicity and ischemia reperfusion injury. *Am. Rev. Respir. Dis.* 136:483–485.

4. Colletti, L., D. Remick, G. Burtch, S. Kunkel, R. Strieter, and D. Campbell. 1990. Role of tumor necrosis factor α in the pathophysiologic alterations after hepatic ischemia/reperfusion in the rat. *J. Clin. Invest.* 85:1936–1943.

5. Dreyer, W. J., L. H. Michael, M. S. West, C. W. Smith, R. Rothlein, R. D. Rossen, D. C. Anderson, and M. L. Entman. 1988. Neutrophil accumulation in ischemic canine myocardium. *Circulation.* 84:400–411.

6. Smith, C. W., S. D. Marlin, R. Rothlein, B. J. Hughes, H. M. Mariscalo, H. E. Rudloff, F. C. Schmalsteig, and D. C. Anderson. 1988. Recognition of an endothelial determinant for CD18-dependent human neutrophil adherence and transendothelial migration. *J. Clin. Invest.* 82:1746–1756.

7. Rothlein, R., M. L. Dustin, S. D. Marlin, and T. A. Springer. 1986. A human intercellular adhesion molecule is distinct from LFA-1. *J. Immunol.* 137:1270–1274.

8. Bevilacqua, M. P., J. S. Pober, M. E. Wheeler, R. S. Cotran, and M. A. Gimbrone. 1985. Interleukin-1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes, and related leukocyte cell lines. *J. Clin. Invest.* 76:2003–2007.

9. Bevilacqua, M. P., J. S. Pober, D. L. Mendrick, R. S. Cotran, and M. A. Gimbrone. 1987. Identification of an inducible endothelial-leukocyte adhesion molecule. *Proc. Natl. Acad. Sci. USA.* 84:9238–9242.

10. Mulligan, M. S., J. Varani, M. K. Dame, C. L. Lane, C. W. Smith, D. C. Anderson, and P. A. Ward. 1991. Role of endothelial-leukocyte adhesion molecule-1 in neutrophil-mediated lung injury in rats. *J. Clin. Invest.* 88:1396–1406.

11. Pober, J. S. 1988. Cytokine mediated activation of vascular endothelium. *Am. J. Pathol.* 133:426–433.

12. Baggiolini, M., A. Walz, and S. L. Kunkel. 1989. Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. *J. Clin. Invest.* 84:1045–1049.

13. Strieter, R. M., S. L. Kunkel, H. L. Showell, D. G. Remick, S. H. Phan, P. A. Ward, and R. M. Marks. 1989. Endothelial cell gene expression of a neutrophil chemotactic factor by TNF-α, LPS and IL-1β. *Science (Wash. DC).* 243:1467–1469.

14. Stern, D., I. Bank, P. Nawroth, J. Cassimeris, W. Kisiel, J. Fenton, C. Dinarello, E. Jaffe, and L. Chess. 1985. Self regulation of procoagulant events on the endothelial cell surface. *J. Exp. Med.* 162:1223–1235.

15. Kurt-Jones, E. A., W. Fiers, and J. S. Pober. 1989. Membrane interleukin-

1 induction on human endothelial cells and dermal fibroblasts. *J. Immunol.* 139:2317–2324.

16. Warner, S. J., K. Auger, and P. Libby. 1987. Interleukin-1 induces interleukin 1 production by adult human vascular endothelial cells. *J. Immunol.* 139:1911–1917.

17. Jaffe, E. A., R. L. Nachman, C. G. Becker, and R. C. Minick. 1973. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Invest.* 52:2745–2756.

18. Thornton, S. C., S. N. Mueller, and E. M. Levine. 1983. Human endothelial cells: use of heparin in long term cloning and serial cultivation. *Science (Wash. DC).* 222:623–625.

19. Maier, J. A. M., P. Voulalas, D. Roeder, and T. Maciag. 1990. Extension of the life span of human endothelial cells by an interleukin-1 alpha antisense oligomer. *Science (Wash. DC).* 249:1570–1574.

20. Ogawa, S., H. Gerlach, C. Esposito, A. Pasagian-Macaulay, J. Brett, and D. Stern. 1990. Hypoxia modulates the barrier and coagulant function of cultured bovine endothelium: increased monolayer permeability and surface coagulant properties. *J. Clin. Invest.* 85:1090–1098.

21. Boyum, A. 1968. Isolation of mononuclear leukocytes and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* 21(Suppl. 97):77–81.

22. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T_4. *Nature (Lond.).* 227:680–685.

23. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

24. Humphreys, M., and S. Hopkins. 1989. Simple, sensitive and specific bioassay of IL-1. *J. Immunol. Methods.* 120:271–276.

25. Shreeniwas, R., S. Ogawa, F. Cozzolino, G. Torcia, N. Braunstein, C. Butura, J. Brett, H. B. Lieberman, M. B. Furie, J. Joseph-Silverstein, and D. Stern. 1991. Macrovascular and microvascular endothelium in long term hypoxia: alteration in cell growth, monolayer permeability, and cell surface coagulant properties. *J. Cell. Physiol.* 146:8–17.

26. Burns, A. R., F. Takei, and C. M. Doershuk. 1991. Expression of ICAM-1 on murine capillary endothelium and alveolar epithelium. *FASEB* (Fed. J 5:3961. (Abstr.)

27. Schindler, R., B. Clark, and C. Dinarello. 1990. Dissociation between IL-1b mRNA and protein synthesis in human peripheral blood mononuclear cells. *J. Biol. Chem.* 265:10232–10237.

28. Schindler, R., J. Gelfand, and C. Dinarello. 1990. Recombinant C5a stimulates transcription rather than translation of IL-1 and TNF. Translational signal provided by lipopolysaccharide or IL-1 itself. *Blood.* 76:1631–1638.

29. Yoshida, N. D., C. Anderson, R. Rothlein, D. N. Granger, and P. R. Kvietys. 1992. Anoxia/reoxygenation-induced neutrophil adherence to cultured endothelial cells. Role of adhesion molecules. *Am. J. Physiol.* 262:H1891–1898.