Hypoxia Mediates Increased Neutrophil and Macrophage Adhesiveness to Alveolar Epithelial Cells

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Leukocyte infiltration is known to play an important role in hypoxia-induced tissue damage. There is a paucity of information on the role of hypoxia in the expression of adhesion molecules on respiratory epithelial cells. The current studies focus on the adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), their expression pattern on alveolar epithelial cells, and their biologic function under hypoxic conditions. Rat alveolar epithelial cells (AEC) were exposed to hypoxia for several time periods. With 5% oxygen, mRNA for ICAM-1 and VCAM-1 rose by 100%, peaking between 0.5 and 1 h. ICAM-1 and VCAM-1 protein showed an increase between 2 and 4 h. Neutrophil adherence to hypoxia-exposed AEC was enhanced by 115%. This increase was reduced by 83% with anti–ICAM-1 antibody. Adherence of alveolar macrophages to AEC increased by 118% and could be blocked by 95% with anti–VCAM-1 antibody. The present study shows for the first time an early increase of ICAM-1 and VCAM-1 expression on rat AEC under hypoxic conditions. These adhesion molecules are involved in increased adhesiveness of neutrophils and macrophages. Such responses might play an important role in the adhesion of leukocytes and macrophages to lung epithelial cells during hypoxic conditions.

Limited information is available about mechanisms of hypoxic lung damage. It is essential that homeostatic mechanisms are maintained in response to acute or chronic hypoxia. Acute exposure to hypoxia results in vasoconstriction of the pulmonary arteries and redistribution of blood from the basal to the apical areas of the lung (1). It is known from previous studies that polymorphonuclear leukocytes (PMN) play a major role in mediating hypoxic injury (2). Both *in vitro* and *in vivo* studies have indicated that during hypoxia adherence of neutrophils to endothelial cells is increased (3). Most hypoxic studies of lungs have concentrated on reoxygenation after hypoxia. Little attention has been directed to the effect of hypoxia alone. In addition, the majority of the studies focused on endothelial and not epithelial cells. Alveolar epithelial cells (AEC) are potential targets for hypoxia in the alveolar space in pathologic conditions such as hypoventilation. Hypoventilation is commonly caused by depression of the respiratory center by drugs, traumatic thoracic cage abnormalities, upper airway obstruction, insults to the medulla of the brain (trauma, hemorrhage), diseases of the nerves to the respiratory muscles (e.g., Guillain-Barré), or drug-induced neuromuscular disorders. The aim of this study was to evaluate changes in the expression pattern of intercellular adhesion molecule-1 (ICAM-1, CD54) and vascular cell adhesion molecule-1 (VCAM-1, CD106) on AEC during hypoxia.

ICAM-1 is an adhesion molecule of the immunoglobulin superfamily. It has two β_2-integrin ligands on leukocytes: LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18). Interactions between ICAM-1 expressed on endothelial cells and β_2-integrins play an important role in a wide variety of immune mechanisms in lung injury (4). Adhesion to vascular endothelium mediated through ICAM-1–β_2-integrin interactions is a key step in emigration of blood leukocytes to sites of inflammation. The relevance of leukocyte–epithelium adhesion provided by ICAM-1, however, has not been defined clearly. Another immunoglobulin superfamily member that interacts with the $\alpha_4\beta_1$-integrin (VLA-4) is VCAM-1 (5). The primary role of this molecule is the promotion of lymphocyte and macrophage adhesion (6).

Under physiologic conditions, tumor necrosis factor-α (TNF-α) has an important role in host defense and tissue repair (7). In lung injury it has been implicated as a proximal mediator (4, 8). In the context of alveolar hypoxia, it is of interest to investigate if VCAM-1 expression is also TNF-α dependent.

The alveolar space with its large surface area covered by AEC is a site of possible interaction of the body with the external environment. AEC have been shown to play a key role in the regulation of the alveolar space because type II AEC synthesize and secrete surfactant, control the volume and composition of the epithelial fluid, and are able to undergo morphologic conversion (at least *in vitro*) to type I AEC (9). AEC also have a role in modulating the activation state of macrophages, fibroblasts, and endothelial cells. Recently, the involvement of AEC in lipopolysaccharide (LPS)-induced lung injury was shown *in vitro* (10). LPS stimulation led to enhanced expression of ICAM-1 on AEC with increased neutrophil adherence, demonstrating that the lower epithelial compartment might play an important role in inflammatory mechanisms during endo-

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Abbreviations: actinomycin D, ACTD; alveolar epithelial cells, AEC; cycloheximide, CHX; Dulbecco's modified Eagle's medium, DMEM; enzyme-linked immunosorbent assay, ELISA; fetal bovine serum, FBS; reduced form of guanosine adenine dinucleotide phosphate, GAPDH; intercellular adhesion molecule-1, ICAM-1; lactate dehydrogenase, LDH; lipopolysaccharide, LPS; *o*-phenylenediamine dihydrochloride, OPD; phosphate-buffered saline, PBS; paraformaldehyde, PFA; polymorphonuclear cells, PMN; reverse transcription-polymerase chain reaction, RT-PCR; sodium dodecyl sulfate, SDS; standard saline citrate, SSC; tumor necrosis factor-α, TNF-α; vascular cell adhesion molecule-1, VCAM-1.

toxin-induced lung injury. Mulligan and coworkers defined a compartmentalized role for ICAM-1 in a model of intrapulmonary deposition of IgG immune complexes (11). We hypothesized that hypoxia may also augment cell surface expression of ICAM-1 and VCAM-1 on respiratory epithelial cells and investigated the potential consequences on neutrophils and alveolar macrophages.

