Increases in oxygen tension stimulate expression of ICAM-1 and VCAM-1 on human endothelial cells

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Willam, Carsten, Ralf Schindler, Ulrich Frei, and Kai-Uwe Eckardt. Increases in oxygen tension stimulate expression of ICAM-1 and VCAM-1 on human endothelial cells. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H2044–H2052, 1999.—Leukocyte infiltration plays a major role in ischemia-associated organ dysfunction and damage. A crucial step for extravasation of white blood cells is binding of leukocyte β-integrins to endothelial adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1). To test for direct effects of oxygen on this process we studied ICAM-1 and VCAM-1 expression in human dural microvascular and umbilical vein endothelial cells (EC) exposed to different oxygen tensions in the absence or presence of tumor necrosis factor-α (TNF-α). Hypoxia (95% N2-5% CO2) resulted in a downregulation of basal but not TNF-α-induced expression of ICAM-1 and VCAM-1. Subsequent rises in oxygen (21, 40, or 95% O2) led to marked increase of ICAM-1 and VCAM-1 cell surface and mRNA expression in both EC types, which after 16 h amounted to about one-third to one-half of maximal TNF-α-induced expression. This increase was greatest after 0.5-h hypoxia and was blunted with prolonged hypoxic preincubation. Exposure of cells preincubated under "normoxic" (21% O2) conditions to hyperoxia (40 or 95% O2) also enhanced expression of both adhesion molecules, but the increase was lower than in cells preexposed to hypoxia. The nitric oxide synthesis inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) enhanced ICAM-1 and VCAM-1 expression under basal and hypoxic conditions, but in the presence of L-NAME, levels in reoxygenated cells were not higher than basal levels. Moreover, the oxygen-induced rise could be mimicked by addition of H2O2 to normoxic cells, and the oxygen-induced expression of VCAM-1 but not of ICAM-1 was inhibited by addition of the free radical scavengers superoxide dismutase, N-acetyl-L-cysteine, and pyrrolidinedithiocarbamate. These data indicate that an increase in oxygen availability stimulates ICAM-1 and VCAM-1 expression on micro- and macrovascular EC, which may contribute to adhesion and transmigration of different leukocyte populations in ischemia-reperfusion injuries.

adhesion molecules; hypoxia; free radicals; reoxygenation

ISCHEMIA IS a central pathomechanism in many acute and chronic diseases. Both persistent ischemia and subsequent organ reperfusion are frequently associated with leukocyte infiltrates (13, 20, 37), and this inflammatory response plays a major role in causing ischemia-associated organ dysfunction and damage. In addition to substrate deprivation and metabolite accumulation, disturbances in oxygen supply are the main consequence of a temporary or persistent impairment of tissue perfusion. Several mechanisms of oxygen-dependent gene regulation were recently characterized (8, 25) and are found to affect an increasing number of cellular functions (16, 18, 19, 28). However, the importance of these mechanisms for induction and maintenance of tissue inflammation remains poorly understood.

Leukocyte infiltration requires a chain of reactions between endothelial cells (EC) and white blood cells that initially retards intravascular leukocyte flow and finally leads to leukocyte transmigration through the endothelial monolayer (see Refs. 4 and 5 for review). These events are mediated by sequential interaction of different endothelial adhesion molecules with cell surface receptors on white blood cells (4, 5). A crucial step between the initial contact and final transmigration of leukocytes is their tight adhesion to EC, which is mainly mediated by the endothelial transmembrane receptors intercellular adhesion molecule-1 (ICAM-1; CD54) and vascular adhesion molecule-1 (VCAM-1; CD106). ICAM-1 serves as the receptor for leukocyte function-associated antigen-1 (LFA-1; CD11a/CD18), a β2-integrin, which is expressed on neutrophils, monocytes, lymphocytes, and natural killer cells (4). VCAM-1 interacts with the β1-integrin very late antigen-4 (CD49d/CD29), which is expressed on lymphocytes and monocytes but in contrast to LFA-1 does not occur on neutrophils (11). Lipopolysaccharide (LPS) and inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interferon-γ stimulate ICAM-1 and VCAM-1 mRNA accumulation and cell surface expression and by this mechanism are thought to promote tissue inflammation (4).

