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Abstract

Molecular mechanisms of the inflammatory reaction in hypoxia-induced lung injury are not well defined. Therefore, effects of alveolar hypoxia were studied in rat lungs, exposing rats to 10% oxygen over periods of 1, 2, 4, 6, and 8 h. An increase in the number of macrophages in bronchoalveolar lavage fluid of hypoxic animals was shown between 1 and 8 h. Extravasation of albumin was enhanced after 1 h and remained increased throughout the study period. NF-kappaB-binding activity as well as mRNA for TNF-alpha, macrophage inflammatory protein (MIP)-1beta, and monocyte chemoattractant protein (MCP)-1 were increased within the first 2 h of exposure to hypoxia. Hypoxia-inducible factor (HIF)-1alpha and intercellular adhesion molecule (ICAM)-1 mRNA were upregulated between 1 and 6 h. Elimination of alveolar macrophages by intratracheal application of liposome-encapsulated clodronate led to a decreased expression of NF-kappaB binding activity, HIF-1alpha, TNF-alpha, ICAM-1, and MIP-1beta. In summary, alveolar hypoxia induced macrophage recruitment, an increase in albumin leakage, and enhanced expression of inflammatory mediators, which were mainly macrophage dependent. Alveolar macrophages appear to have a prominent role in the inflammatory response in hypoxia-induced lung injury and the related upregulation of inflammatory mediators.

Decreased alveolar oxygen induces lung inflammation

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hypoxia; inflammatory mediators; lung injury

ACUTE ALVEOLAR HYPOXIA is a condition that occurs in various clinical situations. Causes are hypoventilation induced, for example, by brain injury, intoxication, or thoracic cage injury. Atelectasis results in a ventilation/perfusion mismatch and alveolar hypoxia as well. Pathophysiologically, acute exposure to hypoxia results in vasoconstriction of pulmonary arteries and a redistribution of blood flow from the basal to the apical portion of the lung (13). It is not well documented whether, at the same time, acute hypoxia also induces changes at the level of pulmonary inflammatory mediators. Most of the in vivo studies performed to date have concentrated on ischemia-reperfusion injury in organs (15, 18). However, the direct effects of hypoxia

without reperfusion on tissue have not been well explored. In addition, studies performed in lungs have focused on lesions after exposure to hypoxia over several days (24, 32). The acute hypoxia-induced lung injury has not been described in detail. One potential reason might be that the lungs have not been regarded as a main target organ of hypoxia. On the basis of evidence from in vitro data, we assumed that short-term hypoxia might lead to lung injury (3).

Lung inflammatory response is regulated by the coordinated function of cytokines, chemokines, and adhesion molecules. Cell adhesion molecules such as the group of selectins, integrins, and the immunoglobulin gene superfamily have been shown to play a key role in the inflammatory response, mediating different steps of leukocyte migration through the endothelium (11). Cytokines are best known for their leukocyte chemoattractant activity, whereas chemokines orchestrate the complex cellular interactions and mediate different steps of migration of leukocytes through the endothelium (11). It has been shown that macrophage inflammatory protein-1 β (MIP-1 β) plays a key role in the IgG immune complex-induced lung injury, whereas monocyte chemoattractant protein-1 (MCP-1) dominates the regulation of IgA immune complex-induced lung injury (5, 14). An important signal transduction element is the transcriptional factor nuclear factor- κ B (NF- κ B). Upon stimulation by viruses, cytokines, lipopolysaccharides (LPS), and also hypoxia, NF- κ B translocates to the nucleus. There it binds to specific promoters and induces gene transcription of various cytokines, chemokines, and adhesion molecules (1). Another transcriptional factor relevant to hypoxia is hypoxia-inducible factor-1 (HIF-1), composed of the oxygen-sensitive HIF-1 α and HIF-1 β subunits. Although the HIF-1 β subunit is expressed constitutively, HIF-1 α expression in the lung is regulated by the inspired oxygen concentration (35). Several target genes of HIF-1 α have been identified, including cyclooxygenase-1, interleukin-6, and vascular endothelial growth factor (28).

Alveolar macrophages are situated at the air-tissue interface in the alveoli and are therefore among the first cells in contact with inhaled organisms or substances. They not only act as phagocytes but also se-

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crete biologically active products, thereby playing an important role in regulating inflammatory reactions (29). Intratracheal instillation of liposomes containing dichloromethylene diphosphonate induces alveolar macrophage depletion (33). The liposome-encapsulated diphosphonate is ingested by phagocytic cells only and results in selective depletion of alveolar macrophages. This intervention allows us to characterize *in vivo* function and role of alveolar macrophages (6, 17).

The following studies were undertaken to evaluate hypoxia-induced lung injury, including changes in inflammatory mediators, and to elucidate the *in vivo* role of alveolar macrophages in this model. We hypothesized that alveolar macrophages might be the main source of cytokines and chemokines in hypoxia-induced lung alteration.