Materials and Methods

AEC

AEC were harvested following a well-established protocol (12). Briefly, male Long Evans rats were anesthetized with subcutane Hypnorm (fentanyl-fluanisone) (0.25 ml/kg) and Domitor (medetomidini hydrochloridum) (0.25 ml/kg). The inferior vena cava was transected for exsanguination, the trachea cannulated, and the bronchoalveolar system lavaged with cold phosphate-buffered saline (PBS) to remove macrophages. Forty milliliters of porcine pancreas elastase (Calbiochem, La Jolla, CA), containing 5 U/ml, were added to the lung and incubated for 20 min at 37°C. Trachea and large airways were discarded and lungs were minced in the presence of 2000 U DNase (Sigma, Buchs, Switzerland). The elastase reaction was stopped with 5 ml fetal bovine serum (FBS) (Life Technologies, Basel, Switzerland). Cells were filtered and incubated for 1 h at 37°C in IgG-coated plastic plates. Unattached cells were then removed, washed, and plated. Cells were cultured for 3 d in Dulbecco's modified Eagle's medium (DMEM) with glutamax (Life Technologies) supplemented with 10% FBS. Cell purity was more than 95%.

Hypoxia

A cell incubator (Bioblock, Ittigen, Switzerland) adjustable to different oxygen concentrations by insufflation of nitrogen (N_2) was used as a hypoxic cell chamber. The concentrations were monitored continuously by an oxygen sensor. Experiments were performed with 10%, 5%, and 2% oxygen and 5% CO_2 at 37°C. For control cells, an incubator with 21% O_2 was used. Before inducing hypoxia, the medium of all cells was changed to high glucose DMEM/1% FBS to stop division of the cells. For RNA studies, cells were grown in 35-mm dishes (Corning, New York, NY); for enzyme-linked immunosorbent assay (ELISA) special 96-well plates with eight-well strip-plates (Costar, Corning, NY) were used.

Cells were exposed to 5% O_2 for 0.5, 1, 2, and 3 h for RNA assays, and for 2, 4, 6, and 8 h for protein detection. Immediately after exposure, Trizol (Life Technologies) or 3% paraformaldehyde (PFA) was added, to avoid a change in oxygen tension, which could influence expression of the adhesion molecules. To study the influence of different O_2 concentrations, cells were placed in the hypoxia incubator for 1 h.

RNA Extraction, Northern Blot Analysis, and Reverse Transcriptase-Polymerase Chain Reaction

Cells were extracted using Trizol, chloroform (Fluka, Buchs, Switzerland) and isopropanol (Fluka). Four micrograms of total cellular RNA was run on a 1% agarose/formaldehyde gel and blotted on a nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). cDNA for ICAM-1 was labeled with ^{32}P dCTP as described previously (4, 13), using a random primers DNA labeling system (Life Technologies). cDNA for VCAM-1 was provided by Dr. P. A. Ward's laboratory (Department of Pathology, University of Michigan, Ann Arbor, Michigan). After incubation of the labeling reaction for 4 h at room temperature, the reaction was stopped and purified over a Sephadex column (Amersham Pharmacia Biotech). The probe was then supplemented with 100 mg/ml final concentration of herring sperm DNA (Roche, Basel, Switzerland), boiled for 5 min, cooled on ice, and added to the blot. The following hybridization solution was used: 1 mM ethylenediamine-tetraacetic acid, 0.5 M Na_2HPO_4, pH 7.2, and 7% sodium dodecyl sulfate (SDS). Blots were hybridized for 24 h after 30 min of prehybridization. To wash the blots, three different wash buffers were used. Low stringency wash was performed with 2× standard saline citrate (SSC), 0.1% SDS, medium stringency wash with 1× SSC, 0.1% SDS, and high stringency wash with 0.1× SSC, 0.1% SDS. The blots were exposed to X-OMAT KODAK film (Sigma) and developed after 2 d of exposure. The same blots were rehybridized with cDNA for β-actin.

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed according to a protocol described earlier (14). Primers had the following configuration: VCAM-1 primer up, 5′- cgg tca tgg tca agt gtt tg -3′; VCAM-1 primer down, 5′- gag atc cag ggg aga tgt ca -3′; TNF-α primer up, 5′- act gaa ctt cgg ggt gat tg-3′; and TNF-α primer down, 5′- gtg ggtgaggagcacgtagt-3′. Annealing temperature of both PCR was 57°C. For each PCR 27 cycles were performed. Equal loading was shown with β-actin or 18S bands.

Cell-Based ELISA

Cells were grown to monolayers and exposed to hypoxia. To ensure unchanged conditions after taking the cells out of the incubator, they were fixed immediately with 3% PFA for 5 min at room temperature (50 μl/well). Each washing step was performed three times with 250 μl/well PBS. Monoclonal mouse anti-rat ICAM-1 antibody (1A29; Serotec Ltd., Oxford, UK) was used as the first antibody at a concentration of 0.5 μg/ml in PBS/10% FBS. For the VCAM-1 ELISA, a polyclonal goat anti-rat VCAM-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (0.4 μg/ml) was incubated with the cells (50 μl/well, 1 h, 4°C). For ICAM-1, the second incubation was performed with a peroxidase-labeled goat anti-mouse IgG (1:2,500 in PBS/10% FBS, 45 min, 4°C) and for VCAM-1, with rabbit anti-goat IgG (1:4,000). To develop color reaction, *o*-phenylenediamine dihydrochloride (OPD) (Sigma) was added to the wells (200 μl/well). The reaction was stopped with 3 M H_2SO_4 and optical density was determined at 492 nm (ELISA reader; Bioconcept, Allschwil, Switzerland).

