Redox regulation of endothelial barrier integrity

XIANGYI ZHAO,1 J. STEVEN ALEXANDER,1 SHU ZHANG,1 YANAN ZHU,1 NOLA J. SIEBER,1 TAK YEE AW,1 AND DONNA L. CARDEN1,2

Departments of 1Molecular and Cell Physiology and 2Medicine, Louisiana State University Health Sciences Center, Shreveport, Louisiana 71130

Received 26 October 2000; accepted in final form 2 May 2001

Zhao, Xiangyi, J. Steven Alexander, Shu Zhang, Yanan Zhu, Nola J. Sieber, Tak Yee Aw, and Donna L. Carden. Redox regulation of endothelial barrier integrity. Am J Physiol Lung Cell Mol Physiol 281: L879–L886, 2001.—Intestinal ischemia-reperfusion is associated with the generation of reactive oxygen metabolites as well as remote, oxidant-mediated lung injury. Oxidants elicit endothelial redox imbalance and loss of vascular integrity by disorganizing several junctional proteins that contribute to the maintenance and regulation of the endothelial barrier. To determine the specific effect of redox imbalance on pulmonary vascular barrier integrity, microvascular permeability was determined in lungs of animals subjected to chemically induced redox imbalance. The effect of redox imbalance on microvascular permeability and endothelial junctional integrity in cultured lung microvascular cells was also determined. Whole lung and cultured pulmonary endothelial cell permeability both increased significantly in response to chemical redox imbalance. Thiols depletion also resulted in decreased endothelial cadherin content and disruption of the endothelial barrier. These deleterious effects of intracellular redox imbalance were blocked by pretreatment with exogenous glutathione. The results of this study suggest that redox imbalance contributes to pulmonary microvascular dysfunction by altering the content and/or spatial distribution of endothelial junctional proteins.

INTESTINAL ISCHEMIA-REPERFUSION (I/R) is an adaptive response to circulatory shock associated with the development of remote pulmonary microvascular dysfunction and the acute respiratory distress syndrome (ARDS) (8, 25, 33, 36, 41). Intestinal I/R-elicited lung injury may involve destruction of endothelial cell-cell junctional proteins such as cadherins, which result in increased permeability edema formation (7). Vascular endothelial (VE)-cadherin, an integral membrane component of endothelial adherens junctions, interacts with the actin cytoskeleton and contributes to the maintenance and regulation of the microvascular barrier by forming homotypic bonds with adjacent endothelial cells (3, 9, 24, 27–30). The fact that soluble cadherin fragments are detectable in the plasma of patients with ARDS supports the concept that disruption of endothelial junctional proteins contributes to microvascular barrier dysfunction in this disorder (7).

Enhanced production of reactive oxygen metabolites within the microcirculation is a well-recognized consequence of intestinal I/R (15) and a documented feature of lung injury in ARDS (4, 24, 31). An important consequence of reactive oxygen metabolite production is intracellular oxidative stress (5, 32). Several mechanisms normally provide protection against this type of injury, including endogenous oxygen radical scavengers, antioxidants, and detoxification enzymes. GSH, the principal intracellular low molecular weight thiol, is a major intracellular antioxidant that protects cells from oxidative stress induced by reactive oxygen metabolites like hydrogen peroxide (H2O2) (9–11, 14, 20–22, 35, 37, 43, 44). However, these defenses can become exhausted if the oxidant load is excessive.

Although oxidants elicit intracellular oxidative stress, the specific contribution of altered endothelial cell redox balance (ratio of GSSG to GSH) to the relevant pulmonary microvascular dysfunction associated with intestinal I/R is unknown. To address this question, we used a chemical model of thiol depletion to address three specific objectives: 1) to define the effect of redox imbalance on microvascular permeability in whole lungs and in cultured lung microvascular endothelial monolayers, 2) to determine the effect of altered GSSG-to-GSH balance on endothelial structural integrity, and 3) to determine the effect of redox imbalance on lung microvascular VE-cadherin content.

MATERIALS AND METHODS

All chemicals and antibodies were purchased from Sigma (St. Louis, MO) unless otherwise specified. Cell culture medium (EGM-2-MV) was purchased from BioWhittaker (San Diego, CA). VE-cadherin antibody was purchased from Immunotech (Sassenage, France).