MATERIALS AND METHODS

Animal model of hypoxia. Male Sprague-Dawley rats (250–300 g) were anesthetized with Hypnorm (fentanyl-fluanisone, 0.25 ml/kg sc) and Domitor (medetomidine hydrochloride, 0.25 ml/kg sc). Animals were placed in a hypoxic chamber with decreasing oxygen tension from 21 to 10% within 60 min. Oxygen was substituted with nitrogen by a Digamix 2M 302/a-F pump (Woesthoff, Bochum, Germany). The gas flow rate was 37 l/min in a closed Perspex chamber. CO₂ tension was maintained normal. Animals remained under 10% oxygen for 1, 2, 4, 6, and 8 h, respectively. Rehydration was ensured by intraperitoneal application of saline. At predefined time points, animals were euthanized. For bronchoalveolar lavage, 10 ml of cold phosphate-buffered saline (PBS) were gently instilled into the lungs, withdrawn, and reinstalled four times and collected. All experimental procedures (determination of extravascular albumin, analysis of interstitial neutrophil accumulation, and evaluation of expression of transcriptional factors and mRNA of various genes) were performed on the same lungs (different lobes) of the respective experimental animal.

All animals were housed in individual isolator cages within the Animal Care Facilities at the University of Zurich until the day of experimentation. The experimental protocols were approved by the animal care committees at the University of Zurich.

Alveolar macrophage depletion. Clodronate-liposomes were prepared as previously described (23, 27). Briefly, liposomes composed of 880 mg soy phosphatidylcholine, 132 mg cholesterol, and 5 mg DL- α -tocopherol were prepared in a clodronate solution (375 mg clodronate in 10 ml Ostac; Boehringer, Mannheim, Germany) by freeze-thawing and filter extrusion. Unencapsulated clodronate was removed with an Amicon ultrafiltration cell, followed by size exclusion chromatography on a Sephadex G25 column. For the *in vivo* experiments, liposomes were diluted in sterile saline in a total volume of 300 μ l. Each animal received a dose of 500 μ g of liposome-encapsulated clodronate. Empty liposomes were used as controls. Rats were anesthetized, and liposomes were administered intratracheally. Hypoxia experiments were started 72 h later.

Bronchoalveolar lavage fluid. Bronchoalveolar lavage fluid (BALF) was centrifuged at 2,000 rpm. Supernatant was aliquoted and frozen at -20°C . Cell pellets from centrifuged BALF were assessed for differential cell counts using cytopins and Diff-Quick (Dade Behring, Duedingen, Switzerland). At the same time, cells were identified with neutrophil- and macrophage-specific antibodies. Cells were blocked with

PBS-10% fetal bovine serum (FBS) and incubated overnight at 4°C with primary antibody. Neutrophils were identified with a monoclonal mouse anti-rat neutrophil antibody (20 $\mu\text{g/ml}$), and macrophages with a monoclonal mouse anti-rat macrophage antibody (5 $\mu\text{g/ml}$) (both from PharMingen, San Diego, CA). A secondary fluorescein isothiocyanate (FITC)- or Cy-3-labeled goat anti-mouse antibody was added for 45 min at 4°C , together with 4,6-diamidino-2-phenylindole (1:500 diluted). The FITC-labeled antibody was diluted 1:50 in PBS-1% bovine serum albumin, the Cy-3-labeled antibody 1:500. All washing steps were performed with PBS. Cells were counted under an epifluorescence microscope.

Albumin extravasation. Extravasation of albumin was determined by a direct albumin enzyme-linked immunosorbent assay (ELISA) according to an earlier protocol (20). BALF from injured lungs (~ 8 ml) was analyzed with the ELISA. A coating carbonate buffer (0.1 M carbonate, pH 9.5) was used to dilute samples (1:1,000), and a standard curve was created with recombinant rat albumin (RDI, Flanders, NJ). A 96-well plate was coated with 100 $\mu\text{l/well}$ and incubated overnight at 4°C . All washing steps (5 times with 200 $\mu\text{l/well}$) were performed with PBS-0.05% Tween 20. To block nonspecific binding, we added 3% dry milk in PBS for 1 h at 4°C . A first polyclonal rabbit anti-rat albumin antibody (RDI) was diluted in PBS-3% dry milk to a concentration of 10 $\mu\text{g/ml}$ and incubated for 1 h at 4°C (100 $\mu\text{l/well}$). A secondary horseradish peroxidase-labeled goat anti-rabbit antibody (Sigma, Buchs, Switzerland) was added to the wells for 1 h at 4°C (100 $\mu\text{l/well}$). To develop color reaction, we added *o*-phenylenediamine dihydrochloride (Sigma) to the wells (200 $\mu\text{l/well}$). The reaction was stopped with 3 M H₂SO₄, and optical density was determined at 492 nm (ELISA reader; Bioconcept, Allschwil, Switzerland). To verify the results, BALF was also run on a SDS-PAGE gel and analyzed with Coomassie staining.

Tissue myeloperoxidase content. Lungs were homogenized in a buffer containing 50 mM potassium phosphate, 0.5% hexadecyltrimethylammonium bromide, and 5 mM ethylenediaminetetraacetic acid (EDTA), sonicated, and centrifuged as described earlier (10). Supernatant (50 μl) was added to 1,450 μl of assay buffer, consisting of 100 mM potassium phosphate, *o*-dianisidine hydrochloride, and 30% H₂O₂. The reaction was assayed every 10 s at 420 nm (ELISA reader). The results are shown as the slope of change in optical density over 360 s. Control values were defined as 1, whereas results from hypoxic lungs were normalized to the value of 1.

Nuclear extracts and electrophoretic mobility shift assay. Nuclear extracts were prepared according to Schreiber et al. (26). Briefly, lungs were homogenized in ice-cold *buffer A* [10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. Lysis was induced with 0.25% Nonidet P-40. After centrifugation, nuclear pellets were resuspended in ice-cold *buffer B* (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM DTT, and 1 mM PMSF) and rocked at 4°C for 1 h. The extracts were centrifuged, and supernatants were frozen at -70°C . The protein concentration of the extracts was determined using the Bradford method (Bio-Rad, Hercules, CA).