Adherence Assay

AEC were grown to confluence in a 96-well plate. PMN from healthy human volunteers were isolated by gradient centrifugation over Ficoll-Paque, followed by dextran sedimentation as described previously (10). To remain consistent with previous investigations, experiments were performed with human neutrophils (10). Before addition to AEC monolayers, PMN were preincubated at 37°C, 5% CO_2, with blocking antibodies to FcRII (mouse anti-human CD32) and FcRIII (mouse anti-human CD16) (10 μg/ml) for 15 min. Both antibodies were purchased from PharMingen (San Diego, CA). AEC were exposed for 2 h to 5% oxygen. Cells were then incubated with 10 μg/ml anti–ICAM-1 antibody (1A29) (Serotec) or mineral oil-induced plasmocytoma cell (MOPC-21) (mouse IgG) as a control antibody for 20 min at 37°C, 5% CO_2, 21% oxygen, together with 10^6 PMN/well. Nonadherent PMN were washed away and remaining cells were counted.

The same experiments were performed using rat alveolar macrophages (15). Macrophages were preincubated with blocking antibodies to FcRII and FcRIII (mouse anti-human CD16 and CD32) as described. AEC were washed and incubated with a polyclonal rabbit anti-rat VCAM-1 antibody or rabbit IgG (10 μg/ml) (Santa Cruz Biotechnology) and 10^6 macrophages/well for 20 min at 37°C with 5% CO_2, as control. The same experiments were performed, using ICAM-1 and VCAM-1 antibody together. Nonadherent cells were washed away and adherent cells were counted.

Assay with Actinomycin D and Cycloheximide

Cells in 35-mm plates or 96-well plates were incubated for 1 h with 0.5 μg/ml final concentration of actinomycin D (ACTD) (Sigma), 5 μg/ml cycloheximide (Sigma), or with both substances in DMEM/1% FBS for 1 h at 37°C, 5% CO_2. For the mRNA experiment, cells were exposed to 5% O_2 for 1 h, whereas cells for protein assays were under hypoxia for 2 h. RNA extraction and Northern blot analysis or ELISA were performed as described.

Assay with Antisense Oligonucleotides for Rat ICAM-1

AEC with a 50% confluence were incubated with sense and antisense phosphorothioate oligonucleotide (2 μg/ml) (Life Technologies) (16) in the presence of 5 μg/ml lipofectin reagent (Life Technologies) in optimal minimum essential medium for 30 min. Cells were washed with optimal minimum essential medium and incubated overnight with the previously described concentrations of oligonucleotides, but omitting lipofectin. Stimulation was performed for 1 h with 5% oxygen, followed by RNA analysis for ICAM-1.

TNF-α Dependency of VCAM-1

AEC were preincubated with a polyclonal rabbit anti-rat TNF-α antibody (Santa Cruz Biotechnology) (10 μg/ml) for 30 min at 37°C, 5% CO_2 or control IgG, followed by a 60-min incubation at 5% oxygen. RNA was extracted and analyzed for VCAM-1.

Lactate Dehydrogenase Assay

To evaluate a possible cytotoxic effect of hypoxia, lactate dehydrogenase (LDH) assays were performed using a nonradioactive cytotoxicity assay (Promega, Madison, WI). LDH content was determined by measuring red formazan resulting from the conversion of tetrazolium salt into this substrate by LDH.

Statistical Analysis

All experiments were performed at least three times. For statistical analysis of Northern blots or RT-PCR, densitometry was performed for mRNA of ICAM-1 or VCAM-1 and β-actin for each blot. mRNA/β-actin ratios were calculated. Each ELISA contained four to six replicates. Again, three different sets of ELISA were analyzed. Each data point in the graphs (mean ± SEM) represents results from replicates from three experiments. The same statistical methods were used for adherence assays. Analysis of variance with post-analysis of variance comparison was performed to assess statistical significance of differences.

Results

Time-Dependent Upregulation of mRNA and Protein for ICAM-1 under Hypoxia

AEC were exposed to 5% oxygen for 0 to 3 h. Control cells were left in an incubator containing 21% oxygen. RNA was extracted and Northern blot analysis performed. An upregulation of mRNA for ICAM-1 peaking at 0.5 and 1 h (81% increase, $P < 0.01$) could be demonstrated (Figure 1A). Cell-based ELISA was performed in special 96-well plates, which allowed incubation with six-well rows separately. As shown in Figure 1B, there was a peak upregulation by 28% ($P < 0.01$) at 2 h, 20% ($P < 0.05$) at 4 h, and 18% ($P < 0.01$) at 6 h. At 8 h, ICAM-1 expression returned to baseline expression.

Time-Dependent Upregulation of mRNA and Protein for VCAM-1 under Hypoxia

Changes in mRNA for VCAM-1 showed a similar pattern, with an 85% increase after 0.5 and 1 h ($P < 0.01$) when compared with baseline levels (Figure 2A). Equal loading was shown with β-actin rehybridization. Figure 2B shows VCAM-1 protein upregulation during hypoxia, peaking between 2 and 4 h (25% increase, $P < 0.01$, and 19% increase, $P < 0.01$, respectively).

