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Effect of steroid on hyperoxia-induced ICAM-1 expression in pulmonary endothelial cells

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Suzuki, Yukio, Kazumi Nishio, Kei Takeshita, Osamu Takeuchi, Kenji Watanabe, Nagato Sato, Katsuhiko Naoki, Hiroyasu Kudo, Takuya Aoki, and Kazuhiro Yamaguchi. Effect of steroid on hyperoxia-induced ICAM-1 expression in pulmonary endothelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 278: L245–L252, 2000.—Intercellular adhesion molecule-1 (ICAM-1) of the vascular endothelium plays a key role in the development of pulmonary oxygen toxicity. We studied the effect of steroid on hyperoxia-induced ICAM-1 expression using cultured endothelial cells in vitro. Human pulmonary artery endothelial cells (HPAECs) were cultured to confluence, and then the monolayers were exposed to either control (21% O_2-5% CO_2) or hyperoxic (90% O_2-5% CO_2) conditions with and without a synthetic glucocorticoid, methylprednisolone (MP). MP reduced hyperoxia-induced ICAM-1 and ICAM-1 mRNA expression in a dose-dependent manner. Neutrophil adhesion to hyperoxia-exposed endothelial cells was also inhibited by MP treatment. In addition, MP attenuated hyperoxia-induced H_2O_2 production in HPAECs as assessed by flow cytometry. An electrophoretic mobility shift assay demonstrated that hyperoxia activated nuclear factor-κB (NF-κB) but not activator protein-1 (AP-1) and that MP attenuated hyperoxia-induced NF-κB activation dose dependently. With Western immunoblot analysis, IκB-α expression was decreased by hyperoxia and increased by MP treatment. These results suggest that MP downregulates hyperoxia-induced ICAM-1 expression by inhibiting NF-κB activation via increased IκB-α expression.

intercellular adhesion molecule-1; nuclear factor-κB; inhibitory protein IκB-α; glucocorticoid

PULMONARY OXYGEN TOXICITY is an important clinical problem that occurs in patients on long-term mechanical ventilation requiring a high inspired oxygen concentration. The lungs of these patients are chronically exposed to a hyperoxic environment. The most common histopathological evidence of pulmonary oxygen toxicity is pulmonary edema, with neutrophil infiltration into the lung parenchyma (13). The mechanism by which hyperoxia causes lung injury has been shown to be through the increased formation of reactive oxygen species (ROS) (14). ROS then produce cellular damage directly through lipid peroxidation and indirectly by increasing the expression of proinflammatory cytokines, adhesion molecules, and other inflammatory mediators. The endothelial cell inflammatory response is believed to play a central role in the pathogenesis of oxidant lung injury and is also considered pivotal in mediating neutrophil migration into the lung parenchyma.

A variety of adhesion molecules on the surfaces of endothelial cells and neutrophils mediate adherence to the vascular endothelium, which must occur for neutrophils to migrate into an inflammatory site. Suzuki et al. (29) previously reported that the exposure of human pulmonary artery endothelial cells (HPAECs) and human umbilical vein endothelial cells (HUVECs) to hyperoxia selectively induces intercellular adhesion molecule-1 (ICAM-1) and ICAM-1 mRNA expression. ICAM-1 induction, which requires de novo protein synthesis, and the interaction with its receptors on neutrophils play important roles in regulating the retention, migration, and activation of neutrophils in the lungs. It has recently been shown (17) that transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) participate in regulating the gene expression of many modulators of inflammatory and immune responses, including ICAM-1. In NF-κB activation, a ROS such as H_2O_2 is considered to serve as one of the key messengers (26).

Pulmonary oxygen toxicity is different from other inflammatory lung diseases such as endotoxin-induced lung injury because the stimuli mediated by oxygen are relatively subacute and moderate compared with those mediated by endotoxin or inflammatory cytokines. Therefore, the interaction between the endothelium and neutrophils in the development of pulmonary oxygen toxicity is not fully understood, and currently, no commonly accepted treatment exists for patients suffering from hyperoxia. Although the effectiveness of steroids in oxidant lung injury remains controversial, several kinds of steroids are used clinically in the treatment of a variety of inflammatory diseases. In particular, a synthetic glucocorticoid, methylprednisolone (MP), has been clinically and widely used as steroid pulse therapy for immunoreactive diseases including rheumatoid arthritis (30), acute allograft rejec-

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tion after kidney transplantation (21), bronchial asthma (8), idiopathic pulmonary fibrosis (10), and bronchiolitis obliterans after lung transplantation (28). In experimental studies, MP inhibited the endotoxin-induced lung vascular permeability in sheep (5), ROS-induced increase in microvascular permeability of isolated rat lungs (15), and zymosan-induced pulmonary damage in rabbits (6). The molecular mechanism of MP in the treatment of oxidant lung injury such as pulmonary oxygen toxicity has not been fully characterized.

This prompted us to study the mechanisms by which MP exerts its anti-inflammatory activity against pulmonary oxygen toxicity in terms of adhesion molecules with the use of cultured endothelial cells. The purpose of this study was to investigate the effect of MP on ICAM-1 expression, ICAM-1 mRNA expression, neutrophil adhesion to endothelial cells, and the DNA binding activity of NF-κB and AP-1 in hyperoxia-exposed endothelial cells.

