Reoxygenation of Endothelial Cells Increases Permeability by Oxidant-Dependent Mechanisms

Hazel Lum, Deborah A. Barr, Jacquelin R. Shaffer, Robert J. Gordon, Alan M. Ezrin, and Asrar B. Malik

We investigated the effects of hypoxia/reoxygenation exposure on the barrier function of endothelial cell monolayers. Bovine pulmonary microvessel endothelial cells were grown to confluence on microporous filters (0.8-μm pore diameter) and exposed to hypoxia (0.1% O₂ or Po₂ = 1 mm Hg) for 2, 4, 12, or 24 hours, followed by reoxygenation with room air for a period ranging from 16 seconds to 2 hours. The transendothelial clearance rate of 125I-albumin was measured to determine the permeability of endothelial monolayers. Permeability increased twofold or fivefold over control values after 1 hour of reoxygenation in monolayers that had been exposed to either 12 or 24 hours of hypoxia. The response occurred within 5 minutes of reoxygenation, increased maximally by 40 minutes, and remained elevated with continuous reoxygenation for up to 2 hours. The increase in permeability was associated with F-actin reorganization, a change to spindelike cells, and injured mitochondria. Immunoblot analysis indicated that neither hypoxia alone nor reoxygenation changed CuZn superoxide dismutase (SOD), MnSOD, and catalase levels. However, release of superoxide anions (O₂⁻) into the extracellular medium increased by twofold within 40-60 minutes of reoxygenation. Treatment of endothelial cells with CuZnSOD (100 units/ml) for the 24-hour hypoxia period prevented O₂⁻ generation and ~50% of the increase in permeability. Higher CuZnSOD concentrations (≥200 units/ml) were not protective. Treatment with catalase (100-1,000 units/ml) inhibited the reoxygenation-induced increase in permeability at the highest catalase concentration (1,000 units/ml), suggesting a critical role of hydrogen peroxide in mediating the response. We conclude that reoxygenation of endothelial cells causes the generation of oxidants and that this mediates the increase in vascular endothelial permeability occurring during reoxygenation. The loss of endothelial barrier function may contribute to the pathogenesis of reperfusion tissue injury. (Circulation Research 1992;70:991-998)

Key Words • hypoxia/reoxygenation • ischemia/reperfusion • superoxide anion • hydrogen peroxide • superoxide dismutase • catalase • endothelial permeability • xanthine oxidase

Tissue edema is a consistent feature of ischemia/reperfusion injury of various organs.¹⁻³ The injury is believed to be mediated by toxic amounts of oxygen free radicals, since treatment with oxygen radical scavengers was often shown to protect against reperfusion injury.¹⁻³ Vascular endothelial cells may be both the source of free radical generation as well as important target cells for these radicals.⁴⁻⁶ However, it is unclear whether endothelium-derived oxygen free radicals are capable of altering the endothelial barrier. Hypoxia and subsequent reoxygenation of endothelial cells may be a critical factor in the mediation of endothelial injury, since reoxygenation can generate superoxide anions, which in turn can lead to the production of hydrogen peroxide and hydroxyl radicals.²⁻⁷ Several studies have also shown that hypoxia is directly capable of injuring the endothelial barrier,⁸⁻¹¹ suggesting oxidant-independent mechanisms of injury. The aims of this study were to examine the consequences of hypoxia followed by reoxygenation on the endothelial barrier function and the basis of altering the endothelial barrier after hypoxia and reoxygenation.

