Intermittent hypoxia-induced endothelial barrier dysfunction requires ROS-dependent MAP kinase activation

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Submitted 4 October 2013; accepted in final form 24 January 2014

LUNG MICROVASCUlAR ENDOTHELIAL cells line the blood vessels of the microcirculatory bed forming a semipermeable barrier, selectively permitting alveolar gas exchange, while preventing fluid and solute flow across the alveolar capillary wall. The barrier function of the endothelial cells is regulated by the structural integrity of the cytoskeleton as well as the tight and adherens junctions with adjacent endothelial cells (8, 23). Given that endothelial cells constitute the first layer of the vasculature that is in direct contact with the blood, any perturbations in blood flow and/or its composition such as hypoxia might affect the barrier function. Systemic hypoxia (i.e., decreases in blood oxygen level) occurs under many different circumstances profoundly impacting a variety of physiological systems (20). Continuous exposure to hypoxia lasting 24 h has been shown to disrupt endothelial barrier function by activating proinflammatory cytokines (1), heat shock protein (HSP)27 (13), and hypoxia-inducible factor (HIF)-1α (21). Intermittent hypoxia (IH) is a hallmark manifestation of recurrent apnea patients with obstructive sleep apnea (OSA). Recent studies showed that OSA patients exhibit pulmonary edema, which could be prevented by treating OSA with continuous positive airway pressure (Refs. 4–6). Mimicking obstructive sleep apnea in anesthetized dogs also produce pulmonary edema (10). The pulmonary edema seen in OSA patients might reflect endothelial barrier dysfunction by IH. However, the effect of IH on endothelial barrier function and the underlying mechanisms are not known.

Emerging evidence suggests that systemic and cellular responses to IH are mediated by oxidative stress resulting from increased generation of reactive oxygen species (ROS) (12, 19). Since oxidative stress results in endothelial barrier dysfunction (3), we hypothesized that IH disrupts endothelial barrier function via ROS-dependent activation of signaling pathways(s). We tested this possibility in human lung microvascular endothelial cells exposed to a previously established in vitro IH paradigm (18), which recapitulates many effects of chronic IH in intact experimental animals.1

MATERIALS AND METHODS

Cell Culture

Human lung microvascular endothelial cells (Lonza Group) were plated at a density of ~1 × 10^6/cm² and cultured in EBM-2 complete medium supplemented with 10% fetal bovine serum, antibiotics, ascorbic acid, and growth factors under 20% O² and 10% CO² at 37°C. Before experiments, the cells were placed in serum-free medium for 18 h.

Exposure of Cells to IH

Endothelial cell cultures were exposed to alternating cycles of 1.5% O² for 30 s followed by 20% O² for 5 min, with a total duration of 330 s per cycle, in a humidified Lucite chamber at 37°C as described previously (31). Briefly, the chamber was equilibrated with gases at a flow rate of 2.4 l/min. The durations of hypoxia and normoxia were maintained using timer-controlled solenoid valves. The O² levels in the culture medium and the ambient O² levels in the chamber were monitored with an O² electrode (Lazar Research Laboratories) and by an O2 analyzer (Beckman LB2), respectively. Cells exposed to repetitive normoxia (i.e., alternating cycles of 20% O² for 30 s followed by 20% O² for 5 min) served as controls. In experiments testing the

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1 This article is the topic of an Editorial Focus by Kimberly A. Smith and Jason X.-J. Yuan (22a).
effects of various drugs, cells were incubated with the indicated concentration of drugs for 30 min before exposure to either repetitive normoxia or IH without changing the medium.

**Determination of Transendothelial Electrical Resistance**

Transendothelial electrical resistance (TEER) was measured in an electrical cell-substrate impedance sensing system (Applied BioPhysics, Troy, NY) as described previously (26). Briefly, endothelial cells were grown to ~95% confluence in polycarbonate wells containing gold electrodes connected to a phase-sensitive lock-in amplifier. Electrodes containing cells were placed in an electrical cell-substrate impedance incubator for 1 h to stabilize basal electrical resistance. The total electrical resistance across the endothelial monolayer was determined by the combined resistance between the basal surface of the cell and the electrode, providing a measure of alterations in cell-cell or cell-matrix adhesion. In the experiments assessing the time course of the response, TEER is expressed as normalized resistance (i.e., ratio of resistance at a given time to resistance at “zero” time).