Nuclear extracts were then analyzed with electrophoretic mobility shift assays (EMSA) for the DNA binding activity of NF- κ B using a double-stranded oligonucleotide probe with the sequence 5'-AGT TGA GGG GAC TTT CCC AGG C-3' (consensus site underlined) (Promega, Madison, WI). End labeling was accomplished by treatment with T4 kinase in

the presence of [γ - 32 P]ATP. Extracts were then incubated for 50 min at room temperature with labeled NF- κ B oligonucleotide in binding buffer in the presence or absence of unlabeled competitor oligonucleotide. DNA-protein complexes were resolved in nondenaturing 5% acrylamide gels in 0.03% Tris-boric acid plus EDTA buffer at 270 V. Gels were dried and exposed to X-OMAT KODAK film (Sigma) and developed after 6 h.

Protein analysis (Western blot) for intercellular adhesion molecule-1. A crude membrane fraction from the lungs was obtained following a protocol described earlier (34). Whole lung protein (20 μ g) was electrophoresed in a 12.5% SDS-polyacrylamide gel. After separation, the proteins were transblotted to a nitrocellulose membrane for 1 h at 150 V (Bio-Rad). Equal loading of proteins was confirmed by Ponceau S staining. The blot was washed in PBS and blocked with PBS-5% low fat milk-0.1% Tween 20 for 1 h at room temperature, followed by an overnight incubation with monoclonal mouse anti-rat intercellular adhesion molecule (ICAM)-1 antibody (Serotec, Oxford, England) in blocking buffer. All washing steps were performed three times with PBS-0.1% Tween 20. A secondary horseradish peroxidase-labeled anti-mouse IgG (1:5,000) in blocking buffer was added for 30 min at room temperature. Signals were detected by enhanced chemiluminescence.

RNA analysis. Total RNA from lungs was extracted as previously described (3) and analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) using a kit (Perkin-Elmer, Branchburg, NJ). Random hexanucleotide primers and murine leukemia virus RT were used for cDNA synthesis. Reverse transcription was performed with 0.8 μ g of RNA at 20°C for 5 min, 42°C for 30 min, and 99°C for 5 min. Specific primers located on separate exons were designed to assess gene expression of TNF- α , ICAM-1, MIP-1 β , MCP-1, and HIF-1 α , (Table 1). RT-PCR products were resolved on 1.5% agarose gels and stained with ethidium bromide. Gels were then photographed under ultraviolet light. PCR was also performed with 18S primers to ensure equal loading.

In vitro assay with alveolar macrophages. Alveolar macrophages were collected as described previously (3). Cells (1×10^7 cells/plate) were cultured for 1 h in DMEM with glutamax (Life Technologies, Basel, Switzerland) supplemented with 10% FBS. After becoming adherent, they were exposed to 5% oxygen for 5 h, while control cells stayed at 21%. A cell incubator (Bioblock, Ittigen, Switzerland) adjustable to different oxygen concentrations by insufflation of nitrogen was used as a hypoxic cell chamber (3). Supernatant was collected and analyzed with a TNF- α and MCP-1 enzyme-linked ELISA purchased from PharMingen.

Statistical analysis. All experiments were performed at least five times. Albumin ELISA contained four to six replicates with five different animals. Each data point in the graphs represents mean \pm SE. Analysis of variance (ANOVA) with post-ANOVA comparison was performed to assess statistical significance of differences.

RESULTS

Depletion of macrophages. Before experiments with clodronate-liposomes were started, different depletion conditions were evaluated. Depletion time was varied between 24 and 72 h, with the most effective depletion observed after 72 h. Several concentrations of clodronate were used (100, 500, 1,000, and 1,500 μ g). Optimal depletion was seen at a dose of 500 μ g, whereas with increased concentrations of clodronate, enhanced neutrophil accumulation was observed. This phenomenon of neutrophil recruitment induced by clodronate is a known observation (6). Depletion was verified with macrophage staining in BALF and tissue (data not shown).

BALF cells. As seen in Fig. 1A, the cell number increased after 1 h (170% increase, $P < 0.05$), being upregulated over a period of 8 h (300% at 2 h, 240% at 4 h, 350% at 6 h, and 150% at 8 h; $P < 0.05$ for all values compared with control). This increase appeared to be almost exclusively due to macrophages as determined by fluorescence staining. Neutrophils were seen after 4 and 6 h, but the contribution to total cell count was only 13% at 4 h and 8% at 6 h ($P < 0.05$). After macrophage depletion, total cell number did not change quantitatively under hypoxia, but the total ratio of cell types changed (Fig. 1B). The increase of the number of neutrophils compared with nondepleted animals after 2 or 4 h of hypoxia was due to clodronate, as verified in normoxic control liposome and clodronate animals (6).

Hypoxia-induced neutrophil activation. Tissue myeloperoxidase (MPO) increased by 230% at 1 h ($P < 0.01$) and by 35–80% between 2 and 8 h ($P < 0.05$) (Fig. 2). Intervention with intratracheally applied clodronate-liposomes did not have an effect on MPO values at 2 and 4 h of hypoxia (data not shown).