ICAM-1 is also upregulated in (post)ischemic organs (13, 20, 31, 36, 37). Antibodies or antisense oligonucleotides against ICAM-1 prevent reperfusion injury in a variety of experimental settings (9, 13, 31, 37). In vitro hypoxia was found to increase leukocyte adhesion to endothelial cells (2, 3, 7, 26, 33, 34). Controversial evidence exists, however, about direct effects of changes in oxygenation on endothelial expression of ICAM-1. Although some investigators reported increased expression under hypoxic conditions (33), other in vitro studies found a decrease in (23) or no effect on (29, 39) ICAM-1 expression. Some groups reported that reoxygenation after hypoxic exposure of EC induces ICAM-1 expression (12, 14, 23, 34), but in other studies reoxygenation was not found to change ICAM-1 protein or mRNA levels significantly (6, 38). The reason for these discrepancies is unknown, and a direct comparison between hypoxia and subsequent reoxygenation has not been attempted in most studies. Moreover, with few
exceptions, these results have relied on cells cultured from human umbilical veins (HUVEC), which differ in many aspects from EC in microvasculature, in which leukocyte trafficking occurs. The effect of oxygen on the expression of VCAM-1, which appears to be of particular importance for lymphocyte and monocyte adhesion, has so far received little attention, but one recent study suggests that its expression is stimulated by hypoxia (33).

To further elucidate the role of changes in oxygen availability on the potential of EC to promote transmigration of different leukocyte populations, we have therefore decided to study in parallel the expression of ICAM-1 and VCAM-1 in HUVEC and immortalized human microvascular EC (HMEC-1) (1) exposed to different continuous or altering oxygen tensions. Our results indicate that in both endothelial cell types the expression of ICAM-1 and VCAM-1 is directly related to oxygen availability in that hypoxia does not stimulate but may even downregulate both adhesion molecules, whereas increases in oxygen tension enhance their expression. This stimulatory effect of oxygen does not require, but is augmented by previous hypoxia and appears to be influenced by the generation of nitric oxide (NO) and reactive oxygen intermediates.

MATERIALS AND METHODS

Cell culture. HUVEC were prepared from umbilical cords by dispase treatment (2.4 U/ml; neutral protease, grade II; Boehringer Mannheim, Germany) as described by Jaffe et al. (15). To confirm the endothelial origin of the harvested cells, we analyzed them microscopically for the typical cobblestone appearance and tested immunohistochemically for the occurrence of von Willebrand factor. HUVEC were grown to confluence in medium 199 supplemented with 20% FCS (GIBCO), penicillin (100 U/ml), streptomycin (100 µg/ml), and glutamine (2 mM) in culture flasks and dishes coated with 1% gelatin. For experiments, the second or third passage of cells were used.

HMEC-1 transfected with the SV-40 large T promoter originally prepared from human neonatal foreskins (1) were grown in DMEM supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 µg/ml), and glutamine (2 mM), epidermal growth factor (0.5 ng/ml; Sigma), and hydrocortisone (1 mg/ml; Sigma).

Experimental protocols. Two to three days before experiments were started, HUVEC and HMEC-1 were plated in 96-well multiwell plates for the performance of cell ELISA or on 8.8-cm² culture dishes (Nunc, Wiesbaden, Germany) for RNA preparation. When confluence was reached, cells were washed and medium with a reduced serum supplementation of 1% was added. Only 50 µl (96-well plates) or 4 ml of medium (culture dishes) were added to reduce the diffusion distance during subsequent changes in partial oxygen pressure.

Exposure to different oxygen tensions was started 12 h later using an air-tight acrylic box that was placed in a conventional tissue culture incubator to keep the temperature at 37°C. The box contained isolated chambers that were gassed separately with prewarmed and humidified gases containing various concentrations of O₂ (0, 3, 21, 40, or 95% O₂), 5% CO₂, and balance N₂. Gas concentrations were adjusted using needle flowmeters (Bailey Fischer and Porter, Göttingen, Germany), and concentrations of O₂ and CO₂ within different chambers were monitored using O₂ and CO₂ electrodes (TINA transcapnode, Radiometer, Copenhagen, Denmark).