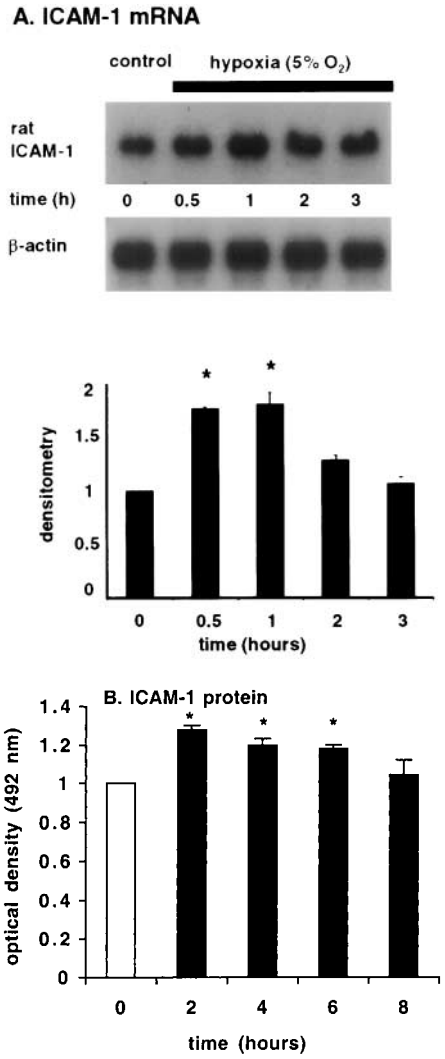

Figure 1. Changes in expression of ICAM-1 mRNA (*A*) and protein (*B*) during hypoxia (5% oxygen). Cells were exposed to 5% O_2 for 0.5, 1, 2, and 3 h. RNA was extracted and Northern blot analysis performed. Results were analyzed by densitometry. Equal loading was shown by β-actin rehybridization. *Asterisks* indicate statistically significant differences from controls at 0 time (*$P < 0.01$). Companion cells were exposed to 5% O_2 for 2, 4, 6, and 8 h and fixed with 3% PFA. ELISA was performed using monoclonal mouse anti-rat ICAM-1 antibody. *Asterisks* indicate statistically significant differences from controls at 0 time (*$P < 0.05$).

Adherence of Neutrophils and Alveolar Macrophages to Alveolar Epithelial Cells after Hypoxia: Role of Epithelial ICAM-1

To define the biologic role of upregulated epithelial ICAM-1, adherence assays were performed using neutrophils and AEC (Figure 3A). AEC were exposed to hypoxia for 2 h. Adherence of neutrophils to hypoxic cells increased by

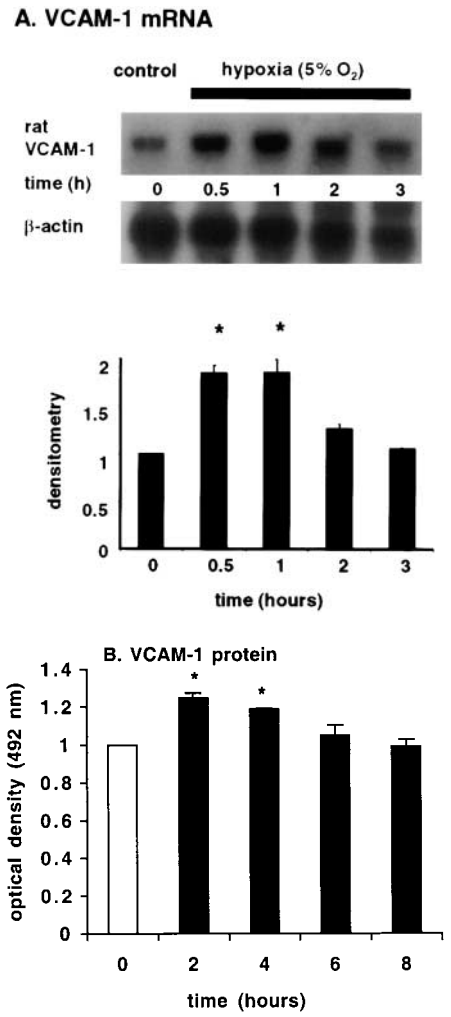

Figure 2. Changes in expression of VCAM-1 mRNA (*A*) and protein (*B*) under hypoxia (5% oxygen). Cells were exposed to 5% O_2 for 0.5, 1, 2, and 3 h. RNA was extracted and Northern blot analysis performed. Results were analyzed by densitometry. Equal loading was shown by β-actin rehybridization. *Asterisks* indicate statistically significant differences from controls ($*P <$ 0.01). Companion cells were exposed to 5% O_2 for 2, 4, 6, and 8 h and fixed with 3% PFA. ELISA was performed using polyclonal goat anti-rat VCAM-1 antibody. *Asterisks* indicate statistically significant differences from controls ($*P < 0.01$).