Extravasation. Exposure to 10% oxygen caused a 100% increase in albumin extravasation as early as 1

Table 1. Optimized conditions for RT-PCR for whole lung mRNA

Gene	Primers	Thermocycle Conditions	
		No. of Cycles	Annealing Temperature, °C
TNF- α	5'-ACT GAA CTT CGG GGT GAT TG-3' 5'-GTG GGT GAG GAG CAG GTA GT-3'	27	57
ICAM-1	5'-AGG TAT CCA TCC ATC CCA CA-3' 5'-CTT CAG AGG CAG GAA ACA GG-3'	24	58
MIP-1 β	5'-CGT GTC TGC CTT CTC TCT CC-3' 5'-CAC AGA TTT GCC TGC CTT TT-3'	32	58
MCP-1	5'-TAT GCA GGT CTC TGT CAC GC-3' 5'-TTC CTT ATT GGG GTC AGC AC-3'	32	58
HIF-1 α	5'-TCA AGT CAG CAA CGT GGA AGG-3' 5'-TAT CGA GGC TGT GTC GAC TG-3'	26	58

ICAM, intercellular adhesion molecule; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; HIF, hypoxia-inducible factor.

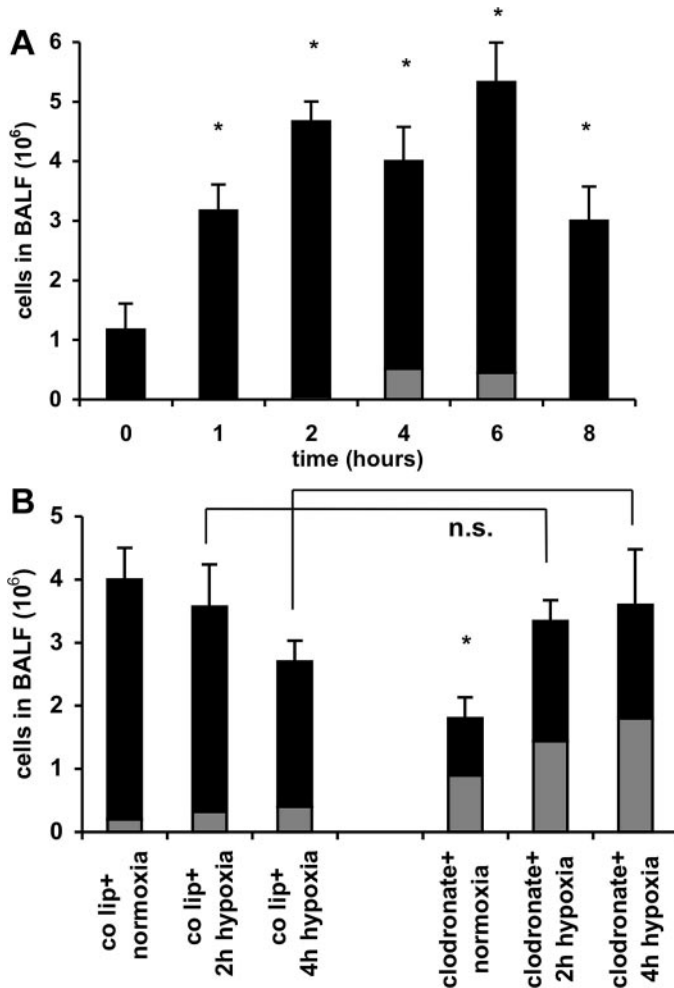


Fig. 1. A: cell analysis in bronchoalveolar lavage fluid (BALF). Rats were exposed to normoxia or hypoxia for 1, 2, 4, 6, and 8 h. The black part of the bars represents macrophages, whereas the gray part reflects neutrophils. Statistically significant difference from animals exposed to hypoxia compared with control animals: $*P < 0.05$. Values are means \pm SE from 5 animals. B: the same analysis was performed in control liposome (co lip)- and clodronate-liposome animals with normoxia and hypoxia for the time points of 2 and 4 h. Statistically significant difference from clodronate-treated animal compared with control liposome animals: $*P < 0.01$. Values are means \pm SE from 5 animals. n.s., Not significant.

and 2 h ($P < 0.05$), as assessed by the transpulmonary flux of albumin (Fig. 3A). After 4 h of hypoxia, albumin concentration even increased by 200% ($P < 0.005$). A similar result was seen in the Coomassie staining of a SDS-PAGE gel performed with BALF (Fig. 3B). To evaluate the influence of alveolar macrophages on vascular permeability, we measured albumin in BALF of normoxic and hypoxic control liposome and clodronate animals. However, there was no difference in vascular permeability between hypoxic animals with or without macrophages (data not shown).

Determination of NF- κ B. NF- κ B as an essential activator of many inflammatory mediators was determined by EMSA. As seen in Fig. 4A, a peak in NF- κ B DNA-binding activity was observed at 1 and 2 h. NF- κ B in LPS-injured lungs was assessed as positive control (4).