TNF-α (1 ng/ml) and LPS (100 ng/ml; from Pseudomonas aeruginosa) were added in parallel to normoxic plates in each experimental setting to assess the cytokine-induced expression of the adhesion molecules.

Cell ELISA for ICAM-1 and VCAM-1. Expression of ICAM-1 and VCAM-1 was quantified by a whole cell ELISA. After incubations with various oxygen tensions, the multiwell plates were removed from the chamber, the cell culture medium was rapidly removed, and the cells were immediately fixed within the plate with ice-cold ethanol (100%) for 10 min. Nonspecific protein binding was blocked subsequently by adding 0.2% casein in PBS for 1 h at room temperature. Primary mouse monoclonal antibodies for ICAM-1 or VCAM-1 (R&D Systems, Wiesbaden, Germany) were added to each well (1:2,000) and incubated overnight at 4°C. Cells were washed in PBS-0.1% Tween, and a second biotinylated antimouse IgG1 antibody (1:2,000) (Amersham, Braunschweig, Germany) was added to each well for 1 h. A streptavidin-peroxidase-conjugated complex (Amersham), diluted 1:5,000 in PBS-0.1% Tween was added for 30 min, and after a further wash with PBS-0.1% Tween the plates were developed by addition of 3,3′,5,5′-tetramethylbenzidine and 0.003% H₂O₂ in citrate-phosphate buffer (pH 5.0).

The reaction was stopped with 4 N sulfuric acid, and color development was measured with a microtiter plate spectrophotometer at 450 nm (Dynatech, Hannover, Germany). On each plate the mean optical density (OD) of at least four wells incubated without primary antibody was considered as background OD and was subtracted from all other readings. For each experimental condition the mean OD of at least four wells on one plate was determined, and the average value was considered as one data point. Recombinant soluble ICAM-1 and VCAM-1 (R&D Systems) were used as standard controls, which showed a linear relation to increasing OD. For each condition at least three independent experiments were performed.

Determination of lactate dehydrogenase release and viability. Lactate dehydrogenase (LDH) release was measured enzymatically by the conversion of pyruvate to lactate using a diagnostic kit (Sigma). LDH release is expressed in relation to maximal release after cell lysis induced by 1% Triton X-100. Furthermore, viability of cells was controlled by trypan blue exclusion.

Measurement of ICAM-1 and VCAM-1 mRNA expression. Semiquantitative PCR was used to assess ICAM-1 and VCAM-1 mRNA expression under different conditions. Total RNA of incubated cells was extracted by lysis with 4 M guanidine thiocyanate and 5.7 M cesium chloride ultracentrifugation. Concentration of RNA was measured spectrophotometrically at 260 and 280 nm and adjusted to 100 ng/ml.

RNA (1 μg) was reverse-transcribed into cDNA by using random hexamers and avian myeloblastosis virus reverse transcriptase (Promega, Serva, Heidelberg, Germany) and 1× PCR buffer (20 mM Tris·HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 100 μM bovine serum albumin). cDNA (100 ng) was amplified by PCR in a total volume of 60 μl using 2.5 U AmpliTaq DNA polymerase (Perkin Elmer-Cetus, Emeryville, CA), 100 μM dATP, dCTP, and dGTP, 50 μM dTTP (Boehringer-Mannheim, Mannheim, Germany), and 0.5 μM of each primer in 1× PCR buffer. Cycles included 1 min each at 95, 60, and 72°C in a microprocessor-driven thermal cycler (Landgraf, Hannover, Germany). Primers for ICAM-1 were 5′: AGA AAT TGG CTC CAT GGT GAT CTC T and 3′: ACA TGC AGC ACC TCC TGT GAC CA; for VCAM-1, 5′: TGA TGA CAG TGT CTC CTT TTG and 3′: ATC CCT ACC ATT GAA
GAT ACT GG; and for GAPDH, 5′: CCA TGG AGA AGG CTG GG and 3′: CAA AGT TGT CAT GGA TGA CC.