MATERIALS AND METHODS

Endothelial cell culture. HPAECs (Kurabo, Osaka, Japan) were cultured in an endothelial cell growth medium supplemented with 10% fetal calf serum, penicillin G (100 U/ml), and streptomycin (100 μg/ml; GIBCO BRL, Life Technologies, Grand Island, NY) at 37°C and 5% CO_2 in a humidified incubator. The cells used for experiments were from *passages 8* to *12*. The endothelial cells were grown on 25-cm^2 tissue culture flasks (Corning, New York, NY) and then subcultured with 0.025% trypsin-0.05 mM EDTA (GIBCO BRL). The endothelial cells were identified by their characteristic cobblestone monolayer appearance, typical ultrastructure, and the presence of von Willebrand factor antigen as confirmed by indirect immunofluorescence staining (DAKO Japan, Tokyo, Japan). Cell viability always exceeded 95% as determined by the trypan blue exclusion test.

Exposure of endothelial cell monolayers to hyperoxia. Endothelial cell monolayers cultured to confluence were exposed to control (21% O_2-5% CO_2, 1 atm) or hyperoxic (90% O_2-5% CO_2, 1 atm) conditions for various periods at 37°C in a humidified multigas incubator (APM-36, ASTEC, Fukuoka, Japan). In some experiments, endothelial cells were exposed to hyperoxia plus various concentrations of MP (11β,17,21-trihydroxy-6α-methyl-1,4-pregnadiene-3,20-dione-21-sodium succinate, $C_{26}H_{33}NaO_8$, mol wt 497; Upjohn, Kalamazoo, MI). MP dissolved in sterile normal saline was added to the culture medium just before hyperoxic exposure. The oxygen concentration was monitored continuously and maintained at 90 ± 0.5% during the entire hyperoxic exposure. P_{O_2} and P_{CO_2} in the medium were measured in preliminary experiments with a blood gas analyzer (model 178, Corning). After a 15-min exposure to hyperoxia, P_{O_2} in the medium was consistently >580 Torr. The culture medium had the same pH value under control and hyperoxic conditions. Viability of the endothelial cells under hyperoxic conditions exceeded 85% within 72 h as measured by the trypan blue exclusion test. Under control conditions, endothelial cell viabilities were >95% up to 48 h without MP and 91 ± 6% at 24 h and 68 ± 5% at 48 h with 10 mM MP. Under hyperoxic conditions, the viabilities were 96 ± 8% at 24 h and 92 ± 7% at 48 h without MP and 89 ± 9% at 24 h and 62 ± 7% at 48 h with 10 mM MP. Under phase-contrast microscopy, endothelial monolayers appeared morphologically normal at 72 h of exposure to hyperoxia.

Flow cytometric analysis. The level of ICAM-1 expression in the endothelial cells was measured by flow cytometry. Endothelial cells with and without MP were detached by treatment with 0.1% EDTA (Sigma, St. Louis, MO) for 1 min at 37°C and washed with Dulbecco's phosphate-buffered saline (DPBS). The suspended cells were incubated with phycoerythrin-conjugated anti-human ICAM-1 monoclonal antibody (LB-2, Becton Dickinson, San Jose, CA) for 30 min at 4°C. The cells were washed three times with DPBS, fixed with 1% paraformaldehyde (Sigma), and then analyzed. The intensity of fluorescence and the light-scattering properties of the cells were determined with a FACScan flow cytometry system equipped with an argon laser (488-nm emission, 15-mW output; Becton Dickinson). Phycoerythrin red fluorescence was collected between 564 and 606 nm with a bandpass filter. All analyses were run simultaneously with a mouse isotype (IgG2) control antibody (Becton Dickinson), and the values obtained were subtracted. In each sample, 10,000 endothelial cells were examined. The list mode was evaluated with the Lysis II program (Becton Dickinson). All experiments were done in quadruplicate, and the results are expressed as the percent intensity of fluorescence compared with that at *time 0*.

ICAM-1 mRNA expression with RT-PCR. The effect of MP on hyperoxia-induced ICAM-1 mRNA expression was analyzed with RT-PCR. Total RNA was extracted from the endothelial cells. First-strand cDNA was synthesized from 10 μg of RNA by SuperScript RT (GIBCO BRL, Life Technologies, Gaithersburg, MD) following the manufacturer's instructions. We amplified synthesized first-strand cDNA with PCR (Perkin-Elmer Cetus, Norwalk, CT) with 50 pmol of the 5′and 3′ primers with 2.5 U of *Taq* polymerase (Takara Biomedicals, Kyoto, Japan) in a total volume of 50 μl. The reaction buffer consisted of 10 mM Tris·HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, and 10 mM deoxynucleotide triphosphates. PCR cycles were allowed to run for 30 s at 94°C, followed by 30 s at 55°C and 1 min at 72°C, and a final extension at 72°C for 10 min. The 5′ and 3′ primers were 5′-TGACCATCTACAGCTTTCCGCC-3′ and 5′-GTCTGAGGTTACACGGTCCGA-3′, respectively (24). Human glyceraldehyde-3-phosphate dehydrogenase primers (Clontech Laboratories, Palo Alto, CA) were used as an internal control. The 5′ and 3′ primers were 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and 5′-CATGTGGGCCATGAGGTCCACCAC-3′, respectively. A 10-μl aliquot of the amplified DNA reaction mixture was fractionated by 2.0% agarose gel electrophoresis, and the amplified product was then visualized by ultraviolet fluorescence after being stained with ethidium bromide. To quantify the levels of mRNA, a standard curve was obtained by titration of RNA from hyperoxia-exposed endothelial cells. The difference in mRNA expression among test samples was determined from the standard curve obtained by running the same number of cycles as unknown samples. The specificity of the amplified products was validated by their predicted size on agarose gel.