Materials and Methods

Reagents

Dulbecco’s modified Eagle’s media (DMEM), fetal bovine serum, and Hank’s balanced salt solution (HBSS) were purchased from Gibco Laboratories, Grand Island, N.Y. Bovine serum albumin (fraction V) (BSA), HEPES, Nonidet P-40, Tween-20, and ferricytochrome C (type III, horse heart) were purchased from Sigma Chemical Co., St. Louis, Mo. Gentamicin was obtained from Whittaker Bioproducts, Walkersville, Md. ¹²⁵I was purchased from New England Nuclear, Boston. Electron microscopy–grade glutaraldehyde, so-
dium cacodylate, and 10% neutral-buffered formalin were purchased from Polysciences Inc., Warrington, Pa. Embed 812, uranyl acetate, osmium tetroxide, and lead citrate were purchased from Electron Microscopy Sciences, Fort Washington, Pa. Rhodamine phalloidin was obtained from Molecular Probes, Inc., Eugene, Ore. The pharmaceutical grade CuZn superoxide dismutase (SOD), obtained from Sterling Research Group, Rensselaer, N.Y.), was essentially endotoxin-free (<0.05 endotoxin units/1,000 units SOD activity). Catalase was purchased from Boehringer Mannheim Corp., Indianapolis, Ind.

**Cell Culture**

Bovine pulmonary microvessel endothelial cells (BPMECs) were cultured as described. Briefly, peripheral lung tissue containing microvessels <100 μm in diameter was finely minced and incubated in 1,000 units/ml collagenase II solution in DMEM with 5% BSA. The material was stirred thoroughly, passed through a nylon mat of 160-μm pore diameter, and centrifuged at 200g for 5 minutes. The cell pellet was resuspended in complete media (DMEM, 20% fetal bovine serum, and 50 μg/ml gentamicin) and plated onto gelatin-coated Petri dishes. After 2–3 days of culture, the contaminating cells were removed with a Pasteur pipette; the remaining cells were allowed to proliferate and used for seeding. The cells were characterized to be endothelial in origin by the presence of angiotensin-converting enzyme activity, factor VIII-related antigen, and uptake of acetylated low density lipoprotein.

**Hypoxia/Reoxygenation Exposure**

Monolayers of BPMECs were placed in a gas-tight controlled atmosphere culture chamber (26×20×18 cm) (Bello Biotechnology, Vineland, N.J.) and connected to tanks of certified mixtures of gases. The hypoxic gas mixture was 95% N₂–5% CO₂; the reoxygenation was room air (21% O₂). The chamber was humidified and kept at 37°C. The chamber O₂ concentration was monitored with an Amatek zirconium-based O₂ analyzer (Thermox Instruments Division, Pittsburgh, Pa.). During hypoxia exposure, chamber O₂ concentration was typically maintained at 0.1% (partial pressure of O₂, 0.76 mm Hg). Before the start of hypoxic exposure, the chamber was flushed at 3 l/min for 15 minutes with the gas mixture used for exposure to cells. After this washout period, the chamber pop-off valve was closed, and gas flow was reduced to <0.5 l/min for the duration of the exposure period. Lactic acid release into the medium was determined from different periods of hypoxic exposure as an index of anaerobic metabolism by the endothelial cells. Lactic acid was assayed using the lactic acid reagent kit (Abbott Laboratories, North Chicago), which was based on a fluorescent attenuation method.

**Transendothelial 125I-Albumin Clearance Rate**

After the prescribed hypoxia and reoxygenation periods, endothelial permeability was determined by measuring the transendothelial 125I-albumin clearance rate as described. In brief, this method measures permeability independent of hydrostatic and oncotic pressures. The system consisted of a luminal (0.7 ml) and an abluminal compartment (25 ml). The cells were seeded (10⁵ endothelial cells per filter) on a polycarbonate microfilter (13-mm diameter, 0.8-μm pore diameter, Nucleopore, Pleasanton, Calif.). The endothelial monolayers were used when cells became confluent (3–4 days). Both compartments contained HBSS with 20 mM HEPES and 0.5% BSA (pH 7.4). The entire system was kept at 37°C by a thermostatically regulated water bath. Crystalized and lyophilized BSA was purified by gel chromatography and labeled with 125I according to the chloramine T procedure. Noncovalently bound iodine was removed by dialysis against 0.1 M NaI in phosphate-buffered saline (PBS) (pH 7.4). The clearance rate of tracer 125I-albumin from the luminal into the abluminal compartment was determined by sampling 400 μl from the abluminal chamber at either 16-second intervals for 10 minutes for determining increased clearance at early time points or 5-minute intervals for determining clearance of later time points. The radioactivities of the samples were used to calculate the volume of luminal chamber activity “cleared” of tracer albumin. The change in volume over time gave the clearance rate (microliters per minute) as determined by weighted least-squares nonlinear regression (BMDP Statistical Software, Inc., Los Angeles, Calif.).