**Measurement of ROS**

Fluorescence microscopy. Cells were loaded with 25 μM 6-carboxy-2',7'-dichloro-dihydrofluorescein diacetate (DCFDA) in EBM-2 medium for 30 min at 37°C. After loading with DCFDA, cells were exposed to IH. At the end of IH exposure, cells were washed twice with warm phosphate-buffered saline (PBS) and examined under a Zeiss Axiovert100TV inverted fluorescence microscope using excitation and emission wavelengths of 495 and 520 nm, respectively. The fields were randomly selected for imaging using a high-resolution, cooled CCD camera system with a ×20 objective lens. In experiments assessing the effect of antioxidant, cells were pretreated with manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP), a membrane-permeable ROS scavenger for 30 min before loading with DCFDA and exposure to IH. The concentration of MnTMPyP was 50 μM, which was based on preliminary experiments.

Malondialdehyde levels. To further confirm ROS generation, malondialdehyde (MDA) levels were determined as a measure of lipid peroxidation in cell homogenates as previously described (22). The results were expressed as microgram of MDA formed per milligram of protein.

**Immunofluorescence Microscopy**

Cells were prepared for immunofluorescence analysis as described previously (28). Briefly, cells were sequentially treated with the following reagents and were rinsed with PBS before each step. The treatments include 1) 3.7% formaldehyde in PBS for 10 min (fixation); 2) 0.25% Triton X-100 in Tris-buffered saline containing 0.01% Tween 20 (TBST) for 2 min (permeabilization); 3) 10% donkey serum in PBS for 60 min (blocking nonspecific binding sites); 4) either rabbit anti-vascular endothelial cadherin (VE-cadherin; 1:200 dilution) or anti-extracellular signal-regulated kinase 1/2 (ERK1/2; 1:1,000 dilution) or anti-phosphorylated JNK (1:1,000 dilution) polyclonal antibody obtained from Cell Signaling and then separated on a 10% SDS-PAGE gel and transferred to PVDF membrane. Cell extracts containing equal amounts of proteins (1 mg/ml) were separated on a 10% SDS-PAGE gel and transferred to PVDF membrane. Membranes were probed with either anti-extracellular signal-regulated kinase 1/2 (ERK1/2; 1:1,000 dilution) or anti-phosphorylated ERK (1:1,000 dilution) or anti-total SAPK/c-jun NH2-terminal kinase (JNK; 1:1,000 dilution) or anti-phosphorylated JNK (1:1,000 dilution) polyclonal antibody obtained from Cell Signaling and then with horseradish peroxidase-conjugated secondary antibody. The immunocomplexes were visualized using an ECL detection system (Amersham). The blots were scanned and quantified using Scion Image Software (National Institutes of Health).

**Western Blot Analysis**

Cells were extracted in lysis buffer containing 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, PMSF, and protease inhibitor cocktail. Cell extracts containing equal amounts of proteins (1 mg/ml) were separated on a 10% SDS-PAGE gel and transferred to PVDF membrane. Membranes were probed with either anti-extracellular signal-regulated kinase 1/2 (ERK1/2; 1:1,000 dilution) or anti-phosphorylated ERK (1:1,000 dilution) or anti-total SAPK/c-jun NH2-terminal kinase (JNK; 1:1,000 dilution) or anti-phosphorylated JNK (1:1,000 dilution) polyclonal antibody obtained from Cell Signaling and then with horseradish peroxidase-conjugated secondary antibody. The immunocomplexes were visualized using an ECL detection system (Amersham). The blots were scanned and quantified using Scion Image Software (National Institutes of Health).

