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Early Albumin Leakage in Pulmonary Endothelial Monolayers Exposed to Varying Levels of Hyperoxia

D. KEITH PAYNE*, MICHAEL W. OWENS* and MATTHEW GRISHAM**

Departments of Medicine and Physiology**, LSU and VA Medical Centers, Shreveport, Louisiana*

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We assessed the effect of varying levels of hyperoxia on ^{14}C-albumin flux across bovine pulmonary artery endothelial cell (BPAEC) monolayers. Endothelialized nitrocellulose filters were mounted in Ussing-type chambers which were filled with cell culture medium (M 199). Equimolar amounts of ^{14}C-labeled and unlabeled albumin were added to the "hot" and "cold" chambers, respectively, and the monolayers were exposed to 3 hours of varying levels of oxygen (16%, 30%, 40%, 60%, and 95%). When compared to 16% O_2, exposure to hyperoxic gas mixtures of 40% or greater progressively increased albumin permeability across endothelial monolayers within 3 hours to a value 2.5 times higher at 95% O_2 compared to 16% O_2 ($p < 0.001$). Hyperoxia-induced permeability increases were prevented by catalase, superoxide dismutase, desferrioxamine, and allopurinol. Our data indicate that hyperoxia induces endothelial permeability changes more rapidly than previously reported even at O_2 concentrations as low as 40%.

Keywords: Endothelium, hyperoxia, oxygen radicals, permeability

INTRODUCTION

Hyperoxia induced cellular damage is thought to result from the intracellular formation of reactive oxygen species (ROS) including superoxide, hydrogen peroxide, and hydroxyl radicals[1]. Most ROS appear to be generated by the reduction of molecular oxygen by mitochondrial metabolism with additional contributions from other cell organelles such as the endoplasmic reticulum[2]. In addition, the xanthine oxidase pathway has been proposed as a source of ROS during hyperoxia[3,4]. Under hyperoxic conditions, ROS production may overwhelm natural antioxidant systems resulting in lipid peroxidation of cellular membranes, protein sulfhydryl oxidation, and DNA damage[5,6,7]. Changes in the cytoskeletal proteins of the vascular endothelium after exposure to hyperoxia have been demonstrated and may lead to changes in endothelial permeability to proteins such as albumin[8].

The lung is exposed to the highest partial pressures of inspired oxygen in the body and is therefore the organ primarily affected by normobaric oxygen toxicity. In particular, the pulmonary vascular endothelium appears to be

Corresponding author: D. Keith Payne. Tel.: 318.675.5920. Fax: 318.675.5959. E-mail: kpayne@lsumc.edu.
Present address: Division of Pulmonary and Critical Care Medicine, LSU Medical Center—Shreveport, 1501 King's Hwy, Shreveport, LA 71130-3932, USA.

more susceptible to oxidant damage than the alveolar epithelium[9]. A "double attack" on the pulmonary endothelium by intracellularly generated ROS from inspired oxygen as well as extracellular ROS from adjacent circulating blood cells has been proposed[10]. There is increasing evidence that permeability increases to macromolecules such as albumin may be one of the earliest markers for endothelial cell dysfunction from hyperoxia, preceding morphological changes by 24–48 hours[11,12,13]. The major objectives of this study were to determine: 1) the time course of albumin leakage by endothelial cell monolayers exposed to varying levels of hyperoxia and 2) the effectiveness of antioxidants, iron chelators, and xanthine oxidase inhibitors in attenuating early hyperoxia-induced albumin leakage across endothelial monolayers.

MATERIALS AND METHODS

Endothelial Cell Culture

Bovine pulmonary artery endothelial cells (BPAEC) from an established cell line (CRL 1733) were obtained from the American Tissue Culture Collection (ATCC, Rockville, Maryland). They were cultured in medium 199 (M199, Gibco Laboratories, Grand Island, New York) supplemented with 10% fetal calf serum, thymidine (2.4 mg/liter; Sigma, St. Louis, Missouri), and gentamycin (50 ug/ml). The cells were seeded into T_{75} flasks, and incubated at 37°C in a humidified 5% CO_2 atmosphere.