**Lactate Dehydrogenase Release**

Cytolysis was monitored by measuring lactate dehydrogenase (LDH) activity (LD-L20 kit, Sigma Diagnostics, St. Louis, Mo.) released into the medium after hypoxia/reoxygenation. LDH activity was based on the reduction of NAD determined by absorbance at 340 nm. The activity present in the medium was normalized to total LDH activity derived from cell lysates. The values are reported as percentage of total activity.

**Ultrastructure**

Confluent monolayers of BPMECs grown on microporous filters exposed to hypoxia/reoxygenation were processed for ultrastructural assessment. The cells were washed in HBSS (pH 7.4) and fixed with electron microscopy grade 2.5% glutaraldehyde in 0.05 M cacodylate buffer. After a wash with the same buffer, the cells were postfixed with 1% osmium tetroxide, stained en bloc with 0.5% uranyl acetate, and dehydrated in ascending concentrations of ethanol. Then four monolayers per group were infiltrated, embedded, and polymerized with Embed 812. Two embedded monolayers were selected and cut into thin (60–70-nm) sections using a diamond knife. The sections were picked up on 200 mesh copper grids, double-stained with 4% uranyl acetate and 0.4% lead citrate, and viewed with an electron microscope (model 100 CX, JEOL U.S.A. Inc., Peabody, Mass.).

**Fluorescent Staining of F-Actin**

Confluent BPMEC monolayers grown on microporous filters were exposed to hypoxia/reoxygenation. The monolayers were washed once with 0.01 M PBS (pH 7.4), fixed in 10% neutral-buffered formalin, and permeabilized with 1% Nonidet P-40. After two washes with PBS, the cell monolayers were incubated at room temperature in the dark with rhodamine phalloidin.
(stock solution, 3.3 μM) diluted 1:20 in 0.01 M PBS. The monolayers were mounted on glass slides with a glycerol/PBS solution (1:1 [vol/vol]). Fluorescence of F-actin was visualized using a Nikon Laphot equipped with epifluorescence.

**Superoxide Anion (O2⁻) Release**

BPMECs were seeded in fibronectin-coated 25-cm² flasks at a density of 10⁵ cells per flask. Confluent monolayers were used for O2⁻ release determination based on O2⁻-mediated reduction of ferricytochrome C.¹⁶ The cells were washed with HBSS, and 1.5 ml HBSS was added to each flask. The cells were exposed to hypoxia for 24 hours, removed from the chamber, and washed twice with HBSS and 1.5 ml HBSS containing 0.3 mM horse heart cytochrome C with or without 300 units/ml SOD. After reoxygenation, the reaction was stopped by placing flasks on ice. The medium was removed and centrifuged at 2,000g for 20 minutes, and the absorbance was read at 550 nm in a spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) against a buffer blank. Cytochrome C reduction was expressed as the difference in absorbance between cells incubated with or without SOD.

**Immunoblot Analysis for Endogenous CuZnSOD, MnSOD, and Catalase**

BPMECs were seeded onto 150-mm-diameter culture dishes and grown to confluence for 3–4 days. The cells were washed once with HBSS containing 20 mM HEPES (pH 7.4) and fresh medium added for hypoxia/reoxygenation exposure. Immediately after exposure to either hypoxia or reoxygenation, the cells were washed in ice-cold 0.01 M PBS, collected, and centrifuged at 2,000g for 10 minutes at 10°C. The cell pellet was quick-frozen in an ethanol–dry ice mixture and kept frozen at −70°C for Western blot analysis.

