Endothelial permeability and IL-6 production during hypoxia: role of ROS in signal transduction

MIR H. ALI,1 SCOTT A. SCHLIDT,2 NAVDEEP S. CHANDEL,3 KAREN L. HYNES,2 PAUL T. SCHUMACKER,2 AND BRUCE L. GEWERTZ3

1Pritzker School of Medicine, 2Section of Vascular Surgery, Department of Surgery, and 3Section of Pulmonary and Critical Care, Department of Medicine, The University of Chicago, Chicago, Illinois 60637

Endothelial permeability and IL-6 production during hypoxia: role of ROS in signal transduction. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L1057–L1065, 1999.—Prolonged hypoxia produces reversible changes in endothelial permeability, but the mechanisms involved are not fully known. Previous studies have implicated reactive oxygen species (ROS) and cytokines in the regulation of permeability. We tested whether prolonged hypoxia alters permeability to increasing ROS generation, which amplifies cytokine production. Human umbilical vein endothelial cell (HUVEC) monolayers were exposed to hypoxia while secretion of tumor necrosis factor-α (TNF-α), interleukin (IL)-1α, IL-6, and IL-8 was measured. IL-6 and IL-8 secretion increased fourfold over 24 h in a pattern corresponding to changes in HUVEC permeability measured by transendothelial electrical resistance (TEER). Addition of exogenous IL-6 to normoxic HUVEC monolayers caused time-dependent changes in TEER that mimicked the hypoxic response. An antibody to IL-6 significantly attenuated the hypoxia-induced changes in TEER (86 ± 4 vs. 63 ± 3% with hypoxia alone at 18 h), whereas treatment with anti-IL-8 had no effect. To determine the role of hypoxia-induced ROS on this response, HUVEC monolayers were incubated with the antioxidants ebselen (50 µM) and N-acetyl-L-cysteine (NAC, 1 mM) before hypoxia. Antioxidants attenuated hypoxia-induced IL-6 secretion (13 ± 2 pg/ml with ebselen and 19 ± 3 pg/ml with NAC vs. 140 ± 15 pg/ml with hypoxia). Ebselen and NAC prevented changes in TEER during hypoxia (94 ± 2% with ebselen and 90 ± 6% with NAC vs. 63 ± 3% with hypoxia at 18 h). N-nitro-L-arginine (500 µM) did not decrease hypoxia-induced changes in dichlorofluorescin fluorescence, IL-6 secretion, or TEER. Thus ROS generated during hypoxia act as signaling elements, regulating secretion of the proinflammatory cytokines that lead to alterations of endothelial permeability.

superoxide; hydrogen peroxide; antioxidants; ischemia; cytokines; human umbilical vein endothelial cells; reactive oxygen species

A better understanding of the basic mechanisms underlying alterations in endothelial permeability may lead to interventions that could preserve barrier function with therapeutic benefits.

Three cytokines have been implicated in the regulation of barrier function in inflammatory states. Tumor necrosis factor-α (TNF-α), interleukin (IL)-1, and IL-6 are increased in blood (2, 11) and edema fluid (31) after tissue injury. Addition of these cytokines to uninjured human umbilical vein endothelial cell (HUVEC) monolayers causes reversible alterations in permeability (9). Furthermore, albumin clearance studies have demonstrated that antibody blockade is effective in preventing changes in endothelial permeability caused by inflammatory cytokines and lipopolysaccharide in vivo (19, 36). Although many studies have focused on neutrophils as the sole source of proinflammatory cytokines, endothelium-derived cytokines may also contribute to the alteration of endothelial permeability via an autocrine or a paracrine mechanism.

Hypoxia that develops in regions of tissue inflammation may amplify the biochemical and functional responses of the vascular endothelium in that state. Proinflammatory cytokines could contribute to the reversible changes in endothelial permeability observed during periods of prolonged hypoxia. Recent studies have begun to implicate reactive oxygen species (ROS) in the cellular responses to inflammatory cytokines (8, 32), whereas other studies have demonstrated that ROS participate in the intracellular signaling initiated during physiological hypoxia (7, 10). The involvement of ROS in both of these cellular responses suggests that cytokines and hypoxia may interact in the regulation of endothelial barrier function during inflammation. The present study sought to clarify the interactions between hypoxia and cytokines in an in vitro model of cultured HUVEC monolayers.