Neutrophil adhesion assay. To determine the effects of MP on neutrophil adherence to hyperoxia-exposed endothelial cells, we conducted adhesion assays. Human peripheral blood neutrophils were obtained from healthy adult volunteers and separated on a discontinuous gradient consisting of Histopaque 1077 and 1119 (Sigma). Neutrophils were resuspended in DPBS at a final concentration of 5×10^5 cells/ml. Neutrophil purity exceeded 98% as confirmed by a modified Wright's stain (Diff-Quik Stain Set, American Scientific Products, McGaw Park, IL). Cell viability by trypan blue exclusion exceeded 98%. One hundred microliters of isolated neutrophils (5×10^5 cells/ml) were applied to HPAEC monolayers in six-well tissue culture plates (Corning) that had previously been exposed to either control (21% O_2-5% CO_2) or hyperoxic

(90% O_2-5% CO_2) conditions in a humidified multigas incubator (APM-36, ASTEC) for 48 h. In some experiments, endothelial monolayers were exposed to hyperoxia with anti-ICAM-1 monoclonal antibody (KM972, Kyowa Medex, Tokyo, Japan) that was added to the culture medium just before the hyperoxic exposure. The plates were then incubated for 60 min at 37°C under 5% CO_2 in a humidified incubator. Nonadherent neutrophils were removed by gently washing the plates three times with prewarmed DPBS. Ten randomly selected fields were read at ×200 magnification under a light microscope. Neutrophil adhesion was evaluated by counting the number of neutrophils adhering to the endothelial cell monolayer.

Intracellular H_2O_2 production in endothelial cells. A ROS such as H_2O_2 is considered to serve as one of the key messengers in NF-κB activation (26). Therefore, to evaluate the effects of MP on intracellular H_2O_2 production in HPAECs, we used 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes, Eugene, OR). DCFH-DA is freely permeable across cell membranes and is oxidized by intracellular H_2O_2, then converted to green fluorescent 2′,7′-dichlorofluorescein (DCF) (29). Endothelial cell monolayers cultured to confluence were incubated with 10 μM DCFH-DA for 60 min at 37°C, washed, and then exposed to either control (21% O_2-5% CO_2) or hyperoxic (90% O_2-5% CO_2) conditions for 4 h at 37°C in a humidified multigas incubator (APM-36). In some experiments, endothelial cells were exposed to hyperoxia plus various concentrations of MP. The endothelial cells were washed with DPBS twice and collected with rubber scraper. Intracellular H_2O_2 production in HPAECs was assessed by measuring DCF fluorescence with flow cytometry. In each sample, 10,000 cells were examined. Fluorescence was collected at 530 nm by employing a band-pass filter, and the list mode was analyzed with the Lysis II program system. All experiments were performed in quadruplicate, and the results are expressed as the percent intensity of fluorescence compared with the control value.

Nuclear protein extracts and electrophoretic mobility shift assay. Endothelial cell monolayers were exposed to either control or hyperoxic conditions and treated with and without MP. The cells were washed with ice-cold DPBS, scraped with a cell scraper, and then collected and resuspended in 2 ml of cold DPBS. The cells were pelleted for 10 s and resuspended in 400 μl of cold *buffer A* [10 mM HEPES-KOH, pH 7.9, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)] by flicking the tube. The cells were allowed to swell on ice for 10 min and were then vortexed for 10 s. The samples were centrifuged for 10 s, and the pellet was resuspended in 20 μl of cold *buffer C* (20 mM HEPES-KOH, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF, and 25% glycerol) and incubated on ice for 20 min. Cellular debris was removed by centrifugation for 5 min at 15,000 rpm and 4°C, and the supernatant fraction was stored at −80°C. The protein concentration was determined with a bicinchoninic acid protein assay (Pierce, Rockford, IL) with BSA (Sigma) as a standard. The yield was 10–20 μg protein/10^6 cells. Double-stranded NF-κB and AP-1 consensus oligonucleotide probes were 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (19) and 5′-CGCTTGATGACTCAGCCGGAA-3′, respectively (18); the binding sites are underlined. The oligonucleotides were annealed with the complementary strand to produce 5′-overhanging ends (*Bam*H I), which enabled labeling by Klenow polymerase (Amersham, Tokyo, Japan) in the presence of deoxynucleotide triphosphates and [^{32}P]dCTP (3,000 Ci/mmol; Amersham). The labeled oligonucleotide was purified on push columns (Stratagene, La Jolla, CA). The typical binding reaction of 20 μl contained 10,000 counts/min of ^{32}P-labeled double-stranded oligonucleotide, 10 μg of nuclear extraction in *buffer C*, 20 μg of BSA, and 2 μg of poly(dI-dC) (Sigma) in gel shift binding buffer. Specific binding was confirmed by competition with a 100-fold excess of unlabeled double-stranded oligonucleotide competitor. For the supershift assay, the antibody against p65 (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the nuclear extracts for 10 min before the addition of radiolabeled probe. After incubation for 20 min at room temperature, samples were analyzed on 4% native acrylamide gels run at 170 V for 1.5 h in 0.5× Tris-borate-EDTA buffer. The gels were then dried and visualized by autoradiography.