The Louisiana State University Health Sciences Center Animal Care and Use Committee approved the animal experiments in advance. At the end of each experiment, the deeply anesthetized animal was euthanized by thoracotomy and exsanguination.

http://www.ajplung.org 1040-0605/01 $5.00 Copyright © 2001 the American Physiological Society
Surgical Preparation

Intestinal I/R. Adult male Sprague-Dawley rats (250–350 g) were anesthetized with ketamine (100 mg/kg) and xylazine (7 mg/kg) intraperitoneally after which the trachea was intubated, the carotid artery was cannulated, and a ventral midline celiotomy was performed. Anesthesia was maintained throughout the experiment with supplemental intraperitoneal ketamine and xylazine. The superior mesenteric artery was completely occluded with a noncrushing microvascular clamp after which the incision was closed. After 120 min of ischemia, the vascular clamp was removed, and the intestine was allowed to reperfuse for 20 min. Control (nonischemic) animals were subjected to an identical protocol without superior mesenteric artery occlusion. Body temperature was maintained at 37 ± 0.5°C throughout the experimental protocol.

Heart-lung isolation. After intestinal I/R or control surgery, the thoracic cavity was incised along both midaxillary lines, 500 U of heparin sodium were injected into the right ventricle, cannulas were placed in the pulmonary artery and left atrium, and the heart and lungs were removed en bloc (1, 7, 8, 13, 41). The isolated lungs were perfused with whole blood (intestinal I/R experiments) or a buffered saline solution (redox imbalance experiments) in an extracorporeal perfusion system with a constant-flow pump. Pulmonary arterial and venous pressures were continuously measured.

Pulmonary capillary pressure. Measurement of pulmonary capillary pressure was estimated with the double-occlusion technique (42) in which the arterial inflow and venous outflow lines are simultaneously occluded and the equilibrium pulmonary arterial and venous pressures were recorded.

Whole Lung Permeability Determination

Capillary filtration coefficient. Pulmonary microvascular permeability was determined by measuring the isogravimetric capillary filtration coefficient as described by Adkins and Taylor (1) and Gaar et al. (13) and is expressed as milliliters per minute per centimeters of water per 100 g of lung tissue.

Cell Isolation and Culture

To determine the mechanism by which redox imbalance contributes to endothelial barrier disruption, cultured lung microvascular cells were employed. Antibodies directed against rat VE-cadherin are currently unavailable. Although the available anti-human VE-cadherin cross-reacts poorly with cultured rat lung microvascular cells, it does cross-react with mouse VE-cadherin. Thus, to determine the effect of redox imbalance on lung microvascular structure and function, mouse lung microvascular endothelial cells (MLMVECs) were used. MLMVECs were isolated from four adult C57BL/6 mice with a modification of the technique reported by Chung-Welch et al. (12).

Permeability Determination in Cultured Lung Microvascular Cells

FITC-albumin. MLMVECs were seeded on fibronectin-coated 8.0-μm Transwell inserts (Becton Dickinson Labware, Franklin Lakes, NJ) at a density of 4 × 10^5/cm² and grown to confluence. Cells grew only on the luminal (coated) surface of the inserts. After exposure to H₂O₂ or thiol-depleting agents (described in Immunofluorescence), medium was removed, and the inserts were transferred to a new 24-well plate containing 1 ml of PBS. Two hundred microliters of FITC-albumin (12.5 mg/ml in PBS) were added to each insert, and the monolayers were incubated at 37°C for 2 h, after which the insert was discarded. Monolayer permeability to FITC-albumin was determined by measuring the absorbance of the solution in each well at 450 nm.

Endothelial Cell Structural Integrity

Immunofluorescence. MLMVECs were cultured on 12-mm round glass coverslips. Two hours after treatment with H₂O₂ or thiol-depleting agents, cells were fixed with 95% ethanol. Two hundred microliters of the primary antibody (mouse anti-VE-cadherin diluted 1:200 in PBS) were added and incubated at 37°C for 1 h. Coverslips were washed with 0.1% fat-free milk powder in PBS, and 200 μl of secondary antibody (Cy3 anti-mouse IgG diluted 1:200 in PBS) were added. Coverslips were mounted on glass slides with mounting medium (Vector Laboratories, Burlingame, CA) and photographed with a computerized microscope recording system.