METHODS

HUVEC culture. Endothelial cells were cultured from human umbilical veins (<24 h postpartum) by a modification of the method described by Jaffe et al. (16). After cannulation of both ends, cords were flushed with 120 ml of HBPESE-buffered saline (50 mM) and incubated with 0.2% collagenase (Sigma Chemical) at 37°C for 15 min. HUVEC were grown to 100% confluence on gelatin-coated T25 flasks (Becton Dickinson) in medium 199 supplemented with 10% human serum, 10% FCS, penicillin, streptomycin, and amphotericin B (Sigma Chemical) at pH 7.4 and 37°C. Cells were studied between the first and second passages after characterization of endothelial phenotype via positive staining for CD31 (platelet-endothelial cell adhesion molecule-1) and factor VIII. After
treatment with 0.1% collagenase and 0.25% EDTA, cells were split 3:1 onto 1) gelatin-coated 25-mm glass coverslips (Fisher Scientific) for ROS determination, 2) -dichlorofluorescin fluorescence (DCFH) studies, 2) gelatin-coated cell culture inserts (12 mm diameter, 3.0 µm pore size; Costar) for permeability analysis and PO2 determination, and 3) gelatin-coated 60-mm dishes (Becton Dickinson) for the collection of supernatants for cytokine measurements.

Induction of hypoxia. Cultured cells were placed in a modular incubator chamber (Billups-Rothenberg) flushed with a gas mixture (1% O2-5% CO2-94% N2) to purge it of atmospheric air. The desired level of hypoxia (P02 = 14 ± 3 Torr) was achieved within 30 min. Samples of medium were analyzed for PO2 and pH at 3-h intervals over 24 h. The PO2 was analyzed using an O2-quenching phosphorescence method with palladium-meso-tetra(4-carboxyphenyl)porphine (15). Phosphorescence decay was detected and calculated using an Oxyspot system (Medical Systems). The pH of aliquots of medium was measured using a blood gas analyzer (Radiometer). The pH of aliquots of medium was measured using a blood gas analyzer (Radiometer).

Measurement of transendothelial electrical resistance as an index of permeability. First-passage endothelial cells were split 3:1 to 12-mm Transwell tissue culture inserts. They were rinsed with HEPES-buffered saline and fed every other day until confluent by visual inspection. The resistances of the monolayers were monitored daily until they reached a steady state. Once stable resistances were obtained (>25 Ω·cm2), the cells were exposed to hypoxia for 3–24 h. Transendothelial electrical resistance (TEER) was measured with a resistance meter (model EVOM, World Precision Instruments) together with the Endohm-12 chamber (World Precision Instruments). Measurements were taken in triplicate and reported as percentage (mean ± SE) for each time point relative to the same insert at time 0.

Cytokine quantification. HUVEC monolayers cultured on gelatin-coated 60-mm plates were exposed to hypoxia as described above. Culture medium was collected at 3-h intervals over 24 h. Hypoxia-induced production of IL-6, IL-8, IL-1α, and TNF-α was assessed by ELISA (R&D Systems). Each sample was measured in duplicate and is expressed as an average of these values.

Exogenous cytokine administration. Human IL-6 (R&D Systems) was added to normoxic HUVEC monolayers at 50, 100, and 150 pg/ml. These concentrations were chosen because they span the range of IL-6 levels observed in cell culture medium during 24 h of prolonged hypoxia. After cytokine administration, TEER was determined at 3-h intervals over a 24-h normoxic period and is expressed as means ± SE for each time point.