Western immunoblot analysis. For immunoblot analysis, 100-mm dishes of confluent cells with and without MP were exposed to either control conditions or hyperoxia for various periods, and then the cells were washed with DPBS and lysed on ice in modified radioimmunoprecipitation assay buffer containing PMSF solution and aprotinin. In some experiments, 1 μg/ml of lipopolysaccharide (LPS; 955:B5; Sigma) was added as a positive control. The lysate was centrifuged at 15,000 rpm for 20 min, and the protein concentration was determined with a bicinchoninic acid protein assay. Samples with equal amounts of protein were mixed with SDS sample buffer consisting of 20% glycerol, 4% SDS, 0.16 M Tris (pH 6.8), 4% β-mercaptoethanol, and 0.5% bromphenol blue and then boiled for 90 s. Ten micrograms of each sample were fractionated on a 10% SDS-polyacrylamide gel, transferred onto an immobilon-polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA), and incubated with an antibody against IκB-α (C-21; Santa Cruz). The primary antibody was counterstained with horseradish peroxidase-conjugated rabbit IgG antibody, visualized with an enhanced chemiluminescence detection kit (Pierce) according to the manufacturer's instructions, and then exposed to photographic films. Hybridization signals on films were quantified by scanning densitometry (NIH Image Program, National Institutes of Health, Bethesda, MD). All experiments were done in quadruplicate, and the results are expressed as the percent density compared with the control value.

Statistical analysis. All data are presented as means ± SD. Two-way analysis of variance and Fisher's paired least significant difference test were used to detect differences among groups (StatView II, Abacus Concepts, Berkeley, CA). A P value of <0.05 indicated significant differences between the means.

RESULTS

The effect of MP on hyperoxia-induced ICAM-1 expression was studied with flow cytometry (Fig. 1). ICAM-1 expression increased in hyperoxia-exposed HPAECs (167.4 ± 25.5%) compared with the control value (100.0 ± 15.2%; $P < 0.01$). Treatment with MP (1 and 10 mM) attenuated hyperoxia-induced ICAM-1 expression in HPAECs (124.7 ± 8.8 and 56.5 ± 11.3%, respectively; $P < 0.01$).

Hyperoxic exposure for 48 h upregulated ICAM-1 mRNA expression in HPAECs as previously reported (29). We therefore examined the effect of MP on hyperoxia-induced ICAM-1 mRNA expression (Fig. 2). Hyperoxia-induced ICAM-1 mRNA was attenuated by MP dose dependently.

We studied whether the treatment of endothelial cells with MP inhibited neutrophil adhesion to endothelial cells that had been previously exposed to hyperoxia

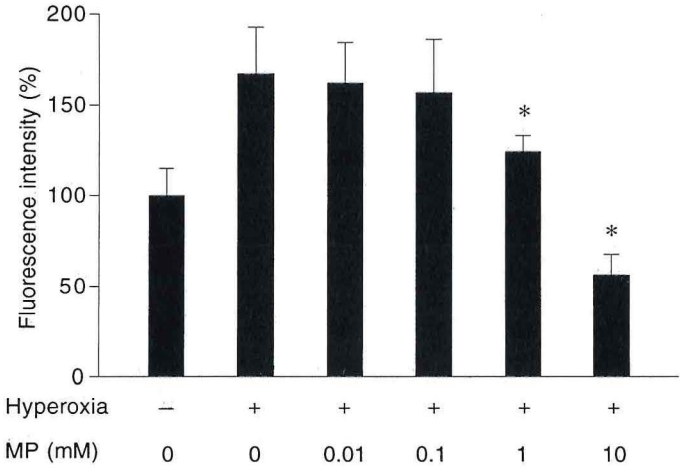

Fig. 1. Inhibition of hyperoxia-induced intercellular adhesion molecule (ICAM)-1 expression by methylprednisolone (MP). Endothelial cell monolayers were exposed to either control conditions (−) or hyperoxia (+) for 48 h with and without indicated concentrations of MP. Level of ICAM-1 expression was assessed with flow cytometry. Treatment with MP (1 and 10 mM) significantly attenuated hyperoxia-induced ICAM-1 expression. $*P < 0.05$ compared with hyperoxia without MP.

for 48 h (Fig. 3). Neutrophil adhesion increased in hyperoxia-exposed HPAECs (17.1 ± 6.0 cells/mm^2) compared with the control value (6.2 ± 2.3 cells/mm^2; $P < 0.01$) and was attenuated by treatment with anti-ICAM-1 monoclonal antibody (11.3 ± 2.5 cells/mm^2). Treatment with 0.1 and 1 mM MP reduced the number of neutrophils adhering to hyperoxia-exposed HPAECs (6.9 ± 2.5 and 3.8 ± 2.1 cells/mm^2, respectively; $P < 0.01$).