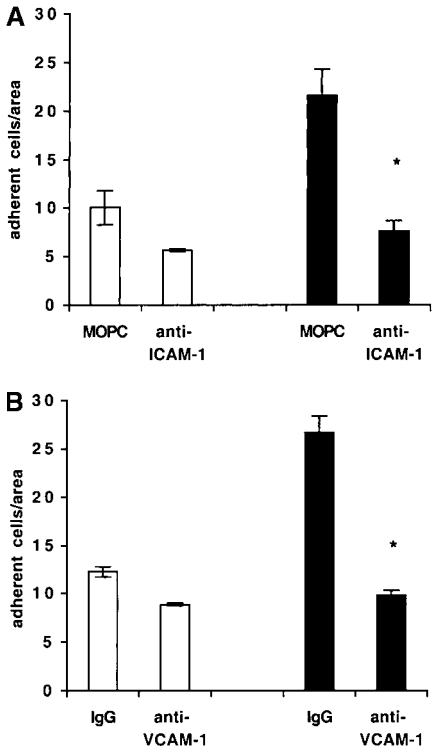

Figure 3. (*A*) Adherence assay with neutrophils. AEC were incubated with either MOPC-21 (control antibody) or with anti–ICAM-1. AEC were exposed to 5% oxygen for 2 h, followed by a 20-min incubation with the neutrophils. Nonadherent cells were removed by washing, and remaining cells were counted. *Asterisk* indicates statistically significant difference from cells exposed to hypoxia with control antibody compared with cell exposed to hypoxia with anti–ICAM-1 ($*P < 0.05$). (*B*) Adherence assay with macrophages. AEC were incubated with either rabbit IgG (control antibody) or with anti–VCAM-1. AEC were exposed to 5% oxygen for 2 h, followed by a 20-min incubation with the macrophages. Nonadherent cells were removed by washing, and remaining cells were counted. *Asterisk* indicates statistically significant differences from cells exposed to hypoxia with control antibodies compared with cells exposed to hypoxia with anti–VCAM-1 ($*P < 0.01$). *Open bars*, 21% oxygen; *solid bars*, 5% oxygen.

115%. This enhanced adherence could be blocked by 83% ($P < 0.05$) in the presence of antibodies to ICAM-1, compared with a control antibody (MOPC-21).

Macrophage adhesion to AEC, previously exposed for 2 h to 5% oxygen, increased by 118% (Figure 3B). To define the role of VCAM-1, AEC were incubated with anti-VCAM-1, and macrophage adherence was evaluated. 95% of the adherence was blocked by anti–VCAM-1 antibody ($P < 0.01$), showing that VCAM-1 is essential for macrophage–AEC adhesive interactions. The incubation of AEC with VCAM-1 and ICAM-1 antibodies led to a 100% blockade of macrophage adhesion to AEC (data not shown). Therefore, it is assumed that ICAM-1 plays only a minor role in macrophage adherence, although neutrophil adherence was ICAM-1 driven.

Oxygen Concentration-Dependent Upregulation of mRNA for ICAM-1 and VCAM-1 under Hypoxia

Monolayers of AEC were exposed to various oxygen concentrations (21%, 10%, 5%, and 2%) for 1 h. Figure 4A shows analysis for ICAM-1 mRNA. An increased expression of ICAM-1 was found with oxygen concentrations of 10% (127%, $P < 0.05$) and 5% (98%, $P < 0.05$), but not with 2%. For VCAM-1, a similar pattern is presented in Figure 4B. There was clearly enhanced VCAM-1 expression at 10% oxygen (98% increase, $P < 0.01$) and 5% oxygen (85% increase, $P < 0.01$), but no increase at 2% oxygen.

Mechanisms of Hypoxia-Induced ICAM-1 and VCAM-1 Synthesis

During hypoxia, mRNA for ICAM-1 and VCAM-1 was upregulated in the first few hours of hypoxia. To obtain more information about transcriptional and translational

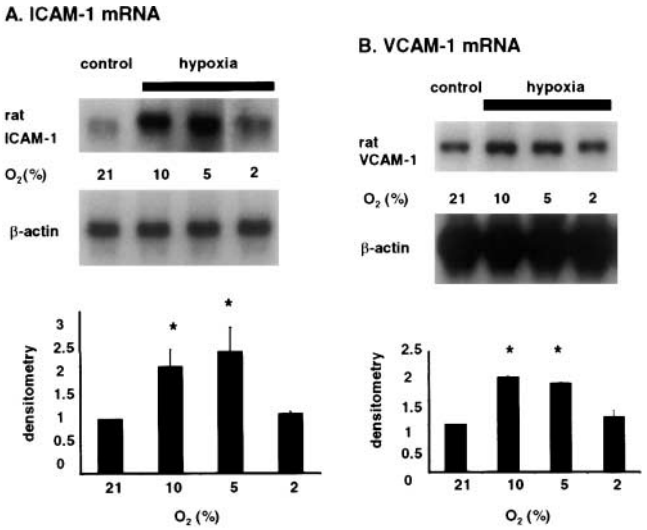

Figure 4. Changes in expression of mRNA for ICAM-1 and VCAM-1 under different oxygen concentrations (21%, 10%, 5%, and 2%) in AEC. Cells were exposed for 1 h to each oxygen tension. RNA was extracted and Northern blot analysis performed. Results were analyzed by densitometry. β-Actin rehybridization showed equal loading. *Asterisks* indicate statistically significant differences from controls (*A*, $*P < 0.05$; *B*, $*P < 0.01$).