Aliquots (10 µl) were taken after different numbers of cycles (usually every 5 cycles starting at 10 cycles) and analyzed on 1.5% agarose gels, and the PCR products were stained with ethidium bromide.

Reagents. Unless otherwise indicated, cell culture reagents were from Seromed and chemicals were from Sigma.

Statistics. For each condition and time point at least three independent experiments were performed. Data are expressed as means ± SE. Student’s unpaired t-test was performed for comparison of data points. P < 0.05 was considered to be significant.

RESULTS

Effect of hypoxia and “reoxygenation” on cellular expression of ICAM-1 and VCAM-1. Both HUVEC and HMEC-1 readily expressed ICAM-1 and VCAM-1 under basal conditions, but the baseline expression of both adhesion molecules on HMEC-1 was only about one-half that on HUVEC. The effects of the addition of TNF-α are shown in Figs. 1 and 2 and Table 1. In both EC types TNF-α resulted in a dose- and time-dependent increase in ICAM-1 and VCAM-1 expression under standard cell culture conditions (21% O2, 5% CO2). After a 12-h exposure to 1 ng/ml TNF-α the increase in ICAM-1 was on average 2.3- and 3.2-fold and the increase of VCAM-1 was 3.1- and 2.5-fold on HUVEC and HMEC-1, respectively, as measured by changes in the OD in cell-bound ELISA (Fig. 2, Table 1). To assess the influence of oxygen deprivation on basal and cytokine-induced expression of both adhesion molecules, cells were incubated for up to 24 h at 21 or 0% O2 in the absence or presence of 1 ng/ml TNF-α. As illustrated in Fig. 2, in the absence of TNF-α hypoxia led to a time-dependent reduction of the cellular expression of ICAM-1 and VCAM-1 on HUVEC. A similar reduction of VCAM-1 expression at low oxygen was seen in experiments with HMEC-1 (Table 1). ICAM-1 expression on HMEC-1, however, did not change significantly under hypoxic conditions. LDH release was slightly increased in cells incubated at 0% O2 for 24 h compared with cells incubated at 21% O2 (28 ± 2.6 vs. 16 ± 2.0% of maximal release after cell lysis).

With the exception of a slight reduction in ICAM-1 expression on HUVEC the response of both EC types to TNF-α was not significantly different in cells exposed to 0% O2 compared with incubation at 21% O2 (Fig. 1, Table 1), although the combination of TNF-α and hypoxia adversely affected cell viability (LDH release 35 ± 3.4%).

We then tested whether the restoration of basal oxygen supply after previous exposure to hypoxia influenced ICAM-1 and VCAM-1 expression. To this end, EC were incubated at 0% O2 for 4 h before the O2 concentration was returned to 21%. This protocol of reoxygenation resulted in an increase in the expression of both ICAM-1 and VCAM-1 in both EC types. The increase in ICAM-1 and VCAM-1 expression reached about one-half to one-third of the maximal response to TNF-α (Fig. 2, Table 1). The stimulatory effect of reoxygenation was not additive to the response achieved with TNF-α (1 ng/ml) (Fig. 2, Table 1). In addition, the response to lower (0.1 ng/ml) or higher (10 ng/ml) concentrations of TNF-α was not affected by reoxygenation (data not shown).

Effect of hypoxia and reoxygenation on ICAM-1 and VCAM-1 mRNA levels. To investigate whether the oxygen-dependent changes in protein levels of both adhesion molecules were achieved at a pre- or posttranslational level, semiquantitative measurements of ICAM-1 and VCAM-1 mRNA were performed in HMEC-1 and HUVEC by RT-PCR. In both EC types hypoxia reduced, whereas subsequent reoxygenation enhanced, ICAM-1 and VCAM-1 steady-state mRNA levels (Fig. 3).