The effect of MP on hyperoxia-induced H_2O_2 production in HPAECs as assessed by using DCFH-DA is shown in Fig. 4. The DCF fluorescence was increased by hyperoxic exposure for 4 h (167 ± 19%) compared with the control value. MP reduced hyperoxia-induced DCF fluorescence dose dependently. One micromolar MP attenuated hyperoxia-induced H_2O_2 production (91 ± 16%; $P < 0.05$) compared with that with hyperoxia without MP.

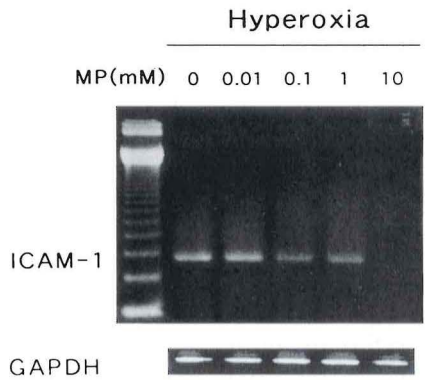

Fig. 2. Downregulation of hyperoxia-induced ICAM-1 mRNA expression by MP. Endothelial cell monolayers were exposed to hyperoxia for 48 h with and without MP. ICAM-1 (351 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 983 bp) mRNA expression were analyzed with RT-PCR. MP downregulated hyperoxia-induced ICAM-1 mRNA expression dose dependently.

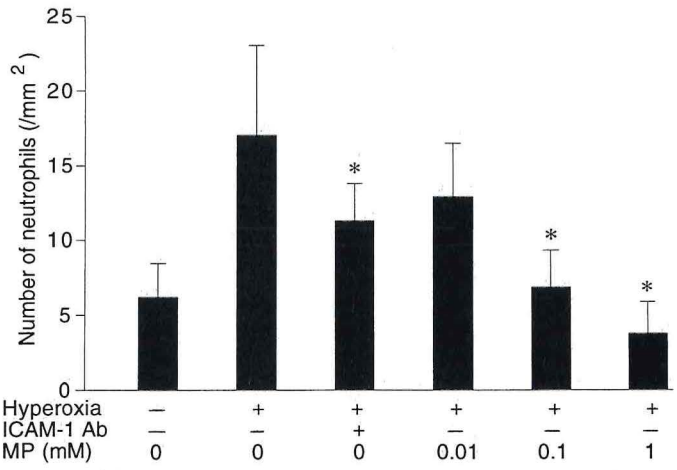

Fig. 3. Inhibition of neutrophil adhesion to hyperoxia-exposed endothelial cells by MP. Isolated human neutrophils were applied to endothelial cell monolayers, which had previously been exposed to either control conditions or hyperoxia for 48 h with and without an anti-ICAM-1 monoclonal antibody and MP. Neutrophil adhesion was evaluated by counting number of neutrophils adhering to endothelial cell monolayers under a light microscope. Ab, antibody. Adhered neutrophils were significantly reduced by treatment with an anti-ICAM-1 monoclonal antibody and MP (0.1 and 1 mM). $*P < 0.05$ compared with hyperoxia without MP.

Transcription factor NF-κB and AP-1 binding sites were identified in the promoter of the ICAM-1 gene, which participates in regulating ICAM-1 expression (17). We therefore investigated the effect of hyperoxia on NF-κB and AP-1 DNA binding activities by electrophoretic mobility shift assay (Fig. 5). NF-κB was activated by hyperoxic exposure for 2 h and returned to baseline level for 4 h. On the other hand, AP-1 was not activated by hyperoxia during the experiment.

Because it has been shown that hyperoxia selectively activated NF-κB, we studied the effect of MP on hyperoxia-induced NF-κB DNA binding activity (Fig. 6). Endothelial cells were exposed to hyperoxia for 2 h with and without MP and subjected to electrophoretic mobil-

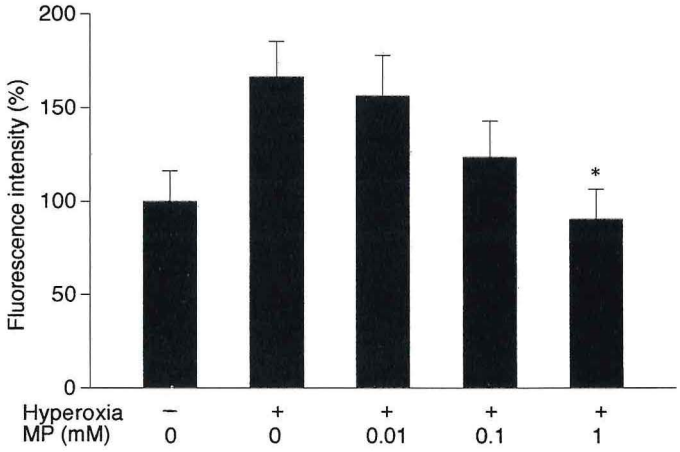

Fig. 4. Attenuation of 2′,7′-dichlorofluorescein (DCF) fluorescence in endothelial cells by MP. Endothelial cell monolayers incubated with 2′,7′-dichlorofluorescin diacetate were exposed to hyperoxia with indicated concentrations of MP. Intensity of DCF fluorescence was measured with flow cytometry. Treatment with MP (1 mM) attenuated hyperoxia-induced DCF fluorescence. $*P < 0.05$ compared with hyperoxia without MP.