processes possibly involved in enhanced upregulation of these adhesion molecules, AEC were preincubated with the transcriptional blocker ACTD, the protein synthesis inhibitor CHX, or both substances. Before exposure to 5% or 21% (control) oxygen, cells were preincubated with the inhibitors for 1 h. Figures 5A and 6A present mRNA upregulation for ICAM-1 and VCAM-1. After 1 h of hypoxia, ACTD prevented the increase in mRNA for ICAM-1 ($P < 0.05$) and VCAM-1 ($P < 0.05$), indicating that upregulation of these two adhesion molecules under hypoxia was probably regulated through a transcriptional pathway. As known from previous studies (17), CHX did not inhibit upregulation at the level of mRNA, which was seen in both control and stimulated cells in this study as well. For the protein assay, AEC were preincubated with ACTD and/or CHX for 1 h, followed by an exposure to 5% O_2 for 2 h. ELISA performed for ICAM-1 and VCAM-1 showed a significant lack of upregulation of ICAM-1 ($P < 0.01$) and VCAM-1 expression ($P < 0.05$) under hypoxia when cells were preincubated with blocking substances (Figures 5B and 6B). As acid does not have specific effects on one single molecule, a potential indirect effect is not completely excluded. To verify eventual toxic effects of the inhibitors, LDH activity in the supernatant was measured at the end of the experiment, showing no changes between unstimulated and stimulated cells, as well as with and without ACTD and CHX (data not shown). These data suggest that *de novo* transcription and translation are both involved in the hypoxia-induced upregulation of ICAM-1 and VCAM-1 in AEC.

Assays with antisense-oligonucleotides for ICAM-1 were performed to show transcriptional mechanisms of hypoxia-induced ICAM-1 upregulation. After incubation of AEC with the antisense-oligonucleotide no enhanced expression of mRNA for ICAM-1 was seen ($P < 0.01$) (Figure 5C).

For further analysis of the molecular mechanism of VCAM-1 upregulation and a possible TNF-α dependency, AEC were treated with an anti–TNF-α antibody. Although upregulation of the adhesion molecule occurred very fast, it could be shown to be TNF-α driven. Addition of the antibody resulted in a 100% blockade ($P < 0.01$) (Figure 7).

Discussion

Hypoxia and its effects on endothelial cells have been evaluated extensively in the literature. Whereas it has been shown that hypoxia does not induce upregulation of ICAM-1 on human umbilical vein endothelial cells (18), endothelial ICAM-1 expression increased dramatically during reoxygenation, not only on human umbilical vein endothelial cells (19) but also on human brain microvascular and on rat coronary microvascular cells (20, 21). Studies on bovine aortic endothelial cells as well as on human retinal capillaries revealed upregulation of ICAM-1 and VCAM-1 under conditions of hypoxia (19). On myocardial cells, however, there was no enhancement of ICAM-1 or VCAM-1 expression under hypoxia (22). These results suggest that adhesion molecule expression is dependent on the cell type under study. The current data focus on the effects of hypoxia on expression of the Ig superfamily of adhesion molecules on respiratory epithelial cells. There is sparse information available about alterations of epithelial cell expression of adhesion molecules under hypoxia. Most studies investigate changes in renal epithelial cells. ICAM-1 upregulation was demonstrated during ischemia on these cells, with a peak between 4 and 12 h for ICAM-1 mRNA (23). Compared with the reduced form of guanosine adenine dinucleotide phosphate (GAPDH), there was a 1.5- to 2-fold increase of ICAM-1 mRNA. In the airway compartment, our study has demonstrated a similar enhancement of ICAM-1, but with a much earlier peak between 0.5 and 1 h. Such differences might be more difficult to interpret by the fact that GAPDH is also altered under hypoxia, as shown recently (24). Escoubet and colleagues pointed out in rat AEC subjected to conditions of anoxia that mRNA for GAPDH rose 2- to 3-fold. However, β-actin levels seemed to be unchanged. Our results did not show any differences in β-actin, which is in concordance with the results of Escoubet and colleagues.

In the current studies, a clear ICAM-1 and VCAM-1 upregulation of both mRNA and protein was shown in AEC during hypoxia. This process of upregulation was time-limited, with mRNA being upregulated only during the first hour and protein upregulation being limited to 4–6 h of hypoxia. As demonstrated in earlier studies in whole lung preparations, the respiratory airway compartment is known to play a role in the inflammatory cascade in parallel to events in the vascular compartment (4, 11). Our current, main investigative target has been the alveolar compartment, using primary cultures of AEC. There is contradictory information about ICAM-1 upregulation on type I/type II cell phenotype of AEC. Enhanced ICAM-1 expression on type II AEC has been detected in several studies.