**Experimental Protocols**

Series 1. The TEER was determined in cells exposed to repetitive normoxia (control) or 10 or 30 or 60 cycles IH (n = 8–16 channels in each group).

Series 2. ROS levels in cells (~5 × 10⁶) exposed to either repetitive normoxia (control) or 30 cycles of IH were monitored by
DCFDA fluorescence and MDA levels \((n = 5 \text{ experiments in each group})\). A double-blinded approach was used to ensure unbiased evaluation of DCFDA fluorescence data.

Series 3. The effect of antioxidant on TEER was determined in cells treated with either vehicle (control) or MnTMPyP \((50 \mu M)\) before exposure to either repetitive normoxia or 30 cycles of IH \((n = 10–15 \text{ channels in each group})\).

Series 4. Cells were exposed to either repetitive normoxia (control) or 30 cycles of IH. The distribution of cortactin, VE-cadherins, and ZO-1 proteins as well as the colocalization of cortactin and actin was assessed by immunocytochemistry using anti-cortactin, anti-actin, anti-VE-cadherins, and anti-ZO-1 antibodies. A double-blinded approach was used for the evaluation of immunostaining patterns \((n = 16–20 \text{ cells in each group})\).

Series 5. In experiments assessing the role of ROS in IH-induced redistribution of cortactin, VE-cadherins, and ZO-1 proteins as well as the colocalization of cortactin and actin, cells were treated with either vehicle (control) or MnTMPyP \((50 \mu M)\) before exposure to repetitive normoxia or 30 cycles of IH \((n = 16–17 \text{ cells in each group})\). The other experimental details are same as described in series 4.

Series 6. To assess the effect of IH on the levels of phosphorylated forms of ERK1/2 and JNK, cells \((\sim 5 \times 10^4)\) were exposed to repetitive normoxia (control) or 30 cycles of IH and the cell extracts were subjected to immunoblot analysis using anti-ERK1/2, anti-JNK, anti-phospho-ERK, and anti-phospho-JNK antibodies \((n = 5 \text{ experiments in each group})\).

Series 7. To assess the role of MAP kinases in IH-induced endothelial barrier dysfunction, PD98059, an ERK1/2 inhibitor \((30 \mu M)\); SP600125, a JNK inhibitor \((30 \mu M)\); or vehicle (control) was added to cell culture medium and the cells were subsequently exposed to either repetitive normoxia or 30 cycles of IH \((n = 10–12 \text{ channels in each group})\).

Series 8. To assess the role of MAP kinases in IH-induced redistribution of cortactin, VE-cadherins, and ZO-1 as well as on the colocalization of cortactin and actin, cells were first treated with PD98059 \((30 \mu M)\) or SP600125 \((30 \mu M)\) or vehicle (control) and then exposed to either repetitive normoxia or 30 cycles of IH \((n = 15–18 \text{ cells in each group})\). The other experimental details are same as described in series 4.

**Data Analysis**

In all experiments, the samples were analyzed in triplicate. The data were expressed as means ± SE. Statistical significance was evaluated by one-way ANOVA followed by Tukey’s test. \(P\) values of < 0.05 were considered significant.

**RESULTS**

**Effect of IH on Endothelial Barrier Function**

Endothelial barrier function was determined by monitoring TEER in lung microvascular endothelial cells before and immediately after termination of IH exposure. Thirty cycles of IH decreased TEER compared with pre-IH, which gradually returned to control levels during 1 h of reoxygenation (Fig. 1, A and B). The effect of IH on TEER was stimulus dependent and the response reached a plateau with 30 cycles of IH (Fig. 1C).

**ROS Mediate IH-Induced Endothelial Barrier Dysfunction**

We then determined whether IH increases ROS levels in lung microvascular endothelial cells. ROS levels were determined by two approaches: one by monitoring DCFDA fluorescence and the other by measuring MDA levels. Cells exposed to IH exhibited increased DCFDA fluorescence and elevated MDA levels, and these effects were prevented by pretreatment with MnTMPyP \((50 \mu M)\), a membrane-permeable ROS scavenger (Fig. 2, A–C). Pretreatment with MnTMPyP markedly attenuated IH-induced reduction in TEER, whereas it had no effect on barrier function in control cells (Fig. 3, A and B).