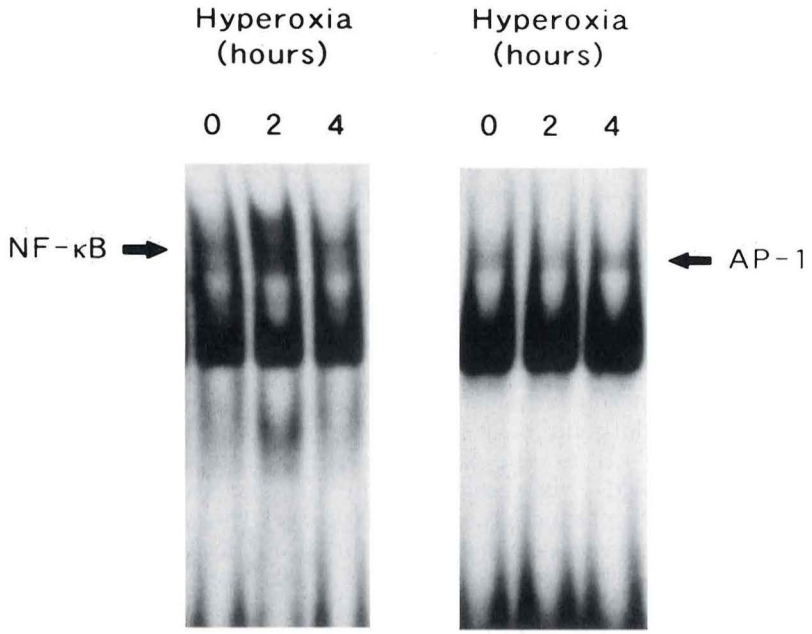

Fig. 5. Differential activation of nuclear factor (NF)-κB and activator protein (AP)-1 by hyperoxia. Endothelial cell monolayers were exposed to either control conditions or hyperoxia, and NF-κB and AP-1 DNA binding activities were examined by electrophoretic mobility shift assay. NF-κB was activated at 2 h of hyperoxic exposure and then returned to baseline level at 4 h. In contrast, hyperoxia did not affect AP-1 activity during experiment.

ity shift assay. Hyperoxia-induced NF-κB activation was reduced by MP dose dependently.

IκB-α is an inhibitory protein that prevents nuclear transport and activation of the transcription factor NF-κB. At first, the time course of IκB-α expression under hyperoxic conditions was studied with Western immunoblot analysis and then compared with LPS treatment as a positive control (Fig. 7). The expression of IκB-α under hyperoxic conditions was unchanged up to 2 h but was attenuated at 4 h and then returned to the baseline level at 6 h. In contrast, IκB-α expression was decreased by treatment with LPS at 1–2 h and then partially returned to the baseline level at 4–6 h.

We further examined the effect of MP on hyperoxia-induced IκB-α expression in endothelial cells (Fig. 8). Endothelial cell monolayers were exposed to either control or hyperoxic conditions for 4 h with and without MP. The level of IκB-α expression was decreased by hyperoxia compared with that under control conditions and was increased by MP treatment. On the other hand, IκB-α expression was strongly inhibited by LPS and recovered by MP treatment.

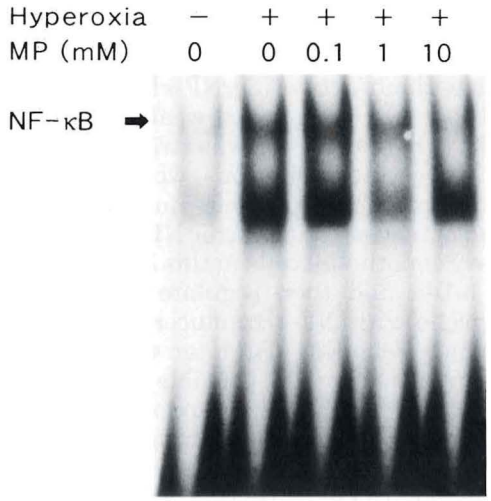

Fig. 6. Attenuation of hyperoxia-induced NF-κB activation by MP. Endothelial cell monolayers were exposed to either control or hyperoxic conditions for 2 h with and without MP. NF-κB binding activity was examined by electrophoretic mobility shift assay. Hyperoxia-induced NF-κB activation was attenuated by MP dose dependently.