Filter Preparation

Nitrocellulose filters (Sartorius, Hayward, California) with a surface area of 2 cm^2 were autoclaved, placed in 24-well plates, and seeded with 1 ml of an endothelial cell suspension (3×10^5 cell/ml). The endothelialized filters were then incubated at 37°C in a humidified 5% CO_2 atmosphere and used in experiments upon reaching confluence (10–14 days after seeding). Following the experiments, the filters were fixed in glutaraldehyde, stained with methylene blue, and the integrity of the monolayers was assessed using a stereomicroscope.

Measurement of Endothelial Permeability

Alterations of endothelial cell permeability in response to varying partial pressures of oxygen (PO_2) in the media were measured with a Ussing-type chamber as previously described. Briefly, endothelialized filters were mounted in acrylic Ussing-type chambers (MRA Corp., Clearwater, Florida) which were connected to water-jacketed glass reservoirs. Filters were placed between two recessed O rings and a minimal amount of silicone high-vacuum grease (Dow Corning, Midland, Michigan) was applied along the rim of each O ring to obtain a tight seal. Both sides of the conical chamber and the connected reservoirs were filled with a total volume of 22 ml of M199. The cross-sectional area of the exposed membrane was approximately 0.95 cm^2. The chambers were maintained at a constant temperature of 37°C by a heating circulator (model FJ, Haake, Saddlebrook, New Jersey) which pumped water through the water jackets around the reservoirs. A bubble lift system was used with a stir rate of approximately 900 bubble/min. This has previously been demonstrated to minimize the formation of unstirred layers[14]. This system was also used to vary the PO_2 within the media by using different mixtures of gases (FIO_2 of 16%, 30%, 40%, 60%, and 95%, all with 5% CO_2).

All permeability experiments were performed using ^{14}C-albumin (New England Nuclear, Boston, Mass.). Approximately 0.25 uCi of ^{14}C-albumin was added to the luminal reservoir (hot chamber) and an equimolar amount (33nM) of unlabeled albumin was added simultaneously to the abluminal reservoir (cold chamber). Samples of 0.5 ml were removed from both chambers at

specified time intervals, diluted in 10 ml of premixed aqueous scintillant (Amersham, Arlington Heights, Ill), and counted in a Beckman LS 8000 scintillation spectrophotometer (Beckman, Irvine, California).

The integrity of the ^{14}C-albumin molecule present in the cold chamber was confirmed by polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate and 2-mercaptoethanol using a 10% polyacrylamide gel[15]. The gel was dried at 62°C for 45 minutes and the location of the ^{14}C-albumin was determined by autoradiography and compared against low molecular weight standards (Biomedical Research Laboratories, Inc., Gaithersburg, Maryland).

In some experiments, the ROS scavengers Cu/Zn SOD (600 U/ml, from bovine erythrocytes, Boehringer Mannheim, Indianapolis, IN) and CAT (1000 U/ml, from beef liver, Boehringer Mannheim, Indianapolis, IN) were added to the medium (luminal and abluminal sides) immediately prior to the beginning of the experiment. These doses of antioxidants were chosen based on the protective effect demonstrated by previous studies[16,17]. In a similar fashion, the xanthine oxidase inhibitor allopurinol (0.1 mM, Sigma, St. Louis) and the iron chelator desferrioxamine (50 uM, Sigma, St. Louis) were also used in certain experiments.