Procedure for immunoblot analysis was performed according to previously reported methods.¹⁷ Cells were sonicated in PBS/1% Triton X-100 and centrifuged (4°C) at 10,000g for 15 minutes. The supernatant was boiled with an equal volume of sodium dodecyl sulfate gel sample buffer, and electrophoresis was performed using denaturing 10% polyacrylamide gels. Samples were then transferred to nitrocellulose. Primary antibodies (diluted 1:400 for anticatalase or anti-CuZnSOD and 1:4,000 for anti-MnSOD) were added and incubated for 2 hours at room temperature. After a wash in PBS/0.1% Tween-20, alkaline phosphatase–conjugated secondary antibodies directed against immunoglobulin G (diluted 1:1,000) were added and incubated for 2 hours. To detect regions of nitrocellulose bound antigen/primary antibody/alkaline phosphatase–conjugated secondary antibody, the nitrocellulose filters were stained for enzyme activity using an alkaline phosphatase detection kit (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.).

**Inactivation of CuZnSOD and Catalase**

SOD was prepared in 50 mM NaCO₃–NaHCO₃ buffer (pH 7.4) and inactivated with 100 mM H₂O₂ for 6 hours at 25°C. The SOD preparation was extensively dialyzed with four changes of HBSS at 5°C to remove the H₂O₂. Inactivated SOD lost 61.5% of enzyme activity when tested by the method of ferricytochrome C reduction of O₂⁻ generated by xanthine oxidase.¹⁶ Catalase was inactivated by heating at 90°C for 30 minutes. Inactivated catalase showed a >95% loss of enzyme activity when measured spectrophotometrically at 240 nm by the rate of reduction of H₂O₂ substrate.¹⁸

**Statistics**

Two-way analysis of variance was used to analyze data with repeated sampling over time (BMDP Statistical Software). Single-sample data were analyzed by two-tailed Student’s t test or a multiple-range test for comparisons within several experimental groups (Scheffe’s test).

**Results**

Exposure for 4 hours of hypoxia did not increase the lactate level in the medium over the control value (Table 1). With 12 and 24 hours of hypoxic exposure, the lactic acid concentrations increased 2.8- and 5.6-fold, respectively, over the normoxic control group, indicative of time-dependent activation of anaerobic metabolism.

The effects of varying hypoxic durations followed by 1 hour of reoxygenation on endothelial permeability are shown in Figure 1. Neither 2 nor 4 hours of hypoxia followed by the 1-hour period of reoxygenation increased endothelial permeability to ¹²⁵¹-albumin. However, a 12-hour period of hypoxia followed by 1 hour of reoxygenation increased (p<0.05) endothelial permeability.
ability over control (i.e., a twofold increase over control). The increase in endothelial permeability was greater (p<0.05) with 24 hours of hypoxia followed by 1 hour of reoxygenation (i.e., a fivefold increase over control).

The time course of the increase in permeability after reoxygenation for 16 seconds to 120 minutes is shown in Figure 2. Endothelial permeability to 125I-albumin increased (p<0.05) within 5 minutes of reoxygenation (Figure 2). The response reached a maximum level within 40 minutes and then remained at approximately this level at 60 and 120 minutes of reoxygenation. In contrast, permeability did not change significantly from baseline values in control monolayers. Neither 24 hours of hypoxia followed by 1 hour of reoxygenation nor 24 hours of hypoxia alone significantly increased LDH release (4.9±2.2% and 4.7±1.0% of total LDH, respectively) compared with the control group (3.5±1.4%) (Table 2).