Effect of different amplitudes of changes in O2 concentration on ICAM-1 and VCAM-1 expression. In regard to the similar responses of both EC types to oxygen availability on ICAM-1 and VCAM-1 expression, subsequent experiments to characterize this response were mainly performed in HMEC-1. To investigate whether
the increase in adhesion molecule expression induced by increasing the O\textsubscript{2} concentration to 21% after a previous 4-h exposure to "0"% O\textsubscript{2} is dependent on the amplitude and/or the magnitude of change in O\textsubscript{2} concentrations, experiments were designed in which HMEC were exposed to three different levels of O\textsubscript{2} concentration (21, 40, or 95%) for 12 h after the exposure to 21% O\textsubscript{2} (standard incubation) or to "0"% O\textsubscript{2} for 4 h. As illustrated in Fig. 4, a significant increase in ICAM-1 and VCAM-1 expression was found under all conditions, but the response was higher after a preincubation at "0"% compared with 21% O\textsubscript{2}. When cells were preincubated at "0"% O\textsubscript{2}, exposure to 95 or 40% O\textsubscript{2} did not lead to a higher increase in adhesion molecule expression compared with a reoxygenation with 21% O\textsubscript{2}.

Effect of duration of hypoxic preincubation on ICAM-1 and VCAM-1 expression after reoxygenation. To assess whether the response to reoxygenation depends on the duration of the hypoxic period, HMEC-1 were preincubated at "0"% O\textsubscript{2} for 0.5–4 h before subsequent exposure to 21% O\textsubscript{2} for 12 h. As shown in Fig. 5, the expression of both ICAM-1 and VCAM-1 was maximal in cells exposed to hypoxia for 0.5 h and declined slightly with prolongation of the hypoxic period.

Effect of cell culture medium exchange on adhesion molecule expression after reoxygenation. To further test for a possible role of putative hypoxia-induced soluble
mediators in the stimulation of VCAM-1 and ICAM-1 during the reoxygenation period the cell culture medium was removed from HMEC-1 after hypoxic exposure for 4 h and was replaced by fresh medium before adhesion molecule expression was assessed after a subsequent incubation at 21% O₂ for 12 h. This exchange of culture medium did not significantly change ICAM-1 (OD 0.35 ± 0.03 vs. 0.40 ± 0.02) or VCAM-1 (OD 0.32 ± 0.03 vs. 0.35 ± 0.04) expression (means ± SE; n = 3).

Effect of N⁶-nitro-L-arginine methyl ester on ICAM-1 and VCAM-1 expression. Because nitric oxide (NO) is a well-known regulator of adhesion molecule expression (35) and NO synthesis and half-life depend on the local effect of hypoxia and reoxygenation (24, 28) the effect of hypoxia and reoxygenation on ICAM-1 and VCAM-1 expression was also tested in the presence of the NO synthesis inhibitor N⁶-nitro-L-arginine methyl ester (L-NAME; 100 µM). As shown in Table 2, L-NAME significantly enhanced the expression of ICAM-1 and VCAM-1 under basal conditions and on hypoxic cells. L-NAME also increased the expression of ICAM-1 in reoxygenated cells, but ICAM-1 levels did not exceed the basal expression in the presence of L-NAME. Moreover, L-NAME did not stimulate VCAM-1 levels of reoxygenated HMEC-1.

Effect of free radical scavengers and H₂O₂ on ICAM-1 and VCAM-1 expression. In view of the stimulation of ICAM-1 and VCAM-1 after increases in oxygen availability, we also tried to obtain an indication for the involvement of oxygen free radicals in this regulation. Thus HMEC were preincubated at "0"% O₂ and thereafter exposed to 40% O₂ in the presence of the free radical scavengers pyrrolidine dithiocarbamate (PDTC; 50 µM), N-acetyl-L-cysteine (NAC; 300 µM) and superoxide dismutase (SOD; 300 U/ml). As shown in Table 3, the three scavengers significantly attenuated the oxygen-induced increase in VCAM-1, irrespective of whether cells were preincubated at "0"% or 21% O₂, but

Table 1. ICAM-1 expression on HMEC-1 exposed to different oxygen tensions in absence or presence of TNF-α