Calculation of Solute Diffusional Permeability

The unidirectional flux of ^{14}C-albumin was measured across the filter while the total solute concentration of both reservoirs remained constant. The volume of liquid in both reservoirs was equal and the initial concentration of the isotope (or unlabeled solute) in the cold (downstream reservoir) was zero. Under these conditions, the following relationship can be derived for the solute permeability coefficient[18]:

$$\frac{Cc}{Cho} = \frac{1 - e^{(-2Pd \times A \times t/V)}}{2}$$

where Cc is the concentration of isotope (or unlabeled solute) in the cold (or downstream) chamber at any time t; Cho is the concentration of isotope (or unlabeled solute) in the hot (upstream) chamber at time zero; Pd is the permeability coefficient; A is the surface area of the exposed filter (0.95 cm^2); and V is the fluid volume in the downstream reservoir. Since the slope of $\ln(1-2Cc/Cho)$ versus time is $-2Pd \times A/V$, then it can be assumed that:

$$Pd = -V \times (slope)/2 \times A.$$

Measurement of PO_2

Following 30 minutes of bubbling with various concentrations of oxygen, 3cc of media was removed with a glass syringe from each Ussing-type chamber and immediately analyzed at 37°C for PO2, PCO2 and pH with a BMS3 MK2 blood gas analyzer (Radiometer, Copenhagen).

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM). Permeability coefficients were compared using a Student's t test for unpaired data and one-way analysis of variance. Statistical significance was defined as $P < 0.05$.

RESULTS

Endothelialized filters markedly reduced albumin permeability compared to non-endothelialized (blank) filters (Fig. 1). Non-endothelialized filters were more than eight times more permeable to albumin than endothelialized filters. No significant difference was noted between permeabilities measured across each side of the endothelialized membrane (luminal to abluminal $Pd = 4.07 \pm 0.65 \times 10^{-5}$ cm/sec, n = 7; abluminal to luminal $Pd = 3.08 \pm 0.46 \times 10^{-5}$ cm/sec, n = 7). Polyacrylamide slab gel electrophoresis of

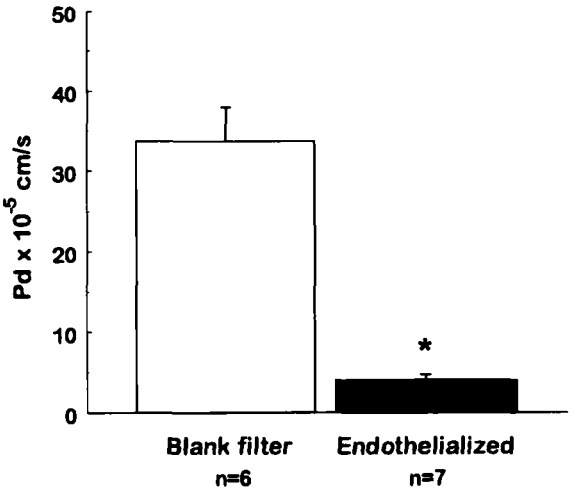

FIGURE 1 Effect of endothelialization on filter permeability. ^{14}C-albumin permeability was determined for blank filters and endothelialized filters exposed to 16% O_2 (PO_2 = 124 mm Hg). *$p < 0.001$ vs. blank filter. All permeabilities were measured from luminal to abluminal side.

^{14}C-albumin collected from the cold chamber revealed a single band corresponding to a molecular weight of 6.8×10^4.

The PO_2 in the media was determined for each O_2 concentration used and ranged from approximately 125 mmHg using 16% O_2 to 540 mmHg with the use of 95% O_2 (Table I). Endothelialized filters exposed to 3 hours of 95% O_2 demonstrated permeability increases to albumin 2.5 times greater than those exposed to 16% O_2 (Figure 2). Significant increases in permeability were also observed using 60% and 40% O_2 (Figure 3). The use of 30% O_2 did not result in a significant increase in permeability during the time course studied. In order to determine if the observed permeability changes were simply due to cell death caused by hyperoxia, endothelial cells were stained with trypan blue after exposure to 3 hours of 95% O_2. Trypan blue dye exclusion was observed in over 95% of the cells exposed to hyperoxia. Monolayer confluence was confirmed after each experiment by examining methylene blue stained filters via stereomicroscopy.