Endothelial Cell Cadherin Content

Western blotting and blot density analysis. Confluent MLMVECs were treated with H₂O₂ or thiol-depleting agents for 2 h after which protein samples were electrophoresed on a 7.5% polyacrylamide gel (36). Resolved proteins were electrophoretically transferred to nitrocellulose membranes. The membranes were incubated with primary antibody (mouse anti-VE-cadherin diluted 1:1,500) and secondary antibody (peroxidase-conjugated anti-mouse IgG diluted 1:1,500) followed by enhanced chemiluminescence (Amer sham Life Science). The density of VE-cadherin staining was measured with a densitometer with an HP Scanjet flatbed scanner. Images were analyzed for density with Image Pro-Plus image analysis software (Media Cybernetics, Silver Spring, MD).

Quantification of GSH and GSSG

Endothelial cell GSH and GSSG levels were determined by the method of Reed et al. (36). Briefly, the assay is based on the initial formation of S-carboxymethyl derivatives of free thiols followed by the conversion of free amino groups to 2,4-dinitrophenyl derivatives. Separation of GSH and GSSG derivatives is achieved by reverse-phase ion-exchange HPLC.

Modulation of Cellular GSH and GSSG

Endothelial cell GSH and GSSG levels were modified in vivo and in vitro with a combination of three chemical thiol-depleting agents as previously described (25). Diamide is a potent cell-permeant oxidant (26) that preferentially oxidizes low molecular weight thiols (e.g., GSH) and free SH groups of proteins (26, 34, 38). The action of diamide induces formation of disulfide bonds via thiol-diamide intermediates (26), thereby promoting the formation of GSSG or disulfide cross-links. Consequently, the redox potential of the cell is shifted in favor of a more oxidized state, usually reflected in an increase in the ratio of GSSG to GSH. Buthionine sulfoximine (BSO) specifically and irreversibly inhibits γ-glutamylcysteine synthetase, the rate-limiting step in GSH synthesis (16). Inhibition of this enzyme causes a marked decrease in the total cellular GSH pool but has minimal effects on the GSSG-to-GSH ratio. 1,3-Bis-2-chloroethyl-1-nitrosourea (BCNU) selectively inhibits GSH reductase, thereby inhibiting the reduction of oxidized glutathione disulfide (GSSG) to the reduced (GSH) form (44).

Treatment Protocols

Animals subjected to intestinal I/R. To determine the effect of intestinal I/R on remote pulmonary redox balance, rats were
subjected to intestinal I/R \((n = 6)\) or control surgery \((n = 6)\), after which the lungs were snap-frozen and stored at \(-80^\circ C\) until analyzed for GSH content and GSSG-to-GSH ratio.

To determine the effect of intestinal I/R on lung permeability edema formation, animals were subjected to intestinal I/R \((n = 6)\) or control surgery \((n = 6)\), after which the lungs were isolated and microvascular permeability was determined. To define the contribution of H\(_2\)O\(_2\) or endothelial GSH content to intestinal I/R-elicited lung injury, catalase \((150,000\, \text{U/kg}; \ n = 6\, \text{rats})\) or the cell-permeant GSH precursor \(N\)-acetyl-L-cysteine \((150\, \text{mg/kg}; \ n = 6\, \text{rats})\) was administered 10 min before intestinal reperfusion and was also added to the isolated lung perfusion reservoir.

**Thiol depletion in whole lungs.** To define the effect of redox imbalance induced by GSH depletion on microvascular permeability in whole lungs, diamide was administered directly to the isolated lung perfusion reservoir to achieve a final concentration of 0.2 mM \((n = 6\, \text{lungs})\), and permeability was measured for 120 min. To further examine the contribution of GSH synthesis and recycling on lung microvascular permeability, BSO \((5\, \text{mM in the extracellular space})\) was administered intraperitoneally to the intact animal 5 h before and BCNU \((50\, \mu\text{M in the extracellular space})\) was administered 1 h before lung removal and diamide administration. To ensure that the effects of diamide were related to GSH depletion and not to direct cytotoxicity, GSH \((2\, \text{mM in the extracellular space})\) was administered 1 h before heart-lung isolation and diamide administration \((n = 6\, \text{rats})\). Lung microvascular permeability was determined at baseline and after drug administration.