Cytokine antibody blockade. Monoclonal antibody to IL-6 (R&D Systems) was added at a concentration sufficient to neutralize >95% of cytokine activity in vitro (3 µg/ml). A polyclonal antibody to IL-8 (R&D Systems) was utilized at 1 µg/ml, which is also sufficient to neutralize >95% of activity. In a parallel series of experiments, human monoclonal anti-IL-1 (R&D Systems) was added at neutralizing concentrations (3 µg/ml) to serve as an irrelevant antibody control. These antibodies were added to the HUVEC cultures 24 h before hypoxic experimentation. Cytokine levels were measured in the culture medium by ELISA every 3 h thereafter along with TEER. Results are expressed as means ± SE for each time point.

Measurement of ROS production-dichlorofluorescin fluorescence. An inverted microscope was equipped for epifluorescence illumination and included a xenon light source (75 W), a 12-bit digital cooled charge-coupled device camera (Princeton Instruments), a shutter and a filter wheel (Sutter), and appropriate excitation and emission filters. Fluorescent cell images were obtained using a ×40 oil-immersion objective (Nikon Plan Fluor). Data were acquired and analyzed using Metamorph software (Universal Imaging). ROS generation in cells was assessed using the probe 2,7-dichlorofluorescin diacetate (DCFH-DA, 10 µM; Molecular Probes). Within the cell, esterases cleave the acetate groups, thereby trapping the nonfluorescent DCFH probe intracellularly. Subsequent oxidation by ROS, particularly H2O2 or the hydroxyl radical (OH·), yields the fluorescent product dichlorofluorescin (DCF) (4). DCF fluorescence was measured using excitation wavelength of 480 nm, dichroic 505-nm long pass, and emitter band pass of 535 nm (Chroma Technology). Neutral density filters were used to attenuate the excitation light intensity. Fluorescence intensity was assessed in clusters of cells (<10 identified as regions of interest, and background was identified as an area without cells or with minimal cellular fluorescence. Intensity values are reported as percentage of initial values after subtraction of background.

Statistics. Data were analyzed with the Minitab II statistical program on the Power Macintosh 7200. Values are means ± SE and analyzed by Student’s t-tests or ANOVA where appropriate. Significance was defined as P < 0.05.

RESULTS

Effects of hypoxia on permeability. The effects of hypoxia (1.5% O2) on TEER were measured in HUVEC monolayers over 24 h. A blank Transwell insert was used as an indicator of background effects on TEER and was consistent at 6 ± 1 Ω·cm2. TEER was measured every 3 h, and values at each time point were reported as a percentage of the original value at time 0. Normoxic controls maintained in a standard incubator environment (5% CO2-95% room air) showed no significant change in TEER over 24 h (data not shown). During prolonged hypoxia (PO2 = 14 ± 3 Torr, range 11–19 Torr) TEER changed significantly over 24 h (Fig. 1). Under these conditions, monolayers demonstrated an initial drop in resistance at 9 h (92 ± 2% of original), with the greatest decreases detected at 18 h (63 ± 3% of...
original, P < 0.01). Thereafter, resistances began to recover until 24 h, plateauing at 88 ± 3% of their original values. By contrast, TEER failed to change significantly over 24 h in cells exposed to 25 or 35 Torr. This suggests that alterations of endothelial permeability are dependent on the length and severity of hypoxia.

Effects of hypoxia on cytokine production. Prolonged hypoxia (PO2 = 14 ± 3 Torr, range 11–19 Torr) caused endothelial cells to secrete several types of cytokines. Over the first 18 h, levels of IL-6 in the medium reached concentrations of 140 ± 15 pg/ml (P < 0.01 compared with normoxic controls; Fig. 2), whereas IL-8 peaked at 1,089 ± 246 pg/ml. However, IL-1α levels remained unchanged (4 ± 6 pg/ml) over a 24-h hypoxic incubation, as did levels of TNF-α (data not shown). These findings suggest a direct temporal correlation between hypoxia-induced IL-6 and IL-8 production and changes in endothelial permeability.