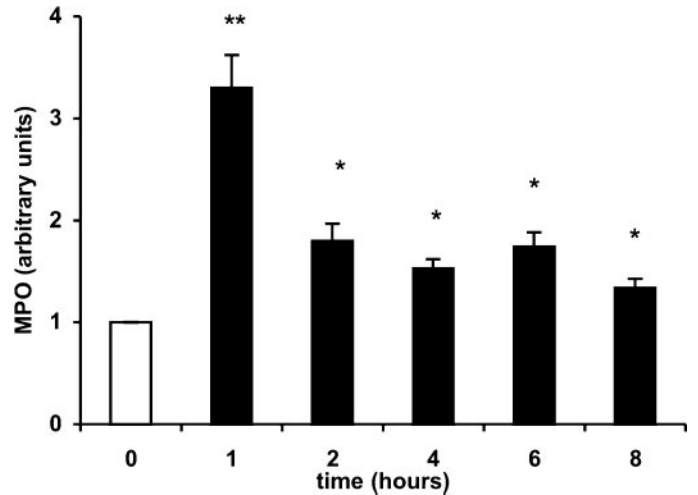


Fig. 2. Hypoxia-induced neutrophil activation in the lung. Lungs of normoxic or hypoxic animals were homogenized, and myeloperoxidase (MPO) was analyzed. Values are means \pm SE from 5 animals. Statistically significant difference from animals exposed to hypoxia compared with control animals: $*P < 0.05$, $**P < 0.01$.

Increased NF- κ B binding was completely inhibited by preincubation of nuclear extracts with an excess of unlabeled consensus oligonucleotide competitor. Clodronate-liposome pretreated animals showed less whole lung NF- κ B binding activity compared with control liposome animals after 2 h exposure to hypoxia (Fig. 4B).

Determination of mRNA for HIF-1 α . RT-PCR of whole lung HIF-1 α showed a baseline expression of HIF-1 α in control lungs. This is also known from mouse brain and skeletal muscle with HIF-1 α expression in normoxic animals (31). HIF-1 α expression was

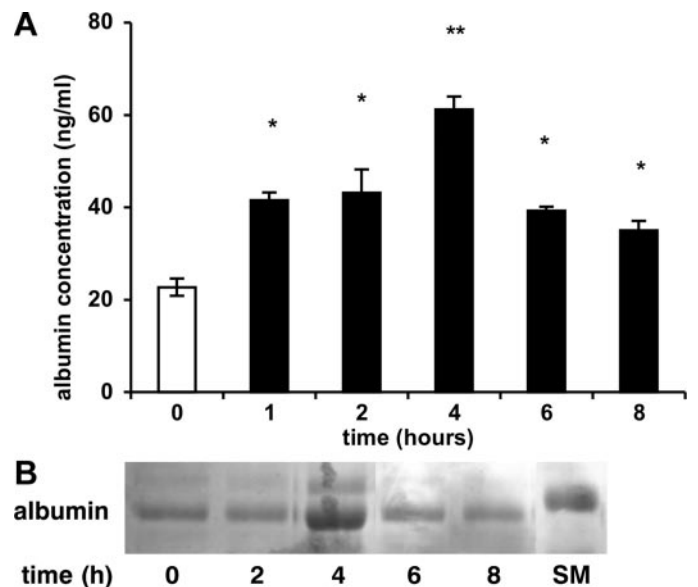


Fig. 3. Albumin extravasation. A: normoxic or hypoxic lungs were lavaged, and albumin was analyzed in BALF by ELISA. Values are means \pm SE from 5 animals. Statistically significant difference from animals exposed to hypoxia compared with control animals: $*P < 0.05$, $**P < 0.005$. B: samples were also electrophoresed on a SDS-PAGE gel and stained with Coomassie blue. SM, size marker.

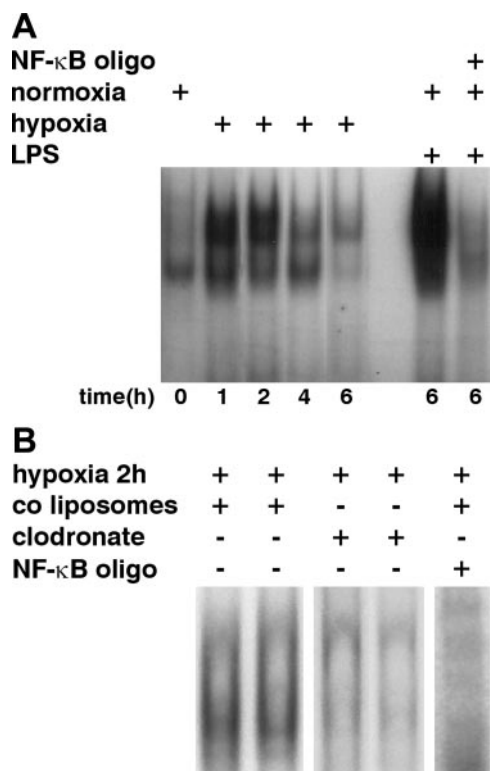


Fig. 4. Determination of NF- κ B DNA-binding activity. Nuclear elements were extracted from whole lungs previously exposed to normoxia or hypoxia for 1, 2, 4, and 6 h (A). As a positive control, nuclear extract from a 6-h LPS lung was taken. EMSA for NF- κ B was performed. The same analysis is shown in animals treated with control (co) liposomes and clodronate-liposome after 2 h exposure to normoxia and hypoxia (B). Specificity for NF- κ B was shown by using competitive unlabeled NF- κ B oligonucleotides. The blots represent 1 of 5 experiments.

increased after 1 h of hypoxia compared with control animals and remained upregulated (Fig. 5A). Interestingly, macrophage depletion led to a downregulation (Fig. 5B).