**Oxidant exposure or thiol depletion in MLMVECs.** Three experiments were performed in each group \((n = 3\, \text{rats})\). Treatment groups consisted of cells exposed to tissue culture medium only (control), H\(_2\)O\(_2\) \((0.5\, \text{mM})\), diamide \((0.2\, \text{mM})\), BSO \((5\, \text{mM})\), BCNU \((50\, \mu\text{M})\), or diamide plus BSO plus BCNU for 1 or 2 h. Cells pretreated with GSH were washed before exposure to H\(_2\)O\(_2\) or the thiol-depleting agents.

**Statistical Analysis**

All values are expressed as means \(\pm\) SE. Data were analyzed with a one-way ANOVA, with Bonferroni posttesting for multiple comparisons or Student’s \(t\)-test where appropriate. A \(P\) value \(< 0.05\) was considered significant.

**RESULTS**

**Intestinal I/R Elicits Lung Redox Imbalance**

Figure 1 demonstrates that intestinal I/R induces a significant reduction in pulmonary GSH content \((A)\) and a significant increase in the lung GSSG-to-GSH ratio \((B)\), indicating marked pulmonary oxidative stress. Although intestinal I/R elicited significant lung redox imbalance, the effect of chemically induced redox imbalance was not examined in the isolated perfused whole lung. Preliminary results in lungs treated with the thiol-depleting agents demonstrated that 2 h of continuous buffer perfusion caused significant washout of GSH, reducing pulmonary GSH content to the extent that quantitative analysis was not feasible. Therefore, the effect of the thiol-depleting drugs on lung redox balance was examined in cultured MLMVECs (see Fig. 4).

**Reactive Oxygen Metabolites Contribute to Intestinal I/R-Elicited Lung Injury**

Intestinal I/R-induced pulmonary oxidative stress was associated with a significant increase in lung microvascular permeability (Fig. 2). Administration of catalase or the GSH precursor \(N\)-acetyl-L-cysteine
blocked the lung injury elicited by gut I/R. These results suggest that reactive oxygen metabolites such as H$_2$O$_2$ contribute to, whereas intracellular GSH blunts, the pulmonary microvascular dysfunction induced by intestinal I/R.

**Redox Imbalance Elicits Pulmonary Microvascular Dysfunction in Whole Lungs**

Figure 3 shows that 15 min after chemical induction of redox imbalance with diamide or diamide plus BSO plus BCNU, there was a significant increase in lung microvascular permeability similar to that observed after intestinal I/R. Importantly, the pulmonary dysfunction induced by redox imbalance was irreversible and progressive 2 h after drug administration. These results demonstrate that depletion of intracellular GSH is sufficient to induce marked pulmonary microvascular dysfunction.

**Thiol-Depleting Drugs Elicit Redox Imbalance in MLMVECs**

Figure 4 illustrates the effects of thiol-depleting drugs and H$_2$O$_2$ on intracellular GSH and the GSSG-to-GSH ratio in cultured MLMVECs. Compared with control-treated cells, treatment with diamide alone, diamide plus BSO plus BCNU, or H$_2$O$_2$ caused a marked reduction in intracellular GSH content (Fig. 4A) and a significant increase in the GSSG-to-GSH ratio (Fig. 4B). The intracellular oxidative stress elicited by diamide and H$_2$O$_2$ was reversed by GSH supplementation.

**Redox Imbalance Increases the Permeability of Lung Microvascular Endothelial Cell Monolayers**

Figure 5 summarizes the effects of diamide alone, diamide plus BSO plus BCNU, and H$_2$O$_2$ on MLMVEC permeability to FITC-labeled albumin. After treatment with diamide, diamide plus BSO plus BCNU, or H$_2$O$_2$, MLMVEC permeability to albumin increased. Diamide significantly increased monolayer permeability for the 2-h duration of the experiment.