Effects of cytokine antibodies on hypoxia-induced changes in permeability. To determine whether cytokines released by endothelial cells contributed to the observed changes in TEER during hypoxia, antibodies to IL-1α, IL-8, or IL-6 were added to the cell culture medium before hypoxic incubation (PO2 = 14 ± 3 Torr, range 11–19 Torr). Monolayers supplemented with anti-IL-1α or anti-IL-8 before hypoxia exhibited a permeability profile similar to that of cells exposed to hypoxia alone (Fig. 3, A and B). However, addition of anti-IL-6 monoclonal antibody significantly attenuated hypoxia-induced decreases in TEER. During prolonged hypoxia, TEER decreased to 63 ± 3% of original levels in controls. However, in the presence of anti-IL-6, the minimum TEER reached was 86 ± 4% at 18 h (Fig. 3C; P < 0.05). These findings suggest that IL-6, but not IL-8, participates as a mediator of hypoxic alteration of endothelial permeability during prolonged hypoxia.

Effects of IL-6 on normoxic HUVECs. To determine the effects of exogenous IL-6 on TEER, recombinant human IL-6 (50 pg/ml) was added to the medium of normoxic HUVEC monolayers. This resulted in an increase in endothelial permeability over the first 9 h, reaching a minimum TEER of 72 ± 2% of original (Fig. 4; P < 0.05). However, unlike the typical biphasic permeability profile observed during hypoxia, no restoration of TEER to baseline values was observed at 24 h with this concentration of cytokine. Similar results were obtained with 100 pg/ml of IL-6, with a minimum TEER of 68 ± 3% (P < 0.01) observed at 9 h. Once again, no reversal of permeability was observed. However, when 150 pg/ml of IL-6 were added to the monolayers, TEER dropped to 66 ± 2% within 6 h (Fig. 4; P < 0.01) and recovered toward baseline by 24 h (P < 0.01). This suggests somewhat paradoxically that concentrations of IL-6 >100 pg/ml are required for the reversal of endothelial permeability.

Effects of hypoxia on DCF fluorescence. ROS generation in HUVECs exposed to hypoxia was investigated using DCFH dye. During hypoxia (PO2 = 14 Torr), the DCF fluorescence signal increased, reaching ~300% of normoxic baseline values at 120 min (Fig. 5; P < 0.01). The increase in fluorescence was reversed rapidly when the monolayers were reoxygenated at 150 min (PO2 = 150 Torr), and within 60 min, the DCF fluorescence had returned to its baseline level. To verify that ROS were responsible for these results, a series of experiments was performed using antioxidants to reduce ROS accumulation. Ebselen [2-phenyl-1,2-benzisoselenazol-3-(2H)-one] is a selenium-containing compound that functions as a glutathione peroxidase mimetic (23). A second compound, N-acetyl-L-cysteine (NAC), enhances the scavenging of ROS by enhancing intracellular pools of reduced glutathione (26). Initial experiments were performed with these agents at various concentrations (10–100 µM ebselen and 100 µM to 1 mM NAC) to determine their effects on ROS levels in HUVECs exposed to PO2 of 14 Torr. Determination of a dose-response relationship for these compounds provided insight into the optimal concentration to be used for subsequent experiments. DCF fluorescence was measured at different concentrations of ebselen or NAC. Addition of 50 µM ebselen abolished the hypoxia-induced increases in DCF fluorescence within 60 min.

![Fig. 2. Secretion of interleukin (IL)-1α, IL-6, and IL-8 by hypoxic (PO2 = 14 ± 3 Torr) HUVECs. Levels of IL-6 and IL-8 increased significantly over first 18 h, plateauing at 140 pg/ml and 3.3 ng/ml, respectively, at 24 h.](image-url)
The antioxidant effects of NAC were evident at 1 mM (Fig. 6A). Because of the possible toxic or nonspecific actions of ebselen and NAC, the lowest concentrations of the compounds that would return DCF fluorescence to normoxic levels within 60 min were used in subsequent experiments.

To determine whether reactive nitrogen species, particularly nitric oxide, are involved in the hypoxia-induced changes in DCF fluorescence, HUVECs were exposed to hypoxia in the presence of N-nitro-L-arginine (L-NNA, 100 µM to 1 mM). Because the DCFH dye can be oxidized by nitric oxide and by H$_2$O$_2$, these experiments were included to distinguish between ROS (Fig. 6A). The antioxidant effects of NAC were evident at 1 mM (Fig. 6B). Because of the possible toxic or nonspecific actions of ebselen and NAC, the lowest concentrations of the compounds that would return DCF fluorescence to normoxic levels within 60 min were used in subsequent experiments.