<table>
<thead>
<tr>
<th>Time of Reading</th>
<th>0 h</th>
<th>4 h</th>
<th>8 h</th>
<th>12 h</th>
<th>16 h</th>
<th>28 h</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ICAM-1</strong></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Hypoxia (&quot;0&quot;% O₂, 4–28 h)</td>
<td>-</td>
<td>0.26 ± 0.02</td>
<td>0.24 ± 0.04</td>
<td>0.24 ± 0.03</td>
<td>0.26 ± 0.02</td>
<td>0.26 ± 0.05</td>
</tr>
<tr>
<td>Hypoxia (&quot;0&quot;% O₂, 4–28 h)</td>
<td>+</td>
<td>0.27 ± 0.01</td>
<td>0.25 ± 0.03</td>
<td>0.79 ± 0.04</td>
<td>0.84 ± 0.06</td>
<td>1.20 ± 0.02</td>
</tr>
<tr>
<td>Reoxygenation (&quot;0&quot;% O₂, 0–4 h)</td>
<td>-</td>
<td>0.25 ± 0.02</td>
<td>0.24 ± 0.02</td>
<td>0.36 ± 0.02*</td>
<td>0.39 ± 0.03*</td>
<td>0.42 ± 0.04*</td>
</tr>
<tr>
<td>Normoxia (21% O₂, 0–28 h)</td>
<td>+</td>
<td>0.27 ± 0.03</td>
<td>0.25 ± 0.02</td>
<td>0.88 ± 0.02</td>
<td>0.86 ± 0.05</td>
<td>1.17 ± 0.05</td>
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<td><strong>VCAM-1</strong></td>
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<td></td>
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<tr>
<td>Hypoxia (&quot;0&quot;% O₂, 4–28 h)</td>
<td>-</td>
<td>0.28 ± 0.03</td>
<td>0.28 ± 0.02</td>
<td>0.24 ± 0.02</td>
<td>0.23 ± 0.02*</td>
<td>0.16 ± 0.02*</td>
</tr>
<tr>
<td>Hypoxia (&quot;0&quot;% O₂, 4–28 h)</td>
<td>+</td>
<td>0.27 ± 0.01</td>
<td>0.28 ± 0.02</td>
<td>0.58 ± 0.02</td>
<td>0.61 ± 0.02</td>
<td>0.75 ± 0.04</td>
</tr>
<tr>
<td>Reoxygenation (&quot;0&quot;% O₂, 0–4 h)</td>
<td>-</td>
<td>0.28 ± 0.03</td>
<td>0.23 ± 0.03</td>
<td>0.25 ± 0.02</td>
<td>0.37 ± 0.03*</td>
<td>0.41 ± 0.02*</td>
</tr>
<tr>
<td>Normoxia (21% O₂, 0–28 h)</td>
<td>+</td>
<td>0.25 ± 0.04</td>
<td>0.23 ± 0.03</td>
<td>0.49 ± 0.03</td>
<td>0.74 ± 0.03</td>
<td>0.82 ± 0.04</td>
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</table>

Values are means ± SE of optical density (OD) of cell-bound ELISA; n = 3 experiments. Before hypoxic exposure and after 4 h of hypoxia in reoxygenation protocol, cells were incubated at 21% O₂. In experiments performed in presence of tumor necrosis factor-α (TNF-α), cytokine was added after 4 h. HMEC-1, human microvascular endothelial cells; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular adhesion molecule-1. *Significant difference from baseline.

Fig. 3. RT-PCR of hypoxic (4 and 8 h at "0"% oxygen), reoxygenated (4 and 8 h reoxygenation at 21% oxygen after 4 h hypoxia at "0"% oxygen), and TNF-α-stimulated (8 h of 1 ng/ml TNF-α) HUVEC. Norm, 8 h at 21% O₂.
had no effect under continuous normoxia. In contrast, basal and reoxygenation-induced ICAM-1 expression was enhanced by PDTC and was not inhibited by NAC and SOD. The addition of $\text{H}_2\text{O}_2$ (1 mM) for 16 h at 21% $\text{O}_2$ led to an increase in ICAM-1 and VCAM-1 that was similar to the rise achieved by reoxygenation (Table 4).