Upregulation of rat mRNA for TNF- α , ICAM-1, MIP-1 β , and MCP-1 in whole lung. To confirm equal loading, we chose 18S as an internal standard. mRNA for TNF- α increased after 1 and 2 h, leveling off within 4 h. ICAM-1 mRNA exhibited a significant increase after 1 h of hypoxia, which persisted up to 8 h after injury. MIP-1 β mRNA and mRNA for MCP-1 both peaked between 1 and 2 h (Fig. 6A). Macrophage depletion led to a clear downregulation of mRNA for TNF- α , ICAM-1, and MIP-1 β compared with control liposome animals (Fig. 6B), whereas mRNA for MCP-1 was not changed (data not shown).

To assess a potential correlation of the response of mRNA for ICAM-1 to hypoxia with ICAM-1 protein expression, we determined whole lung ICAM-1. As shown in Western blot analysis, ICAM-1 protein increased after 2 h and remained upregulated over 8 h (Fig. 6C). These results demonstrate that enhanced expression of ICAM-1 protein occurs at a transcriptional and translational level.

TNF- α and MCP-1 production of alveolar macrophages under hypoxic conditions. To analyze the in vitro expression pattern of TNF- α and MCP-1 of alveolar macrophages, we exposed cells to hypoxia for 5 h. TNF- α and MCP-1 proteins were measured in the supernatant. As seen in Fig. 7A TNF- α was increased from 54 up to 89 pg/ml (68% increase, $P < 0.01$), whereas MCP-1 production during hypoxia was enhanced by 50% ($P < 0.01$) (Fig. 7B).

DISCUSSION

These studies demonstrate for the first time that acute exposure of rats to moderate hypoxia results in a mild lung injury. The injury was characterized by the accumulation of macrophages, a modest neutrophil influx, and an increased accumulation of extravascular albumin. On the level of inflammatory mediators, DNA-binding activity of NF- κ B and expression of mRNA for HIF-1 α , TNF- α , ICAM-1, MIP-1 β , and MCP-1 were increased.

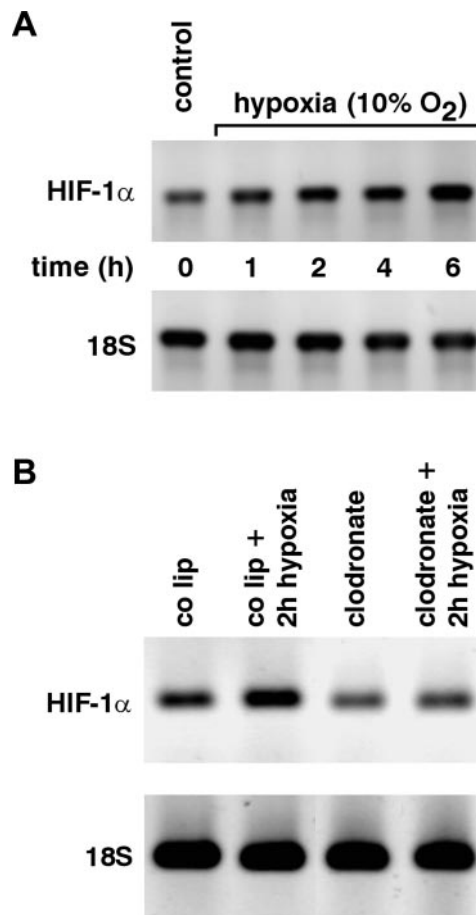


Fig. 5. Determination of hypoxia-inducible factor (HIF)-1 α mRNA. Whole lung RNA was extracted, and RT-PCR was performed according to the primers and annealing thermocycle conditions shown in Table 1. Equal loading was shown with 18S bands. A: analysis of mRNA for HIF-1 α in rat lung after 1, 2, 4, and 6 h of exposure to hypoxia. B: analysis of HIF-1 α in rat lung in control liposome (co lip) and clodronate-liposome animals after 2-h exposure to hypoxia. The blots represent 1 of 5 experiments.