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and reactive nitrogen species generated during hypo-
oxia. In the presence of 500 µM and 1 mM L-NNA, the DCF signal was mildly reduced (16%) but never abol-
ished (Fig. 6C). That this return toward baseline was achieved with the addition of 1 mM NAC, 50 µM
ebselen, or normoxia suggests that nitric oxide is minimally involved in hypoxia-induced changes in DCF fluorescence.

Effects of ROS on IL-6 production during hypoxia. To assess the effects of ROS generated during hypoxia on the release of cytokines from endothelial cells, the concentrations of cytokines present in the medium of hypoxic cells were compared with the concentrations in cells pretreated with 50 µM ebselen or 1 mM NAC. Ebselen reduced the hypoxia-induced IL-6 production from a maximum of 140 pg/ml at 18 h to 13 pg/ml (Fig. 7A; P < 0.01). The maximum concentration detected in the ebselen-treated cells was not different from that in normoxic controls at 24 h (25 vs. 20 pg/ml, P > 0.2). Similar attenuation of cytokine release was seen in cells pretreated with 1 mM NAC before hypoxia (Fig. 7B). Pretreatment with 500 µM L-NNA did not significantly alter hypoxia-induced IL-6 secretion (Fig. 7C). These findings suggest that ROS, not nitric oxide, are involved in the enhanced release of IL-6 during hypoxia.

Effects of antioxidants on endothelial permeability. To determine the effects of ROS generation on the changes in TEER during hypoxia, HUVEC monolayers were treated with 50 µM ebselen before hypoxia. Ebselen attenuated the hypoxia-induced changes in TEER (Fig. 8A; P < 0.01). The minimum TEER level at 18 h increased from 63 ± 3 to 95 ± 3% of original, and the typical decrease in TEER between 6 and 18 h was not observed. After 24 h, the ebselen-treated cells exhibited higher resistances than untreated controls (94 ± 2 vs. 88 ± 3%). Similar results were obtained from cells pretreated with 1 mM NAC; the minimum TEER at 18 h was 90 ± 6% of control (Fig. 8B; P < 0.05), whereas the value at 24 h was 95 ± 4% of original TEER. The effects of nitric oxide on these changes were tested by pretreating HUVECs with 500 µM L-NNA; these results indicate that inhibiting nitric oxide production during hypoxia does not alter changes in TEER (Fig. 8C; P > 0.2).

DISCUSSION

Many investigators have studied the effects of hypoxia on the vascular endothelium. Ogawa et al. (25) reported that prolonged hypoxia caused reversible, selective changes in barrier and coagulant functions that were not lethal to endothelial cells. Others (3, 22, 27) have reported that prolonged hypoxia alters monolayer permeability and cell surface properties. However, the mechanisms linking the decrease in cellular

Fig. 6. Effects of hypoxia (2% O₂) on DCF fluorescence in HUVEC monolayers treated with antioxidants. A: addition of ebselen (10, 25, or 50 µM) during hypoxia significantly reduced DCF fluorescence. However, return of DCF fluorescence was achieved only at 50 µM. B: addition of N-acetyl-L-cysteine (NAC; 100 µM, 500 µM, and 1 mM) during hypoxia significantly reduced DCF fluorescence. However, return of DCF fluorescence was achieved only at 1 mM. C: addition of N-nitro-L-arginine (L-NNA; 100 µM, 500 µM, and 1 mM) did not return DCF fluorescence to normoxic levels. However, return of 21% O₂ to HUVEC monolayers caused a brisk attenuation of DCF signal.
Fig. 7. Effects of antioxidants on HUVEC IL-6 secretion during hypoxia. A: pretreatment of HUVECs with 50 µM ebselen significantly decreased hypoxia-induced IL-6 secretion. B: pretreatment with 1 mM NAC also significantly attenuated IL-6 secretion during hypoxia. C: treatment of HUVEC monolayers with 500 µM L-NNA had no significant effect on hypoxia-induced IL-6 secretion.