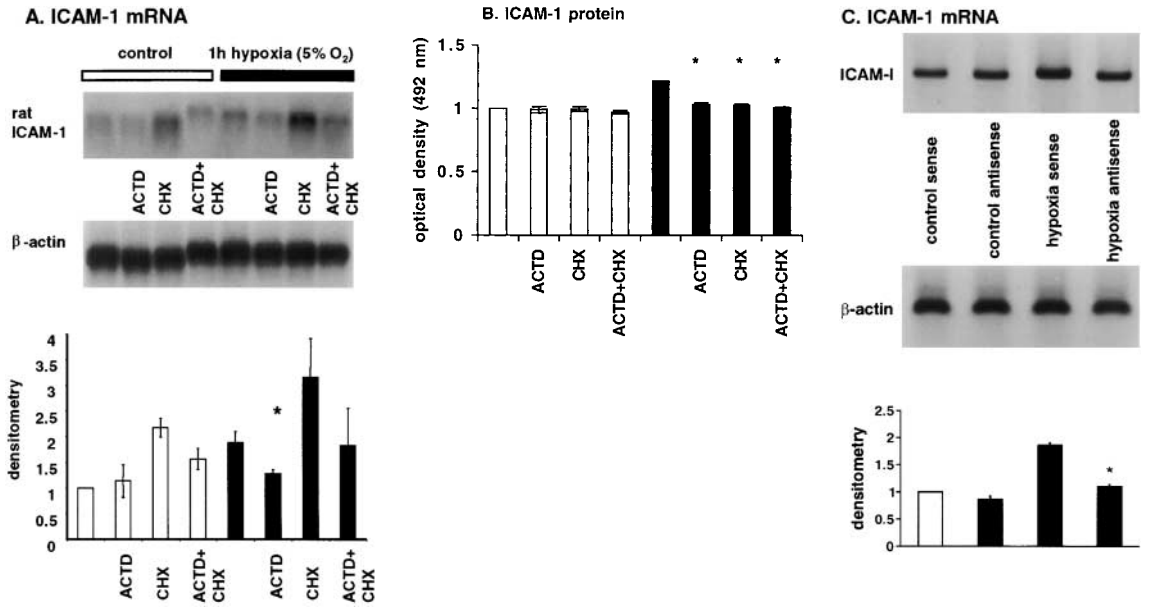

Figure 5. Changes in expression of mRNA and protein for ICAM-1 in AEC under hypoxia after preincubation with ACTD and CHX. Cells were incubated with ACTD and/or CHX for 1 h in DMEM/1% FBS at 37°C with 5% CO_2, followed by an incubation of 1 h at 5% oxygen. Control cells were incubated for 1 h at 21% oxygen. RNA was extracted and Northern blot analysis performed (*A*). Companion cells were preincubated with appropriate substances, followed by an incubation for 2 h at 5% oxygen. ELISA was performed using monoclonal mouse anti-rat ICAM-1 antibody (*B*). *Open bars*, 21% oxygen; *solid bars*, 5% oxygen. *Asterisks* indicate statistically significant difference from cells exposed to hypoxia compared with cells exposed to hypoxia and blocking substances (*A*, $^*P < 0.05$; *B*, $^*P < 0.01$). Changes in expression of mRNA for ICAM-1 in AEC under hypoxia after preincubation with ICAM-1 antisense oligonucleotides. Cells were preincubated with antisense– and sense–ICAM-1 oligonucleotides before exposure to hypoxia (1 h, 5% oxygen). RNA was extracted and RT-PCR performed (*C*). β-actin bands showed equal loading. *Asterisk* indicates a statistically significant difference from cells exposed to hypoxia with a preincubation with sense oligonucleotides compared with cells exposed to hypoxia with antisense oligonucleotide preincubation ($^*P < 0.01$).

Kang and associates showed that hyperoxia *in vivo* caused changes in the distribution pattern of ICAM-1 on mouse type I alveolar epithelial cells and at the same time an induction of ICAM-1 expression on type II alveolar epithelial cells (25). In addition, an *in vitro* study demonstrated LPS-induced upregulation of ICAM-1 on human alveolar type II cells (26). A cell line consisting of rat alveolar epithelial cells with type II phenotypic features showed a clear upregulation of ICAM-1 upon stimulation with LPS (10). These *in vitro* findings are in contrast to previous *in vitro* studies on type II cells, where no enhanced expression of ICAM-1 could be detected (27). Possible explana-

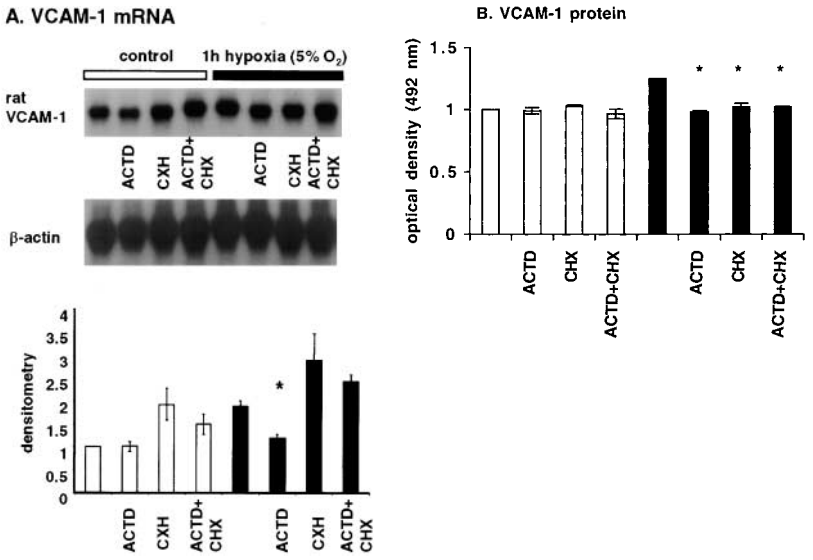

Figure 6. Changes in expression of mRNA and protein for VCAM-1 in AEC under hypoxia after preincubation with ACTD and CHX. Cells were incubated with ACTD and/or CHX for 1 h in DMEM/1% FBS at 37°C with 5% CO_2, followed by an incubation of 1 h at 5% oxygen. Control cells were incubated for 1 h at 21% oxygen. RNA was extracted and Northern blot analysis performed (*A*). β-actin rehybridization showed equal loading. *Asterisk* indicates a statistically significant difference from cells exposed to hypoxia alone ($^*P < 0.05$). Companion cells were preincubated with appropriate substances, followed by an incubation for 2 h at 5% oxygen. ELISA was performed using polyclonal goat anti-rat VCAM-1 antibody (*B*). *Open bars*, 21% oxygen; *solid bars*, 5% oxygen. *Asterisk* indicates statistically significant difference from cells exposed to hypoxia ($^*P < 0.05$).