Exposure of endothelial cells to 24 hours of hypoxia followed by reoxygenation increased the release of superoxide anion (O$_2^-$) into the medium (Figure 3). Reoxygenation for 5 or 20 minutes did not increase the release of O$_2^-$, whereas reoxygenation for 40 or 60 minutes increased O$_2^-$ release approximately twofold over the control value. Treatment of endothelial cells with CuZnSOD (100 units/ml) for the 24-hour hypoxic period prevented the reoxygenation-induced O$_2^-$ release (Figure 3). Western blot analysis indicated that hypoxic exposure for 24 hours alone or hypoxia for 24 hours followed by 1 hour of reoxygenation did not alter the endothelial contents of CuZnSOD, MnSOD, and catalase (Figure 4).

Exposure to 24 hours of hypoxia followed by 1 hour of reoxygenation caused reorganization of F-actin distribution, and the cells became spindleshape (Figure 5). The normally distinct peripheral actin bands of normoxic cells (Figure 5a) became less structured and distinct after hypoxia/reoxygenation (Figure 5b). Bundles of intensely fluorescent, centralized stress fibers were evident in the hypoxia/reoxygenated cells (Figure 5b). Endothelial cells exposed to hypoxia alone contained more prominent stress fibers than normoxic cells, but the peripheral bands were as apparent as those of normoxic cells (Figure 5c).

Ultrastructural features of normoxic and hypoxic/reoxygenated endothelial cells are illustrated in Figure 6. The normoxic endothelial cells contained the typical constituents of mitochondria, vesicles, and nuclei (Figure 6a). Figure 6b illustrates a region of normal cell-to-cell contact with overlapping of the cytoplasmic extensions in normoxic cells. After 24 hours of hypoxia and 1 hour of reoxygenation, the endothelial cells contained numerous cytoplasmic filaments (Figure 6c), whereas the cell-to-cell (junctional) contacts were unaltered (Figure 6d). The reoxygenated endothelial cells had an increased number of injured mitochondria (Table 3). In contrast, exposure of endothelial cells to 24 hours of hypoxia alone did not alter mitochondrial morphology, which remained typical of normoxic cells (Figures 6a and 6e). An increased frequency of large (−2-μm-diameter) vacuoles was evident after both hypoxia and hypoxia/reoxygenation (Figures 6c and 6e), which was not noted in normoxic cells (Figure 6a).

**Table 2. Release of Lactate Dehydrogenase Into the Endothelial Cell Medium After Hypoxia/Reoxygenation**

<table>
<thead>
<tr>
<th>Group</th>
<th>LDH activity in medium* (%)</th>
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<tbody>
<tr>
<td>Normoxia (24 hours)</td>
<td>3.47±1.45</td>
</tr>
<tr>
<td>Hypoxia (24 hours)</td>
<td>4.67±1.02</td>
</tr>
<tr>
<td>Hypoxia (24 hours)+reoxygenation (1 hour)</td>
<td>4.95±2.25</td>
</tr>
<tr>
<td>Normoxia (24 hours)+reoxygenation (1 hour)</td>
<td>3.05±0.82</td>
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</table>

*Values are mean±SEM determined from three experiments done in triplicate. LDH, lactate dehydrogenase.

**Figure 2.** Graph showing the effects of 24-hour hypoxia and different reoxygenation periods on transendothelial 125I-albumin clearance rates. Values are mean±SEM. The number of monolayers ranged from eight to 24. *Significant difference (p<0.05) compared with the respective normoxic controls.