To assess the role of hyperoxia generated free radicals in the observed permeability changes, the free radical scavengers superoxide dismutase (SOD, 600 U/ml) and catalase (CAT, 1000 U/ml) were added to the medium immediately preceding the 3 hour exposure to 95% O_2. At these concentrations, both enzymes were protective when used together as well as separately (Figure 4). The use of the iron chelator desferrioxamine (50 uM) and the xanthine oxidase inhibitor allopurinol (0.1 mM) also prevented hyperoxia-induced permeability changes. Boiling of SOD and CAT at 100°C for 45 minutes resulted in a loss of protection ($Pd = 12.0 \pm 2.2 \times 10^{-5}$ cm/sec, n = 6 and $13.4 \pm 4.4 \times 10^{-5}$ cm/sec, n = 5, respectively).

DISCUSSION

The major findings of this study are: 1) exposure of endothelial monolayers to hyperoxic gas mixtures as low as 40% O_2 resulted in a rapid (less than 3 hours) increase in permeability to albumin; 2) permeability to albumin over a 3 hour period increased progressively as the PO_2 in the media increased and 3) hyperoxia-induced permeability changes were prevented by pre-treat-

TABLE I PO_2, PCO_2, and pH in Media for Different O_2 Concentrations*

% O_2	16%	30%	40%	60%	95%
PO_2	123.4 ± 0.6	184.2 ± 1.6	252.2 ± 1.2	347.9 ± 2.1	538.5 ± 5.4
PCO_2	37.0 ± 0.6	34.6 ± 0.3	34.3 ± 0.6	36.8 ± 1.1	40.9 ± 1.7
pH	7.45	7.44	7.42	7.40	7.39

* n = 4 for all values

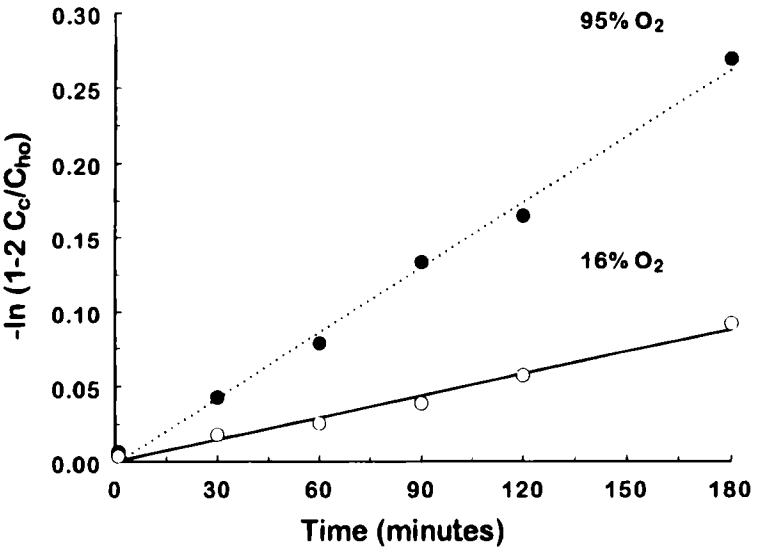

FIGURE 2 Representative 3 hour experiments with 16% O_2 (solid line, open circles) and 95% O_2 (dotted line, closed circles). Diffusional permeability is proportional to the slope of the regression lines. In these experiments exposure of the filters to 16% O_2 and 95% O_2 yielded permeability coefficients to albumin of 4.1×10^{-5} cm/s and 10.9×10^{-5} cm/s, respectively.