**Structural Integrity of the Lung Microvascular Endothelial Barrier**

Immunofluorescent staining (Fig. 6) showed normal lung endothelial cell morphology consisting of homogeneous VE-cadherin distribution to endothelial cell-cell junctions under control conditions. In monolayers exposed to H$_2$O$_2$, diamide alone, or diamide plus BSO plus BCNU (Fig. 6, B–D, respectively), the cell-cell junctional integrity is disrupted, as manifested by diminished cell apposition, gap formation, and a heterogeneous distribution of VE-cadherin at cell junctions.

---

Fig. 3. Chemical induction of redox imbalance with thiol-depleting agents. Diamide and diamide + buthionine sulfoximine (BSO) + 1,3-bis-2-chloroethyl-1-nitrosourea (BCNU) elicited a progressive increase in lung microvascular permeability compared with results from control surgery. Lung edema formation induced by diamide was blocked by administration of GSH (diamide + GSH) 1 h before diamide administration. Significantly different from control: *P < 0.05; **P < 0.01; †P < 0.001.

Fig. 4. Change in GSH (A) and GSSG/GSH (B) in mouse lung microvascular endothelial cells (MLMVECs) exposed to thiol-depleting agents, H$_2$O$_2$ and diamide (D). Diamide and diamide + BSO + BCNU significantly reduced lung GSH content and increased the pulmonary GSSG-to-GSH. Significantly different compared with control: *P < 0.05; **P < 0.01.
**Endothelial Cell Cadherin Content**

Immunostaining of the 130-kDa cadherin band on Western blots of MLMVECs exposed to diamide or diamide plus BSO plus BCNU demonstrates that endothelial cell exposure to the thiol-depleting drugs induced a loss of cadherin content (Fig. 7).

**DISCUSSION**

Reperfusion of the ischemic intestine is associated with microvascular dysfunction in the gut and in remote organs such as the lung. In the pulmonary microcirculation, this dysfunction is manifested as increased permeability edema formation, progressive hypoxemia, and respiratory failure or ARDS.

Considerable evidence has accumulated over the past several decades to support a pivotal role for reactive oxygen metabolites in the local and remote lung injury initiated by intestinal I/R (15, 36, 41). Substantial evidence also exists to support a critical role for oxidant-mediated pulmonary damage in ARDS. For example, the breath condensate of patients with ARDS contains substantial quantities of H$_2$O$_2$ compared with that of control patients or patients mechanically ventilated for reasons other than ARDS (4, 19, 31, 40). Currently, the levels of H$_2$O$_2$ that are formed at or near the endothelial surface are widely debated, but many studies (20, 22, 23) have employed concentrations as high as 1 mM; these concentrations are thought to represent levels of peroxide released at the neutrophil-endothelial interface.

Glutathione is a major intracellular antioxidant that appears to be a specific target of oxidative damage in ARDS because these patients have reduced total GSH defenses and increased GSSG in their alveolar fluid (6, 28, 35). The results of this study extend previous observations in that we have shown that detoxifying...
Redox imbalance may participate in the regulation of endothelial cadherins through other mechanisms. First, redox imbalance may induce cadherin endocytosis similar to that described in H$_2$O$_2$-challenged endothelial cells (23). Second, redox shifts could activate redox-sensitive cysteine proteins such as caspases that mediate the proteolytic cleavage of endothelial cell cadherins, resulting in loss of endothelial apposition (6, 17). Alternatively, redox shifts may contribute to diminished homotypic cadherin binding, resulting in decreased cell apposition and increased vascular solute flux (2). Regardless of the exact mechanism, our data are consistent with the redox-induced loss of cadherin content at endothelial cell borders that is associated with enhanced fluid flux across the vascular barrier and is reversed by restoring intracellular GSH. Thus redox imbalance renders endothelial cells vulnerable to loss of junctional proteins and disruption of barrier integrity.

In summary, I/R-mediated pulmonary microvascular injury appears to be critically dependent on intracellular redox status and is attenuated by therapies that target intracellular GSH stores. By altering the composition and spatial distribution of the endothelial cell junctional complex, redox imbalance promotes micro-
vascular dysfunction that is manifested as enhanced solute permeability.

This work was supported by National Institutes of Health Grants P02-DK-43785, 1-R03-AG-17348-01, DK-44510, and HL-47615.

REFERENCES