**DISCUSSION**

Endothelial cells are exposed to a wide range of different oxygen tensions, depending on both their position within the circulation and alterations in ventilation and perfusion. In fact, EC are exquisitely resistant to hypoxia, as shown in previous studies (14, 29, 38) and also in the present investigation, in which both HUVEC and HMEC-1 could be gassed with 95% N$_2$-5% CO$_2$ for up to 24 h with only a moderate reduction in cell viability. This resistance to hypoxia is apparently caused solely by a loss of cell viability or cell detachment. Although the functional relevance of this downregulation of the adhesion molecules during hypoxia remains unknown, subsequent upregulation of ICAM-1 after hypoxic exposure is likely to enhance reperfusion and reoxygenation injury, because blocking the interaction of ICAM-1 and leukocyte β$_3$-integrins reduces organ damage (9, 13, 30, 37). It has also been shown that leukocyte adhesion receptors are upregulated by hypoxia, which may amplify the effect of temporary hypoxia on EC-leukocyte interaction (7, 26, 32, 38).

In accordance with our results, an upregulation of ICAM-1 was also found in previous studies applying different protocols of hypoxia and reoxygenation (12, 14, 23, 34), whereas Clark et al. (6) and Yoshida et al. (38) found no significant change under similar conditions. At variance with our findings, another group reported that hypoxia per se stimulates ICAM-1 and also VCAM-1 (33). In that investigation, however, fluorescence-activated cell sorter analysis was used to measure both adhesion molecules, and the incubations of nonfixed cells included in this protocol may have implied some degree of reoxygenation. In contrast to ICAM-1, a role of VCAM-1 for leukocyte infiltration in ischemic syndromes has so far not been assessed. However, hypoxia was found to induce not only neutrophil but also monocyte transmigration through the endothelium (17).

Upregulation of VCAM-1, which serves as a ligand for monocytes, may contribute to this process. Hypoxia and inflammation interact in a complex fashion under pathophysiological conditions. Although ischemia appears to induce and promote tissue inflammation, the inflammatory response, once established, may further impair oxygenation through edema and coagulation disturbances. Moreover, severe systemic inflammation, such as in septic shock, can be a primary cause of hypoxia. Despite the coincidence of inflammatory mediators and changes in oxygenation under these conditions, we did not find synergistic or additive effects of changes in oxygenation and TNF-α when the cytokine was applied at concentrations that achieved a

**Table 2. Effect of L-NAME on ICAM-1 and VCAM-1 expression**

<table>
<thead>
<tr>
<th></th>
<th>ICAM-1</th>
<th>VCAM-1</th>
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<tbody>
<tr>
<td>Normoxia (21% O$_2$)</td>
<td>0.27 ± 0.03</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-NAME</td>
<td>0.50 ± 0.02*</td>
<td>0.42 ± 0.03*</td>
</tr>
<tr>
<td>Hypoxia (16 h “0”% O$_2$)</td>
<td>0.25 ± 0.03</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>L-NAME</td>
<td>0.36 ± 0.02*</td>
<td>0.30 ± 0.02*</td>
</tr>
<tr>
<td>Reoxygenation (4 h “0”% O$_2$ + 12 h 40% O$_2$)</td>
<td>0.37 ± 0.04</td>
<td>0.39 ± 0.04</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-NAME</td>
<td>0.47 ± 0.03*</td>
<td>0.37 ± 0.03</td>
</tr>
</tbody>
</table>

Values are means ± SE of OD from cell-bound ELISA in HMEC-1; n = 3 experiments. *Significant difference between cells exposed to different oxygen tensions in absence or presence of N$^{\text{G}}$-nitro-L-arginine methyl ester (L-NAME, 100 μM).
maximal response (1–10 ng/ml; Fig. 1, Table 1). Similarly, LPS-induced expression of ICAM-1 and VCAM-1 could not be further enhanced by reoxygenation protocols (data not shown). Also, hypoxia per se did not change the TNF-α response, and for unknown reasons these findings are in contrast to a recent report by Zünd et al. (39), who observed that hypoxia augmented the LPS-induced rise of ICAM-1.