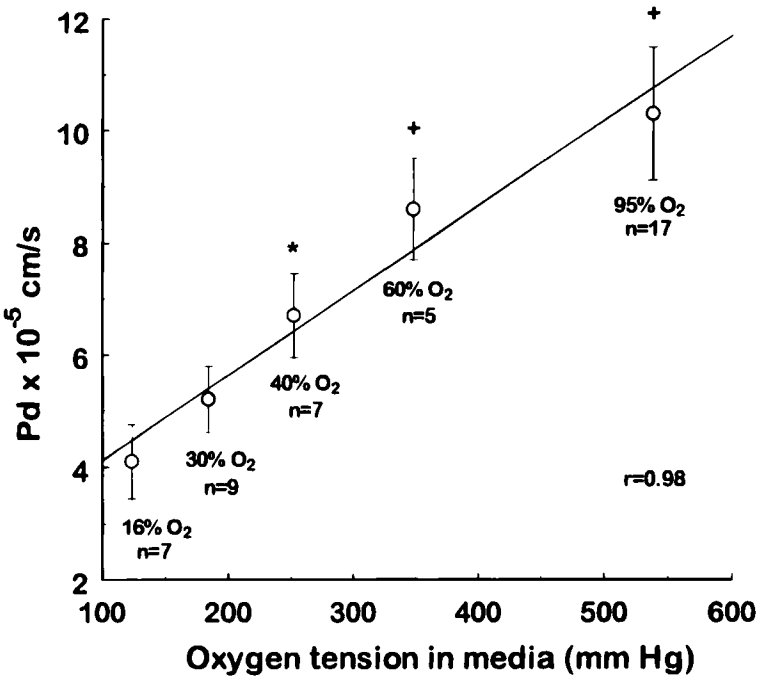

FIGURE 3 Effect of media PO_2 on albumin permeability of endothelialized monolayers. Permeability increased in a linear fashion with increasing PO_2. *$p < 0.05$ vs. 16% O_2. +$p < 0.01$ vs. 16% O_2.

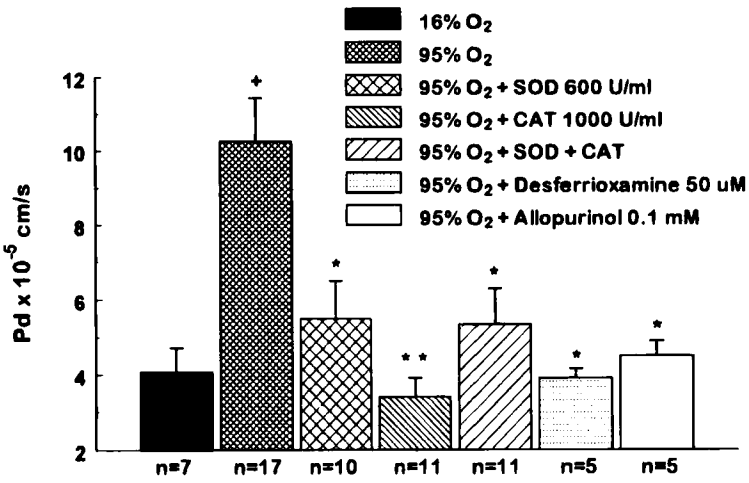

FIGURE 4 Pretreating endothelialized filters with the antioxidants SOD and CAT, the iron chelator desferrioxamine, and the xanthine oxidase inhibitor allopurinol protect against hyperoxia-induced permeability changes (each antioxidant was added to both luminal and abluminal sides). +$p < 0.001$ vs 16% O_2. *$p < 0.01$ vs. 95% O_2. **$p < 0.001$ vs. 95% O_2.

ment with antioxidants (SOD, CAT), iron chelators, and xanthine oxidase inhibitors.

We used a well-established *in vitro* system to detect changes in endothelial permeability to the tracer molecule albumin. Other investigators have also found that endothelialized filters provide a useful *in vitro* model for permeability assays[19,20,21]. Although permeability values in these systems tend to be 10–100 times higher than measurements made across endothelial barriers *in vivo*, reproducible relative changes in measured parameters (e.g. permeability) more than absolute values have provided useful insights into *in vivo* physiology[22,23].