**Figure 3.** Bar graph showing endothelial cell release of superoxide anion (O$_2^-$) after 24 hours of hypoxia and different durations of reoxygenation. SOD, superoxide dismutase. Values are mean±SEM. The study was done in duplicates consisting of two to six experiments. *Significant increase (p<0.05) over the normoxic group.
Endothelial cells were treated with CuZnSOD (range, 10–400 units/ml) during the 24 hours of hypoxic exposure to assess the possible role of $O_2^-$ generation in mediating the increase in permeability. The reoxygenation-induced increase in endothelial permeability was reduced in a dose-dependent manner with increasing concentrations of CuZnSOD from 10 to 100 units/ml (Figure 7a). However, at concentrations of $\geq 200$ units/ml, the permeability increase was equal to the control reoxygenated group (i.e., without CuZnSOD treatment) (Figure 7a). CuZnSOD alone at the highest concentration used (400 units/ml) had no effect on basal endothelial permeability values ($^{125}$I-albumin clearance rate, 0.024±0.003 $\mu$l/min for the CuZnSOD-treated group and 0.017±0.001 $\mu$l/min for the control group). The effect of CuZnSOD was specific, since inactivation of CuZnSOD did not reduce the reoxygenation-induced increase in permeability (Table 4). Inactivated CuZnSOD alone had no effect on the basal transendothelial $^{125}$I-albumin clearance rate (normoxia control, 0.011±0.002 $\mu$l/min; normoxia plus inactivated CuZnSOD, 0.011±0.001 $\mu$l/min [Table 4]).

The protective effects of treating endothelial cells with CuZnSOD during different phases of hypoxia/reoxygenation exposure were also investigated (Figure 7b). Continuous treatment of endothelial cells with 100 units/ml CuZnSOD (i.e., the concentration shown to be most effective in Figure 7a) during the 24-hour hypoxia and 1-hour reoxygenation period reduced the reoxygenation-induced increase in permeability by 65% (Figure 7b). This reduction was similar (53%) ($p>0.05$) to that observed when CuZnSOD was only administered during the hypoxic period (Figure 7b). In contrast, treatment of endothelial cells with 100 units/ml of CuZnSOD during the reoxygenation period did not reduce the permeability increase (Figure 7b).

Treatment with catalase (100–1,000 units/ml) during the 24 hours of hypoxia prevented the reoxygenation-induced increase in permeability at 1,000 units/ml, whereas catalase concentrations of 100 and 200 units/ml were ineffective (Figure 8). In contrast, inactivated catalase (1,000 units/ml) had no effect on the increase in permeability (Table 4).

**Discussion**

The present study indicates that reoxygenation of previously hypoxic endothelial cells induced an increase in endothelial permeability to $^{125}$I-albumin. This required a minimum of 12 hours of hypoxic exposure, and the response was further augmented after 24 hours of hypoxia. The dependence of the increase in permeability on the duration of hypoxia may be related to the severity of hypoxia, since lactic acid release into the...
medium increased 2.8-fold with 12 hours of hypoxia and 5.6-fold with 24 hours of hypoxia. The prolonged 12–24-h hypoxic periods may be required to degrade ATP to hypoxanthine, a substrate for the generation of superoxide anions during reoxygenation. A hypoxic period of at least 12 hours may also be needed to convert xanthine dehydrogenase to its oxidase form.

Since some studies have suggested that exposure to hypoxia alone can increase endothelial permeability, we investigated the possibility that the response occurred during a period of hypoxia. The present results indicate that the increase in permeability is likely the result of reoxygenation, since the permeability increased gradually during the reoxygenation period; i.e., the permeability value was only slightly elevated at -5 minutes of reoxygenation and peaked at 40 minutes of reoxygenation. The reoxygenation-induced peak increase in endothelial permeability was associated with a twofold increase in superoxide anion ($O_2^-$) release into the extracellular medium. The observation that there were increased numbers of abnormal mitochondria (e.g., those with missing cristae) after reoxygenation suggests that mitochondria may be primary sites of $O_2^-$ production during this period.