Fig. 8. Effects of hypoxia on endothelial permeability in presence of antioxidants. A: 50 µM ebselen added to confluent HUVEC monolayer cultures before hypoxia (PO$_2$ = 14 ± 3 Torr) prevented hypoxia-induced changes in endothelial permeability, as shown by TEER. B: treatment with 1 mM NAC also attenuated changes in TEER during 24 h of hypoxia. C: treatment with 500 µM L-NNA did not significantly affect hypoxia-induced changes in TEER over 24 h.
P02 to the functional response have not been established. This investigation focused on the role of endothelium-derived ROS and the manner in which they mediate alterations in endothelial permeability during periods of prolonged hypoxia. Our findings demonstrate that hypoxia induces the generation of ROS in endothelial cells, which increases cytokine secretion and leads to changes in permeability.

In our HUVEC studies, prolonged hypoxia (P02 = 14 Torr) resulted in a transient increase in endothelial permeability between 6 and 24 h, reaching maximum levels at 18 h. These changes corresponded to increases in the levels of IL-6 and IL-8 secreted by the endothelial monolayers, suggesting an autocrine effect of these proinflammatory cytokines on permeability. The addition of a polyclonal antibody to IL-8 did not alter the permeability profile of cells subsequently exposed to hypoxia, but the permeability increase was substantially attenuated in cells pretreated with an anti-IL-6 monoclonal antibody. These findings demonstrate that the hypoxic alteration of endothelial permeability is mediated, at least in part, by IL-6. The addition of exogenous IL-6 at concentrations observed during 24 h of hypoxia produced time-dependent changes in permeability, supporting this assertion. Although lower concentrations of IL-6 increased permeability, only 150 pg/ml mimicked the biphasic changes in endothelial permeability seen during hypoxia. These findings suggest that a threshold level of IL-6 is required for the permeability reversal, with the critical level in the range of 100–150 pg/ml. Further studies are required to determine the specific mechanisms by which higher levels of IL-6 mediate the temporal changes in endothelial permeability.

These data also demonstrate that hypoxia induces the generation of cellular ROS, which appear to participate in the signaling responsible for cytokine secretion. Many studies have demonstrated that ROS participate in the signaling cascades induced by lipopolysaccharide (30) and cytokines such as TNF-α (29). Increased ROS levels have also been shown during periods of physiological hypoxia (5, 6, 21), suggesting that ROS signaling may be a common factor involved in cellular hypoxic detection and cytokine responses. Our DCFH studies revealed a threefold increase in ROS levels during hypoxia. On the basis of the attenuation of the DCF signal by ebstein or NAC and the inability to decrease this signal with L-NNA, we conclude that H2O2 generation is required for the cytokine response. Pretreatment of HUVEC monolayers with ebstein or NAC also attenuated the changes in permeability observed during hypoxia. These findings lead to the conclusion that ROS generated during hypoxia act as intracellular signals responsible for the secretion of IL-6, an important modulator of endothelial permeability.

The effects of cytokines on endothelial cells have been extensively studied in ischemia-reperfusion models, and these molecules have been implicated as mediators of the hypoxic alteration of endothelial permeability. A host of cytokines, particularly TNF-α and IL-1α, have been shown to increase permeability in a variety of cell types (28). When determining which of these mediators might be responsible for the observed changes in permeability of HUVEC monolayers during hypoxia, the list of candidates becomes much shorter. Our studies demonstrate that HUVECs secrete very little TNF-α and IL-1α during prolonged hypoxia; this is consistent with previous studies demonstrating that the major source of these cytokines in the microcirculation is the polymorphonuclear leukocytes (17). Our model consisted of only HUVECs exposed to hypoxia, so it is still possible that TNF-α and/or IL-1α may contribute to changes in endothelial permeability in vivo. However, the increase in IL-6 levels in our HUVEC system mirrored increases in vascular permeability during prolonged hypoxia, suggesting that it may play an important role in these changes.