Our findings indicate that permeability changes across an endothelial monolayer in response to hyperoxia occur considerably earlier than previously reported. In contrast, Phillips and Tsan reported that no significant albumin leakage across an endothelial monolayer occurred before 3 days of exposure to 95% O_2[8]. As in our study, they found no evidence of cell death or detachment during the course of their experiments. No change in the size of the endothelial cells was detected until day 3 of hyperoxia. Interestingly, these investigators described subtle changes in actin filament distribution at 2 days of hyperoxic exposure, one day earlier than they detected albumin leakage. In contrast to Phillips and Tsan, we used a Ussing-type chamber for the measurement of permeability. In a previous investigation, we demonstrated the ability of this system to detect changes in albumin flux across an endothelial monolayer exposed to hypoxia/reoxygenation over a 3–4 hour period[16]. A bubble-lift system enabled us to stir both sides of the endothelial monolayers during the experiments, thus decreasing the formation of unstirred layers[14]. These differences in methodologies between the two systems may account for our different measurement outcomes.

Our findings are supported by the work of Davis *et. al.* who detected increased levels of albumin in the bronchoalveolar lavage fluid of normal human subjects after 17 hours of breathing 95% O_2[11]. These subjects achieved an average PaO_2 (partial pressure of arterial oxygen) of 506 ± 4 torr, similar to our value in the culture media of 538.5 ± 5 torr. In another recent in vivo study, Weir *et al.* used sensitive quantitative immunocytochemistry techniques to demonstrate that rats exposed to 100% oxygen for 24 hours had five times more albumin in the interstitial spaces of the lung compared to

controls[13]. Light microscopic sections of the lungs of the experimental rats after 24 hours of hyperoxia demonstrated minimal morphologic changes despite the increase in interstitial albumin. Gross interstitial edema was seen at 60 hours, a morphologic time course similar to other reports[24,25,26]. More recently, Boyce *et al.* reported increased lung albumin in rats exposed to 100% O_2 for as little as 24 hours[12]. Thus it appears that albumin leakage across the small vessels of the lung occurs well in advance of gross morphologic changes. There is also evidence that certain metabolic activities of the vascular endothelium may be affected by short periods of hyperoxia. Block *et al.* reported that serotonin uptake was significantly depressed in bovine endothelial cells after 20 hours of 95% O_2 and that plasma membrane fluidity was reduced in as little as 4 hours of exposure to 95% O_2[27,28].

Our findings support the hypothesis that hyperoxia produces cellular damage through the excessive generation of free radical species. Other investigators have shown that free radical production by lung cells, including $O_2^{\bullet-}$, H_2O_2, and $OH^{\bullet}$, are increased under hyperoxic conditions[29,30,31]. In particular, lung mitochondrial production of H_2O_2 increases linearly with increasing levels of oxygen up to 60% with evidence of an even greater rate of H_2O_2 production at higher O_2 levels[31]. In our system, SOD and CAT, either alone or together were effective in preventing permeability changes in response to hyperoxia. The relatively large size of both molecules (SOD, 31,000 mol wt; CAT 210,000 mol wt.) would seem to make them ineffective against the initial intracellular sites of oxidant damage. It is possible that these antioxidants may be intercepting free radical species (such as H_2O_2, or $O_2^{\bullet-}$) as they exit the cell, before they are able to react with neighboring cells. Conceivably, this could provide some protection against degradation of cell-cell junctions via free radical interaction with Fe^{3+} bound to extracellular tight junctional proteins. Alternatively, these antioxidants may be binding to the cell membrane and confer protection by preventing lipid peroxidation.

Iron appears to play a major role in free radical induced tissue injury via a number of mechanisms including participation in the Haber-Weiss chemical reaction that generates $OH^{\bullet}$ from $O_2^{\bullet-}$ and H_2O_2. In our system, the iron chelator desferrioxamine provided protection equal to that observed with SOD and CAT, indicating that the generation of $OH^{\bullet}$ may be of key importance in the early phase of hyperoxia-induced endothelial permeability increases. M199 contains iron and we cannot exclude the possibility that free iron in the media may have contributed to the observed results. Iron bound to lipid and protein components of the cell membrane, particularly near the intercellular junctions, could contribute to local generation of ROS with resultant damage of key intercellular adhesion molecules. At relatively high concentrations (>0.5 mM), desferrioxamine may act as a free radical scavenger for a variety of ROS including $O_2^{\bullet-}$ and $OH^{\bullet}$, however, this is much less likely at the relatively low concentration (50 uM) used in these experiments[32,33].