Treatment of endothelial cells with CuZnSOD prevented 53% of the reoxygenation-induced increase in permeability at the optimal CuZnSOD concentration of 100 units/ml. This effect of CuZnSOD was likely the result of removal of the generated $O_2^-$ by the CuZnSOD-catalyzed dismutation reaction, since $O_2^-$ generation during reoxygenation was inhibited by CuZnSOD treatment. The protective effect of CuZnSOD supports the hypothesis that $O_2^-$ generation contributes to the increase in permeability during reoxygenation. It is also possible that the prolonged period of hypoxia decreased the levels of intracellular antioxidant enzymes, thereby creating an imbalance between the release of oxidants during reoxygenation and the oxidant scavenging potential. However, this is unlikely, since the intracellular contents of endogenous MnSOD, CuZnSOD, and catalase were not altered by the 24 hours of hypoxia or the 1-hour reoxygenation period.

The effect of CuZnSOD in reducing the reoxygenation-induced increase in permeability was critically dependent on the treatment protocol and on the particular SOD concentration used. CuZnSOD was effective only when it was present in the medium during the 24-hour hypoxic period. It was ineffective if the enzyme was added at the beginning of the reoxygenation period. In a study investigating the effects of oxygen radicals generated by hypoxia/reoxygenation on the synthesis of tissue-type plasminogen activator, Shatos et al. observed that SOD restored tissue-type plasminogen activator synthesis to control levels when SOD was present during a 24-hour hypoxic period. The requirement of a prolonged SOD incubation with endothelial cells suggests that this period is needed for SOD to permeate the intracellular sites (e.g., mitochondria) where oxidants are likely generated during reoxygenation.

Higher CuZnSOD concentrations (≥200 units/ml) paradoxically were not protective. This may be explained by the production of $H_2O_2$ as the result of the dismutation reaction catalyzed by SOD. Overproduction of CuZnSOD in transfected cells and high concentrations of exogenous CuZnSOD have been implicated in mediating tissue injury, which is consistent with the lack of protection observed at the high SOD concentrations in the present study. Another reason for this effect of high SOD concentrations may be that the enzyme can increase nitric oxide levels because the generated $O_2^-$ normally inactivates nitric oxide. The increased nitric oxide may lead to the production of the potentially toxic oxidants, peroxynitrite and hydroxyl radical.

In contrast to the partial protection observed at the optimal CuZnSOD concentrations, catalase at 1,000 units/ml abrogated the increase in permeability during reoxygenation, suggesting that $H_2O_2$ is needed for the response. The finding that catalase inhibited the increase in permeability supports the notion that the dismutation reaction catalyzed by SOD generates $H_2O_2$. 

![Figure 6](http://circres.ahajournals.org/)

**Figure 6.** Photomicrographs showing ultrastructural features of endothelial monolayers grown on microporous filters and exposed to 24 hours of normoxia (panels a and b), 24 hours of hypoxia and 1 hour of reoxygenation (panels c and d), and 24 hours of hypoxia (panels e and f). Left panels (a, c, and e) compare organellar characteristics, and right panels (b, d, and f) compare the junctional integrity in the three different exposure protocols. Magnification, ×7,500 for panels a, c, and e and ×12,000 for panels b, d, and f.

<table>
<thead>
<tr>
<th>Table 3. Mitochondrial Alterations After Hypoxia/Reoxygenation</th>
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<tr>
<td>Group</td>
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<tr>
<td>--------------------</td>
</tr>
<tr>
<td>Normoxia (24 hours)</td>
</tr>
<tr>
<td>Hypoxia (24 hours)</td>
</tr>
<tr>
<td>Hypoxia (24 hours)+reoxygenation (1 hour)</td>
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</tbody>
</table>

Values indicate the number of mitochondria (n) containing any one of the abnormal features. Numbers were tabulated from randomly selected electron micrographs at an original magnification of ×10,000. Mitochondria were considered abnormal because of loss of membrane cristae, presence of electron-lucent spaces in the matrix, and membrane injury. Percentage of abnormal mitochondria was determined as follows: abnormal (n)/[normal (n) + abnormal (n)].
which in turn can increase permeability directly and/or through the production of other oxidants such as the hydroxyl radical.