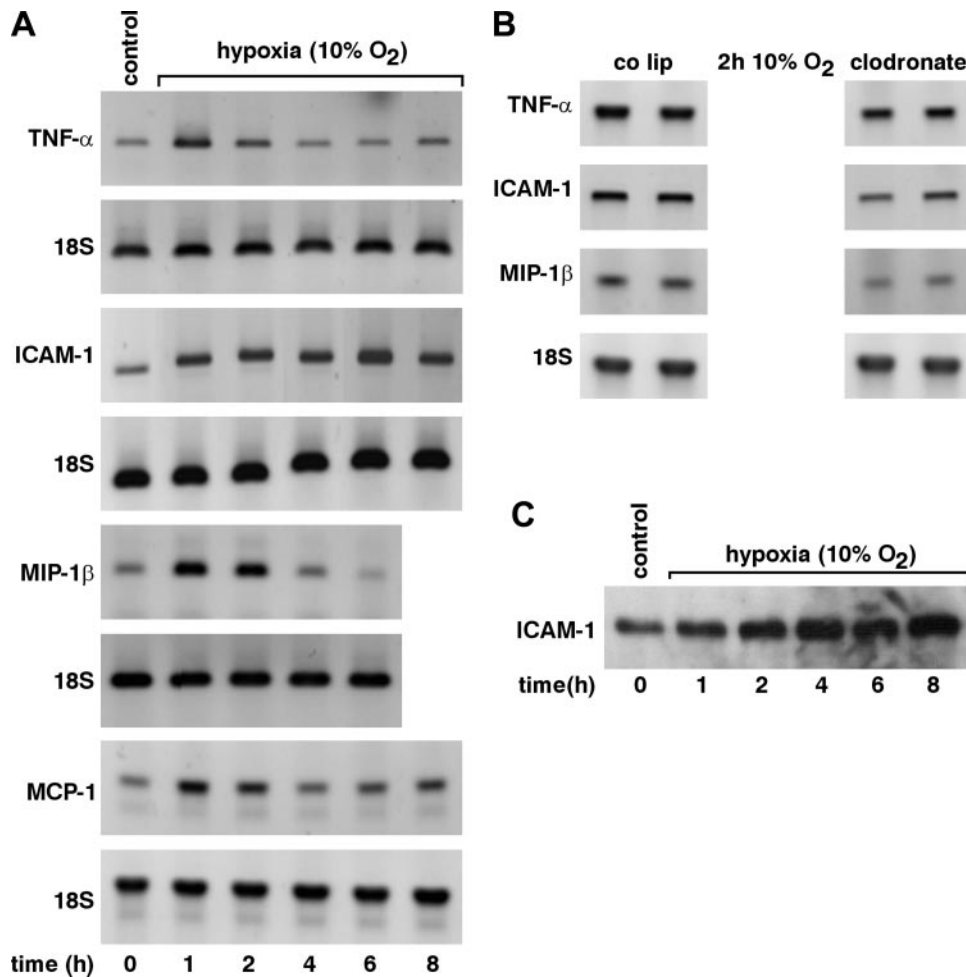


Fig. 6. Determination of whole lung mRNA of TNF- α , intercellular adhesion molecule (ICAM)-1, macrophage inflammatory protein (MIP)-1 β , and monocyte chemoattractant protein (MCP-1) (A and B) and ICAM-1 protein (C). Whole lung RNA was extracted, and RT-PCR was performed. Equal loading was shown with 18S bands. A: analysis of mediators in rat lung after 1, 2, 4, 6, and 8 h of exposure to hypoxia. B: analysis of mediators in rat lung in control liposome (co lip) and clodronate-liposome animals after 2 h of exposure to hypoxia. C: whole lung ICAM-1 protein in rat lungs exposed to normoxia or hypoxia. Membrane proteins were extracted, electrophoresed on a SDS-polyacrylamide gel, and transblotted to a nitrocellulose membrane. ICAM-1 was determined after 1, 2, 4, 6, and 8 h of exposure to hypoxia. The blots represent 1 of 5 experiments.

Compared with other acute inflammatory processes in the lung such as LPS-induced or immune complex-induced lung injury, only a slight increase of mRNA and protein for ICAM-1 under hypoxia was observed (4, 19, 25). Whole lung mRNA for ICAM-1 was increased by 300% in the LPS lung injury and by 250% in the IgG immune complex-induced lung injury. In the hypoxia-induced lesion, however, only a 77% increase of mRNA for ICAM-1 was measured. Our previous *in vitro* studies support these data: alveolar epithelial cells (AEC) under LPS stimulation showed a 600% increase of mRNA for ICAM-1, whereas hypoxia led to a 100% upregulation (3). One reason for the observed differences could be the severity of the injury, which is high in the IgG- and LPS-induced inflammation models with capillary leakage and a recruitment of neutrophils. The hypoxia-induced injury, however, results in a mild lesion. Another explanation could be a different functional role of ICAM-1 in the hypoxia-induced lung injury. O'Brien et al. (21) recently showed that ICAM-1 on AEC played an important role in host defense against *Klebsiella pneumoniae*. A protective function of the upregulated adhesion molecule ICAM-1 could also play a role in the hypoxic lesion. Furthermore, it could be hypothesized that the inflammatory changes caused by hypoxia represent a priming condition and gain in

severity in conjunction with an ensuing superimposed injury. It could be shown in a cell model with alveolar macrophages that LPS stimulation under hypoxia increased injury compared with LPS stimulation alone (16). Similar results were seen in a sepsis model of lung injury. Under sublethal conditions, there was no evidence of an inflammatory response in lungs of animals with cecal ligation/puncture-induced sepsis alone. However, after a direct intrapulmonary insult, enhanced lung injury in septic animals was observed (7).

The fact that vascular leakage is increased by chronic hypoxia is not new. Stelzner et al. (30) showed *in vivo* increased pulmonary extravasation of albumin in rat lungs after 48 h of exposure to hypobaric hypoxia. However, short-term exposure to hypoxia (1, 3, 6, and 13 h) did not cause significant increases in extravasation. These findings are not in accordance with our results. Differences between the experimental systems may partly explain this, since we were exclusively investigating under decreased oxygen concentrations. Stelzner et al., however, applied hypobaric hypoxia. Although we determined extravascular albumin, our results, however, do not explain the origin of the albumin (exudate, transudate). Theoretically, the interstitial accumulation of albumin might be due to an enhanced inflammation-induced vascular permeability,