With respect to the mechanisms of increased expression of ICAM-1 and VCAM-1 on raising the O2 concentration, both the amplitude of rise in oxygen tension and the absolute levels of oxygen tension appear to be relevant. Noteworthy in this respect, raising atmospheric oxygen to supranormal levels of 40 or 95% in cells previously kept at 21% O2 led to a significant increase in ICAM-1 and VCAM-1, although the difference was less marked than after raising oxygen from "0" to 21% (Fig. 4). On the other hand, independent of whether cells were kept at "0" or 21% oxygen, we found no difference between ICAM-1 and VCAM-1 expression induced by subsequent exposure to 40 or 95% oxygen, so that the sensitivity of the underlying mechanisms appears to correlate inversely with oxygen availability. Although these data show that hypoxic preincubation is not a precondition for the oxygen-induced stimulation of adhesion molecules, the question remains of how preincubation at low oxygen tension enhances the response of ICAM-1 and VCAM-1 expression to subsequent increases in oxygen tension (Fig. 4).

The generation of reactive oxygen species is considered to be a central mechanism in the cellular effects of increased oxygenation and may affect the expression of certain genes through redox-sensitive transcription factors (25). It has been suggested that hypoxia may increase the sensitivity toward oxygen by stimulating synthesis and activity of free radical-producing enzymes such as xanthine oxidase (10). However, in the present study the reoxygenation response was inversely related to the duration of hypoxic exposure (Fig. 5) and maximal after 0.5 h of hypoxia, the shortest time point investigated. This suggests that any putative synthetic process that increases the sensitivity toward a subsequent rise in oxygen must be rapid and that counterregulatory mechanisms attenuate the sensitivity toward reoxygenation with prolonged hypoxia. It has also been suggested that endothelial cells release cytokines such as IL-1 during hypoxia that may stimulate ICAM-1 expression during reoxygenation (34). However, we were unable to confirm an important role of soluble mediators in inducing ICAM-1 and VCAM-1 expression after hypoxia, because this increase was only slightly reduced by an exchange of culture medium after the hypoxic period.

Table 3. Effect of scavengers on ICAM-1 and VCAM-1 expression

<table>
<thead>
<tr>
<th></th>
<th>ICAM-1</th>
<th>VCAM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>21% O2 (16 h)</strong></td>
<td>0.23 ± 0.04</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td><strong>PDTC (50 µM)</strong></td>
<td>0.31 ± 0.02*</td>
<td>0.28 ± 0.02</td>
</tr>
<tr>
<td><strong>NAC (300 µM)</strong></td>
<td>0.24 ± 0.01</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td><strong>SOD (300 U/ml)</strong></td>
<td>0.24 ± 0.01</td>
<td>0.31 ± 0.04</td>
</tr>
</tbody>
</table>

Values are means ± SE of OD from cell-bound ELISA on HMEC-1; n = 3 experiments. Controls were incubated in parallel to same oxygen concentrations in absence of scavengers. PDTC, pyrrolidinedithiocarbamate; NAC, N-acetyl-L-cysteine; SOD, superoxide dismutase.

* Significant difference from controls.

Table 4. Effect of H2O2 on ICAM-1 and VCAM-1 expression

<table>
<thead>
<tr>
<th></th>
<th>ICAM-1</th>
<th>VCAM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>0.26 ± 0.02</td>
<td>0.27 ± 0.03</td>
</tr>
<tr>
<td><strong>16 h 21% O2 + H2O2 (1 mM)</strong></td>
<td>0.42 ± 0.03*</td>
<td>0.37 ± 0.02*</td>
</tr>
<tr>
<td><strong>4 h &quot;0&quot;% O2 + 12 h 21% O2</strong></td>
<td>0.40 ± 0.03*</td>
<td>0.38 ± 0.04*</td>
</tr>
</tbody>
</table>

Values are means ± SE of OD from cell-bound ELISA on HMEC-1; n = 3 experiments. Controls were incubated at 21% O2 in absence of H2O2. * Significant difference from controls.
REFERENCES