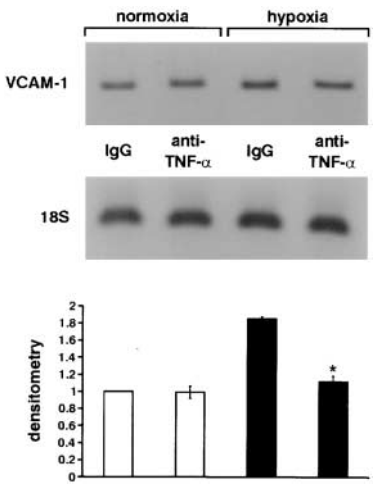

Figure 7. Changes in expression of mRNA for VCAM-1 in AEC under hypoxia after preincubation with a polyclonal anti–TNF-α antibody. Cells were incubated with the antibody (10 μg/ml) or rabbit IgG for 30 min in DMEM/1% FBS at 37°C with 5% CO_2, followed by an incubation of 1 h at 5% oxygen. Control cells were incubated for 1 h at 21% oxygen. RNA was extracted and RT-PCR was performed. 18S bands showed equal loading. *Asterisk* indicates statistically significant difference from cells exposed to hypoxia with control antibody compared with cells exposed to hypoxia with TNF-α antibody (*$P < 0.01$).

tions of these different *in vitro* findings may include a change during culture of the phenotype of type II cells moving toward features of type I cells or improvements in harvesting techniques and culture conditions during the recent years, resulting in a more stable phenotype of type II cells.

To date, there is only limited information available about VCAM-1 on alveolar epithelial cells. VCAM-1 expression has not been detected on the surface of isolated human type II pneumocytes (28). However, recent studies have demonstrated that constitutive VCAM-1 expression was enhanced by rhinovirus infection of alveolar epithelial cell lines (29). In addition, increased VCAM-1 expression has been noted on isolated human alveolar epithelial cells stimulated by TNF-α (30). These results give evidence for increased VCAM-1 expression on AEC.

Because previous studies have raised the question about the biologic function of ICAM-1 on alveolar epithelial cells, adherence assays were performed. We could show that adherence of neutrophils to stimulated (hypoxic) AEC increased by more than 100% when compared with cells maintained under normoxic conditions. This enhanced adherence was at least in part mediated by ICAM-1, similar to cells after endotoxin stimulation (10). Whatever the route of emigration of neutrophils into the alveolar compartment (31), the presence of ICAM-1 on AEC would be expected to enhance the adhesiveness of neutrophils to AEC, putting AEC at added risk of injury. To evaluate the role of macrophage adhesion to AEC, adherence assays were performed. The results provide strong evidence for VCAM-1–induced adhesion of alveolar macrophages to AEC. Therefore, it is assumed that the presence of VCAM-1 facilitates AEC-induced injury by lung macrophages.

Compared with other experimental systems of lung inflammation, such as LPS-induced injury, there was a less intense increase of mRNA and protein for ICAM-1 during hypoxia. AEC under LPS stimulation showed a 700% increase of mRNA for ICAM-1 (10), whereas hypoxia only led to a 80–90% upregulation. This could be due to a different functional role of ICAM-1 in the hypoxia-induced lung injury. O'Brien and coworkers showed recently that ICAM-1 on AEC played an important role in host defense against *Klebsiella pneumoniae* (32). A protective function of the upregulated adhesion molecules ICAM-1 and VCAM-1 could also be hypothesized in the hypoxic lesion.

Our studies demonstrate a fast transcriptional and translational pathway of the enhanced expression of the adhesion molecules. Protein expression on the surface of macrophages is also a fast process (4). Whether the upregulation of these adhesion molecules is regulated by additional factors remains to be determined. It is known from other studies that the transcription factor hypoxic inducible factor-1 (HIF-1) is involved in the upregulation of several genes like nitric oxide synthase (33). Such factors may play a role in hypoxia-induced expression of ICAM-1 and VCAM-1 in AEC.

An interesting observation is the lack of upregulation of the adhesion molecules under 2% oxygen, whereas there was a clearly enhanced expression under 10% and 5% in our studies. Cell death was excluded because LDH concentrations in the supernatant fluids did not increase. It would appear that extreme hypoxia precludes gene activation, which is not surprising because there is abundant evidence that NFκB activation requires the formation of intracellular oxidants (34).

Although the upregulation of the adhesion molecules appeared to be a fast response, strong TNF-α dependency was still demonstrable. This is surprising on the one hand because of the speed of the reaction; on the other hand, it has been shown previously in human bronchial epithelial cells that the expression of these adhesion molecules was cytokine inducible (35).

In summary, this work demonstrates upregulation of ICAM-1 and VCAM-1 on rat AEC *in vitro* during hypoxic conditions, promoting adherence of neutrophils and macrophages to AEC. Understanding the sequence of events in the hypoxic induction of gene expression and the biologic consequences may provide future opportunities for therapeutic interventions.

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