DISCUSSION

In the present study, we showed that the synthetic glucocorticoid MP attenuated hyperoxia-induced

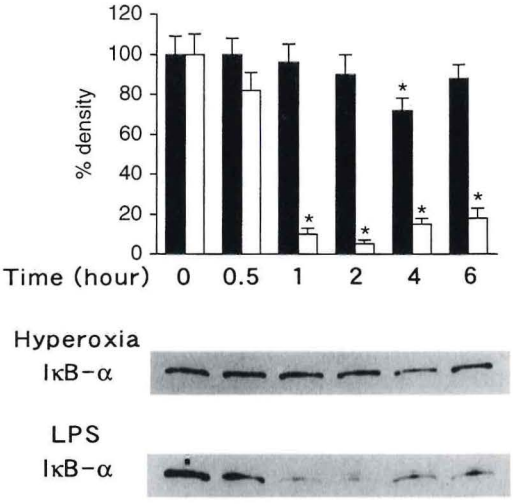

Fig. 7. Changes in inhibitory protein IκB-α expression by hyperoxia. Endothelial cell monolayers were either exposed to hyperoxia (solid bars) or stimulated with lipopolysaccharide (LPS; open bars) for indicated periods, and IκB-α expression was examined by Western immunoblot analysis (*bottom*). Quantitative analysis was performed by densitometric scanning of blots (*top*). Values are expressed as percentage of *time 0*. Expression of IκB-α was unchanged up to 2 h of hyperoxic exposure, attenuated at 4 h, and then returned to baseline level at 6 h. In contrast, IκB-α expression with LPS treatment was decreased at 1–2 h and then partially returned to baseline level at 4–6 h. $*P < 0.05$ compared with *time 0*.

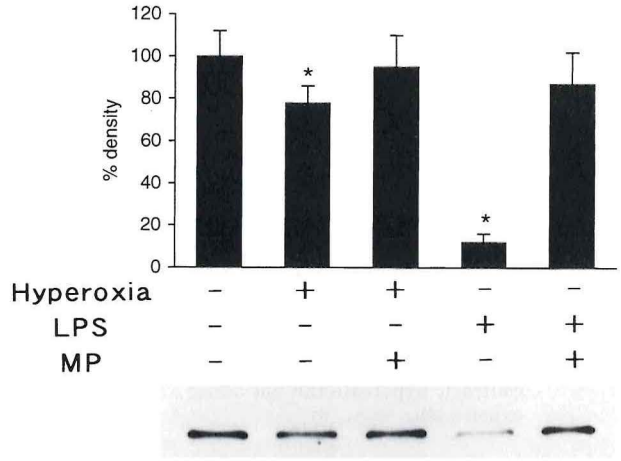

Fig. 8. MP increased IκB-α expression. Endothelial cell monolayers were exposed to either control or hyperoxic conditions for 4 h with and without MP (1 mM). IκB-α expression was determined by Western immunoblot analysis (*bottom*). Quantitative analysis was performed by densitometric scanning of blots (*top*). Values are expressed as percentage of control value. Expression of IκB-α was decreased by hyperoxic exposure and then recovered with MP treatment. IκB-α expression was strongly suppressed by LPS and recovered with MP treatment. $*P < 0.05$ compared with control value.

ICAM-1 and ICAM-1 mRNA expression and reduced neutrophil adhesion to endothelial cells (Figs. 1–3). Hyperoxia-increased neutrophil adhesion was inhibited by treatment with an anti-ICAM-1 monoclonal antibody, suggesting that, at least partially, neutrophil adhesion was mediated by ICAM-1. In addition, we have shown that MP attenuated hyperoxia-induced intracellular H_2O_2 production in HPAECs by applying DCFH-DA (Fig. 4). Suzuki et al. (29) previously showed that the exposure of cultured HPAECs and HUVECs to hyperoxia for 48–72 h increased ICAM-1 and ICAM-1 mRNA expression followed by increased neutrophil adhesion. An antioxidant, *N*-acetylcysteine, inhibits hyperoxia-induced ICAM-1 expression in endothelial cells by increasing extracellular glutathione concentration in vitro (1). Taken together, these results suggest that increased ROS production, including H_2O_2, by hyperoxia enhances ICAM-1 expression and thus stabilizes and prolongs neutrophil adhesion. Decreases in ICAM-1, ICAM-1 mRNA, and neutrophil adhesion to hyperoxia-exposed endothelial cells by MP suggested that this may be a mechanism by which MP ameliorates oxidant lung injury including pulmonary oxygen toxicity (5, 6, 15). MP might thus be useful in the treatment for oxidant lung injury such as pulmonary oxygen toxicity at some clinical condition. Koizumi et al. (16) have shown that dexamethasone improved survival and decreased lung damage if given when exposure to hyperoxia was to be soon terminated; however, dexamethasone worsened lung damage and diminished survival if given early during exposure to hyperoxia. On the other hand, Halpern et al. (11) reported that rats receiving MP ($10–60\ mg \cdot kg^{-1} \cdot day^{-1}$) survived for less time than control rats.