An increase in IL-6 secretion in response to hypoxia has been demonstrated in a variety of cell types ranging from vascular smooth muscle cells (33) to monoclonal antibodies to IL-6 monoclonal antibody. These studies show that hypoxia-induced acute-phase reaction overlaps with, but is not identical to, the IL-6-induced acute-phase reaction. This is consistent with our observation that blocking the effects of IL-6 with a monoclonal antibody does not completely eliminate hypoxia-induced changes in permeability. Thus IL-6, although a major mediator, is not the only participant in the hypoxia-induced response. It follows that any attempt to completely abolish changes in permeability during hypoxia must act upstream of the cytokine response, preferably at the level of the O2 sensor. Indeed, our studies showed that the permeability changes could be abolished by antioxidants, which appear to act proximal to the cytokine step in the signaling response to hypoxia.

An interesting aspect of this cascade of events is the cellular detection of hypoxia and subsequent changes in signaling, which lead to the increase in IL-6 secretion. The responses to hypoxia in the present study are not likely to reflect cell injury inasmuch as they were reversible and not associated with loss of viability. Indeed, Farber and Rounds (12) showed that endothelial cells can withstand long-term exposure to anoxic conditions without loss of viability or apparent loss of function. We therefore propose that the results in the present study represent physiological responses rather than pathophysiological effects of cellular injury. Recent work has identified a role for ROS in mediating other hypoxia-induced changes in cell physiology (14). For example, ROS have been shown to be necessary for the upregulation of hypoxic genes [vascular endothelial growth factor (VEGF) (20) and erythropoietin (13)] and the activation of hypoxia-induced transcription factor-1 (7). Kuroki et al. (18) used antioxidants to demonstrate that ROS control is critical to the expression of VEGF in vivo and in vitro. Chandel et al. (7) found that the
activation of mRNA for erythropoietin, glycolytic enzymes, and VEGF depends on the ROS generated by the mitochondria during hypoxia. Moreover, in mitochondria-deficient cells, the increase in the transcription for these hypoxic genes was not observed during prolonged hypoxia. In addition, Vanden Hoek et al. (34) demonstrated that ROS are produced by mitochondria during hypoxia and are critical to the induction of cardiac preconditioning in cardiomyocytes. Duranteau and colleagues (10) found that ROS generation in cardiomyocytes was increased during physiological hypoxia and that these ROS mediate a decrease in contractile activity, a response that was prevented by pretreatment with antioxidants. Collectively, these results underscore a growing awareness that low levels of ROS generated during hypoxia function as signaling molecules affecting gene expression and cell function during hypoxia. The findings of the present study indicate that ROS are also responsible for changes in endothelial permeability, although the specific source of these molecules in HUVECs was not demonstrated.

A major functional consequence of ROS production during hypoxia is the increase in IL-6 secretion, which contributes to the changes in endothelial permeability. Ala et al. (1) demonstrated that hypoxia-reoxygenation increases IL-6 production via ROS; this trend was prevented by pretreatment with superoxide dismutase or glutathione peroxidase. In hypoxia-reoxygenation models, the increase in ROS is thought to occur at reoxygenation and may be responsible for increases in monolayer permeability. In our study, changes occurred during continuous hypoxia before reoxygenation. Our DCFH studies help clarify this theory by demonstrating that an increase in ROS generation occurs during hypoxia before reoxygenation. We suggest that ROS generated in response to physiological hypoxia contribute to the regulation of endothelial permeability and cytokine release, whereas ischemia and reperfusion may affect permeability by enhancing the generation of ROS at reoxygenation. Regardless of the site of production of ROS during hypoxia or reoxygenation, changes in permeability are attenuated by the use of antioxidants. Future studies are required to fully clarify the mechanisms by which hypoxia augments intracellular ROS generation and the specific signaling system by which these ROS augment cytokine release.

Address for reprint requests and other correspondence: B. L. Gewertz, Dept. of Surgery, The University of Chicago, 5841 S. Maryland Ave., MC 5029, Chicago, IL 60637.

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