The reoxygenation-induced increase in permeability was not accompanied by any visible ultrastructural alterations in intercellular junctions. However, we cannot exclude the possibility that subtle junctional changes could account for the increased permeability of albumin. It is clear that increased permeability was not caused by cytolysis, as indicated by absence of LDH release by the endothelial cells subjected to 24 hours of hypoxia followed by 1 hour of reoxygenation. Other studies have similarly reported that hypoxia and reoxygenation exposure of endothelial cells does not produce cell detachment or cytolysis.

The increase in permeability after hypoxia/reoxygenation was accompanied by the reorganization of the F-actin cytoskeleton. This reorganization has been causally linked to a loss of the barrier function in response to proinflammatory mediators such as α-thrombin. Oxidants generated during reoxygenation may alter the depolymerization/polymerization state of actin microfilaments in endothelial cells and thus cause reorganization of the cytoskeleton. Interestingly, H$_2$O$_2$ activates protein kinase C in endothelial cells, suggesting the possibility that the oxidant-induced cytoskeletal rearrangement is the result of phosphorylation of specific actin-binding proteins.

In conclusion, exposure of endothelial cell monolayers to hypoxia for 12–24 hours followed by reoxygenation for 1–2 hours increased permeability of the endothelium to albumin. The response was triggered by reoxygenation, since the permeability increase occurred gradually during this period. The response was accompanied by the loss of peripheral actin band and severe mitochondrial injury. Treatment of endothelial cells with CuZnSOD during the hypoxic period with an optimal concentration of 100 units/ml prevented the O$_2$- generation during reoxygenation and produced a 53% reduction in the increase in permeability; in contrast, higher CuZnSOD concentrations were not protective. Catalase at the concentration of 1,000 units/ml prevented the increase in permeability. Therefore, generation of oxidants (in particular H$_2$O$_2$) by endothelial cells mediates the increase in endothelial monolayer permeability during the period of reoxygenation. These results suggest that reperfusion of organs can directly increase vascular endothelial permeability by an oxidant-dependent mechanism without the involvement of inflammatory cells such as the neutrophil.

### Table 4. Effects of Inactivated Superoxide Dismutase and Catalase on $^{125}$I-Albumin Clearance Rate

<table>
<thead>
<tr>
<th>Group</th>
<th>Inactivated SOD</th>
<th>Inactivated catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rate (μl/min)</td>
<td>n</td>
</tr>
<tr>
<td>Normoxia</td>
<td>0.011±0.002</td>
<td>8</td>
</tr>
<tr>
<td>Normoxia + inactivated enzyme</td>
<td>0.011±0.001</td>
<td>8</td>
</tr>
<tr>
<td>Hypoxia/reoxygenation</td>
<td>0.056±0.015*</td>
<td>8</td>
</tr>
<tr>
<td>Hypoxia/reoxygenation + enzyme</td>
<td>0.070±0.02*</td>
<td>7</td>
</tr>
</tbody>
</table>

Values are mean±SEM. SOD, superoxide dismutase; rate, $^{125}$I-albumin clearance rate; n, number of monolayers. Endothelial cells were treated with either 100 units/ml inactivated SOD or 1,000 units/ml inactivated catalase (see “Materials and Methods” for details).

*Significant ($p<0.05$) increases over normoxic group, which was not affected by pretreatment of monolayers with the inactivated enzymes.
FIGURE 8. Bar graph showing effects of catalase (CAT) treatment during 24 hours of hypoxia on the increase in transendothelial $^{125}$I-albumin clearance rate during reoxygenation. Values are mean ± SEM. The number of monolayers per group ranged between eight and 16. *p<0.05 for the catalase-treated group (1,000 units/ml) vs. the control group (no treatment, 0 units/ml) exposed to hypoxia/reoxygenation.

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