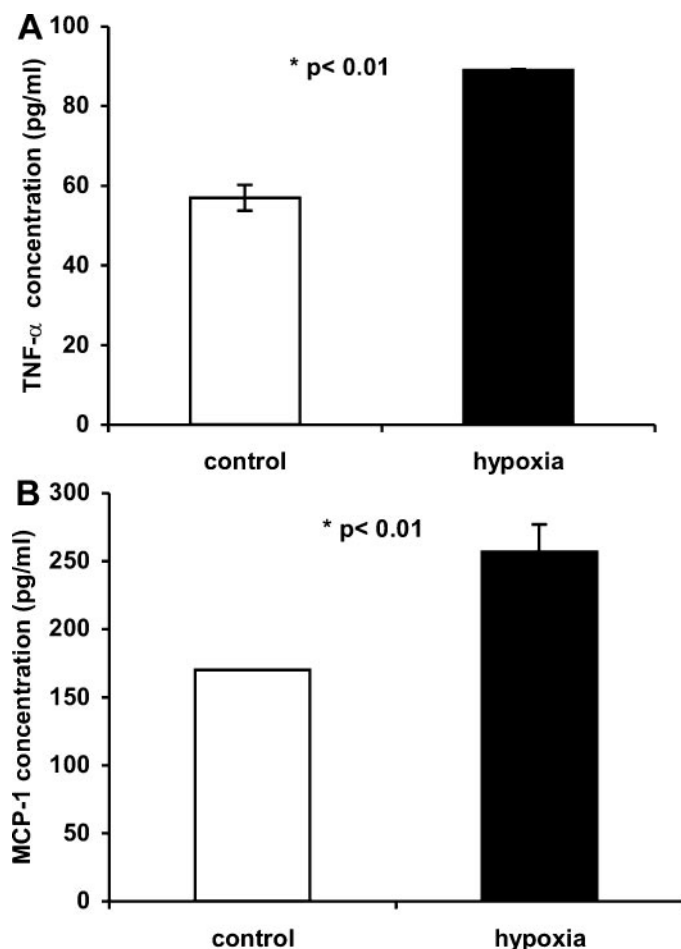


Fig. 7. Determination of TNF- α and MCP-1 protein produced by alveolar macrophages *in vitro*. TNF- α (A) and MCP-1 (B) were measured by ELISA in the supernatant of hypoxia-exposed alveolar macrophages (5% oxygen, 5 h) and in control supernatants (21% oxygen, 5 h). Values are means \pm SE of 5 different experimental sets with duplicates.

but also to hypoxic vasoconstriction with transudation. This question was previously analyzed with the help of a special experimental setup (30). Stelzner et al. measured hemodynamic changes in the pulmonary vascular system as well as protein leakage in hypoxic rat lungs. Although mean pulmonary pressures in hypoxic animals were increased compared with pressure in normoxic animals, no significant differences in protein leakage index were seen in the two different groups, assuming that hypoxic vasoconstriction is not responsible for increased extravasation of albumin.

An interesting finding in our studies was the transient increase of mainly macrophages in BALF under hypoxia. Compared with other models such as LPS- or IgG-induced lung injury, neutrophil recruitment was minimal. To evaluate whether these alveolar macrophages were the main source of mRNA of inflammatory mediators in the case of hypoxia-induced lung injury, we performed macrophage depletion. Theoretically, other potential sources such as interstitial macrophages or nonmacrophage cell types might be responsible for the production of mRNA of inflammatory me-

diators. Macrophage depletion, however, showed that mRNA of the mediators were produced in alveolar macrophages or at least induced by them. Hypoxia-induced NF- κ B activation in whole lung tissue seems to be macrophage dependent as well. This implies that products of activated alveolar macrophages are required to stimulate nuclear translocation of NF- κ B, which has been shown in the IgG model (17). The same hypothesis can also be applied to enhanced whole lung HIF-1 α , which is also attenuated by macrophage depletion. Again, activation of HIF-1 α might be triggered by alveolar macrophages or even produced by macrophages. The fact that mRNA of inflammatory mediators is not completely abolished suggests the presence of other sources for inflammatory mediators such as interstitial macrophages, AEC, or endothelial cells. It is well known that AEC are able to endocytose liposomes (12). In view of the less than perfectly complete macrophage depletion, a small residual fraction of alveolar macrophages could be another source of inflammatory mediators. Uneven distribution of the intratracheal liposomes cannot be fully excluded in this context. An interesting finding was the observation that macrophage depletion did not affect MCP-1 expression compared with other inflammatory mediators. Macrophages thus do not appear to be a main source of MCP-1. Epithelial cells might be a more important production site for MCP-1 as has been previously shown in the kidney in tubular epithelial cells (2).

A very important aspect of the data in this model is not only the inflammatory component, but also physiological mechanisms. Alveolar hypoxia might increase ventilation and therefore cause a mechanical distension of lung tissue. Few publications examine the impact of hyperpnea on inflammatory mechanisms. Most of these studies, however, were performed with dry air, and experiments evaluated the effect of repetitive hyperpnea (8, 9, 22). Their results do not correlate with our experiments. A potential impact of hyperpnea in our model cannot be fully excluded.

MPO data provided valuable information. It could be shown that hypoxia induced a slight interstitial accumulation of neutrophils at an early time point of inflammation. Compared with other models of lung inflammation such as LPS-induced lung injury, however, the magnitude of neutrophil infiltration was small. These results support our interpretation that neutrophils are not the main effector cells in this model.

To further demonstrate the importance of alveolar macrophages in the hypoxia-induced inflammation, we studied the *in vitro* expression pattern of TNF- α of alveolar macrophages. Interestingly, the increase of TNF- α production was not impressive compared with the *in vivo* data. Therefore, we assume that not only the direct input of alveolar macrophages is central in the hypoxic inflammatory reaction but also the indirect influence of these cells on other cell types in the lung. Similar results were also seen in IgG lung injury model (17).

In conclusion, this study demonstrates that acute hypoxia results in inflammatory changes in the lung representing a mild lung injury, whereby alveolar mac-

rophages are the main effector cells during this inflammatory response. The precise interaction of effector cells with target cells in this model of acute hypoxia will be further investigated.

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