NF-κB was originally identified as a heterodimeric complex consisting of 55-kDa (p55) and 65-kDa (p65) subunits. NF-κB is present in cytoplasm complexed with its inhibiting protein IκB, which prevents NF-κB from being translocated to the nucleus and binding to DNA. Many inducers of NF-κB, including tumor necrosis factor-α, LPS, and interleukin-1, phosphorylate IκB-α at Ser^{32} and Ser^{36} contained within the NH_2 terminus of the protein. Phosphorylated IκB-α is then ubiquitinated on Lys^{21} and Lys^{22} and subsequently degraded by the 26S proteasome. The degradation of IκB unmasks the nuclear localization sequences in the remaining NF-κB dimer, which, in turn, translocates to the nucleus, binds to the specific promoter sites, and activates gene transcription. We demonstrated that hyperoxia increased the NF-κB DNA binding activity in endothelial cells (Fig. 5), although we did not identify NF-κB as the molecule responsible for regulating ICAM-1 expression in the present study. Increased ROS production such as H_2O_2 by hyperoxia is believed to serve as the messenger for NF-κB activation. Schreck et al. (26) showed, using H_2O_2-treated Jurkat T cells, that NF-κB was rapidly activated within 30–60 min in vitro. Shea et al. (27) reported in mice that it took 24 h for NF-κB activation in pulmonary lymphocytes with hyperoxic (100% O_2) exposure in vivo. The difference in onset of NF-κB activation between cell culture and the animal model may be related to the time required to overwhelm the endogenous antioxidants such as superoxide dismutase and glutathione present in the lungs.

AP-1, a heterodimer of protooncogene proteins c-Fos and c-Jun, is induced by many types of stimuli, including 12-*O*-tetradecanoylphorbol 13-acetate (TPA), growth factors, cytokines, and ultraviolet irradiation (9). AP-1 DNA binding activity in endothelial cells was not affected by hyperoxic exposure (Fig. 5). Our result suggested that hyperoxia induces ICAM-1 expression through activating NF-κB but not AP-1. The types of transcription factors involved in target gene expression appear to depend on the experimental conditions such as cell type and type of stimuli. For example, Roebuck et al. (23) reported that H_2O_2 activates ICAM-1 expression in HUVECs through AP-1. Bradley et al. (4) observed that H_2O_2 increases ICAM-1 expression in HUVECs without activating NF-κB. Meyer et al. (20) reported that AP-1 responded weakly in H_2O_2-treated HeLa cells, whereas NF-κB was strongly activated.

Under the control conditions, we detected IκB-α, an inhibitory protein that prevents nuclear transport and activation of transcription factor NF-κB (Figs. 7 and 8). Pulmonary endothelial cells in this in vitro system thus express IκB-α and then regulate NF-κB activation. Treating cells with NF-κB inducers rapidly disperses IκB-α, which reappears at later time points. Rapid IκB-α degradation was responsible for initially releasing NF-κB to the nucleus, whereas subsequent IκB-α resynthesis terminated NF-κB transcription in induced cells. In our study, the expression of IκB-α was suppressed by treatment with LPS at 1–2 h (Fig. 7). In contrast with LPS, hyperoxia moderately attenuated IκB-α expression at 4 h. The difference in IκB-α degradation between hyperoxia and LPS may depend on the intensity of stimuli or the signal transduction pathway.

Glucocorticoids exert their potent anti-inflammatory action by binding to the intracellular glucocorticoid receptor (GR), which belongs to the steroid/thyroid hormone receptor superfamily. After ligand binding, GR is translocated to the nucleus and is thus able to activate the transcription of several genes by binding to a specific DNA sequence, termed glucocorticoid response element (3). Glucocorticoids are believed to inhibit NF-κB activation in at least two ways. First, GRs interact directly with p65 to inhibit DNA binding via a protein-protein interaction (7, 22). Second, glucocorticoids increase IκB-α production, which, in turn, prevents NF-κB translocation to the nucleus. We showed in this study that MP attenuated hyperoxia-induced NF-κB activation and increased IκB-α expression (Fig. 8). Our results suggested that MP attenuated ICAM-1 expression by inhibiting NF-κB activation through IκB-α synthesis. Auphan et al. (2) reported that Jurkat T cells treated with TPA partially dispersed IκB-α and that dexamethasone increased IκB-α expression. Scheinman et al. (25) also observed that dexamethasone enhanced IκB-α synthesis and attenuated NF-κB activation stimulated by tumor necrosis factor-α. On the other hand, Heck et al. (12) reported that a GR mutant, which does not enhance IκB-α, represses NF-κB activity and that glucocorticoid analogs, which competently enhance IκB-α synthesis, do not repress NF-κB activity. They concluded that glucocorticoid-mediated IκB-α induction and glucocorticoid-mediated NF-κB repression are two events that are not related each other.

In conclusion, MP reduced hyperoxia-induced ICAM-1 and ICAM-1 mRNA expression and neutrophil adhesion to endothelial cells. MP attenuated hyperoxia-induced NF-κB activation, with increased IκB-α expression. These results indicated that hyperoxia upregulates ICAM-1 expression through NF-κB activation by reducing IκB-α expression and that MP downregulates hyperoxia-induced ICAM-1 expression by inhibiting NF-κB activation with increased IκB-α expression. MP might be potent in the treatment of oxidant lung injury at some clinical condition. Transcription factor NF-κB plays an important role in controlling gene expression of many modulators of inflammatory and immune responses, including ICAM-1. Because of its central role in acute inflammation, the transcription factor NF-κB should provide a key for modulating the endothelial adhesion molecule expression in response to inflammatory stimuli.

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