The potential importance of xanthine dehydrogenase (XDH) to xanthine oxidase (XO) conversion as a source of ROS under hyperoxic conditions is supported by our experiments. Pretreatment with allopurinol was effective in preventing hyperoxia-induced increases in albumin leakage across the endothelial monolayers. We have previously reported significant amounts of XDH/XO in our BPAEC cell line (13 mU/mg wet weight) and have observed significant protection from ischemia/reperfusion permeability changes in endothelial monolayers with the use of oxypurinol[16]. Both allopurinol and oxypurinol in relatively high concentrations ($\geq$ 1mM) may act as direct $OH^{\bullet}$ and $O_2^{\bullet-}$ scavengers[34,35]. Allopurinol may also quench free radicals by acting as an electron transfer agent at concentrations $\geq$ 0.37 mM[35]. While we cannot rule out the possibility that part of the protective effect we observed against hyperoxia induced permeability changes was due to these mechanisms, our use of a much lower dose of allopurinol (0.1 mM) makes these explanations less likely.

Our findings using allopurinol are supported by several other investigations. Jenkinson, *et al.* reported a beneficial effect of allopurinol on pulmonary dynamics and lung morphology of premature primates exposed to 100% oxygen[36]. Rodell *et al.* reported that $O_2^{\bullet-}$ production by tungsten (a XO inhibitor) treated BPAEC was decreased and that endothelial monolayer permeability to albumin was similarly decreased in BPAEC monolayers pretreated with tungsten prior to exposure to neutrophil elastase[3]. Furthermore, rats fed a tungsten-rich diet had greatly lowered lung XO activity and displayed much less acute edematous lung injury in response to hyperoxia. Decreases of XO in response to hyperoxia occur relatively quickly. Terada *et al.* reported the XO activity in both rat lungs and BPAEC decreased significantly by 6 hours in response to 95–100% O_2[4].

Our findings differ from two recent studies in which no significant role of cellular XO in the generation of ROS was demonstrated[37,38]. In contrast to our use of bovine pulmonary artery endothelial cells, these investigators used bovine aortic endothelial cells. It is possible that tissue differences in the role of XDH/XO in the generation of ROS exist. Experimental endpoint differences may also explain our divergent findings. These investigators used cell detachment after 48 hours of hyperoxic exposure. We assessed albumin leakage during 3 hours of hyperoxic exposure. We believe our endpoint is a more sensitive measure of early oxidant induced endothelial cell dysfunction.

The mechanism by which oxygen derived free radicals increase endothelial monolayer permeability to albumin remains unclear and the present study does not address this issue directly. A likely explanation is the creation of paracellular pathways for tracer movement by cytoskeletal mediated endothelial cell retraction. Reversible changes of endothelial cell actin peripheral bands induced by oxyradical species have been associated with increases in endothelial monolayer permeability to albumin[39,40]. Changes in the distribution of actin filaments within endothelial cells and a shift from G actin to F actin in response to hyperoxia have been described[41]. Agents such as phalloidin which induce polymerization of actin appear to enhance endothelial barrier function and reduce endothelial monolayer permeability to a variety of agents[42,43].

In conclusion, our data indicate that a rapid increase in endothelial cell permeability to albumin occurs within 3 hours of exposure to oxygen levels as low as 40%. Albumin leakage induced by these ROS is preventable by pretreating BPAEC monolayers with a variety of agents including antioxidants, iron chelators, and XO inhibitors. Further work is needed to elucidate the mechanisms by which ROS increase endothelial permeability to macromolecules.

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