**Reorganization of the Cytoskeleton and Junction Proteins by IH**

Endothelial barrier function depends on the integrity of cytoskeleton and junction proteins \((8, 23)\). We, therefore,
determined the effects of IH on actin and cortactin, the major cytoskeletal proteins. Immunofluorescence analysis revealed reorganization of cortactin and actin as well as stress fiber formation in cell exposed to IH (Fig. 4A). Quantitative analysis of the fluorescence showed that cortactin redistributed to cell periphery (Fig. 4B). Colocalization of actin and cortactin was markedly increased in IH-exposed cells compared with controls (Fig. 4, A and C). Reoxygenation for 1 h restored the effects of IH on actin and cortactin (Fig. 4, A and C). Pretreatment with MnTMPyP prevented IH-induced reorganization of actin and cortactin (Fig. 4, A and C).

VE-cadherins and ZO-1 representing tight and adherens junction proteins, respectively, regulate endothelial cell permeability by modulating membrane adhesion of neighboring cells (8, 27, 28). In control endothelial cells, ZO-1 and VE-cadherins are primarily localized at the cell boundaries exhibiting a continuous distribution, which was markedly disrupted in IH-exposed cells (Fig. 5A). Both ZO-1 and VE-cadherins were redistributed partially to the cytoplasm in IH-exposed cells (Fig. 5, B and C). As a consequence, intercellular gaps and regions of membrane ruffling appeared on the cell-cell interface in IH-exposed cells (Fig. 5A), and these effects disappeared following reoxygenation for one h (Fig. 5, A–C). MnTMPyP prevented reorganization of junction proteins (Fig. 5, A–C).

**MAP kinase Activation by IH**

ERK1/2 and JNK contribute to endothelial cell barrier function via phosphorylation of junction proteins (27). Therefore, the roles of ERK1/2 and JNK in IH-induced alteration in endothelial barrier function were examined. Cells exposed to IH showed increased levels of phosphorylated forms of ERK1/2 and JNK. Either reoxygenation or treatment with MnTMPyP decreased IH-induced ERK1 and 2 phosphorylation, and completely prevented JNK phosphorylation (Fig. 6, A and B). Treatment of cells with either 30 µM of PD98059, an ERK1/2 inhibitor, or 30 µM of
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SP600125, a JNK inhibitor, prevented IH-induced decrease in TEER (Fig. 7, A and B) and the reorganization of cytoskeleton and junction proteins (Fig. 8, A–D).

DISCUSSION

In this study, we determined the impact of IH on lung microvascular endothelial barrier function and assessed the underlying mechanisms. Our results demonstrate that exposure to IH disrupts the endothelial barrier dysfunction via ROS-dependent activation of ERK1/2 and JNK-mediated reorganization of cytoskeleton and junction proteins. The effects of IH were reversed following reoxygenation. IH-induced reorganization of cytoskeleton and junction proteins is unlikely due to shear stress caused by gas flows, because alternating cycles of normoxia, which were used as controls, virtually had no effect on endothelial barrier function. It would have been ideal to monitor TEER during exposure to IH, but technical difficulties precluded performing these experiments. However, we believe that TEER measurements performed immediately after the last episode of hypoxia represents the impact of IH on endothelial permeability. The cumulative, total duration of hypoxia during 30 cycles of IH is 15 min (30 s/cycle × 30 cycles). Despite this short duration, IH exposure led to marked changes in barrier function and reorganization of cytoskeleton and junction proteins. In sharp contrast, 24 h of continuous hypoxic exposure is required to produce similar changes in barrier function and cytoskeletal reorganization (2, 21). These observations demonstrate that IH is a more potent stimulus than continuous hypoxia in disrupting endothelial barrier function.

What makes IH a more potent stimulus than continuous hypoxia? Using two independent approaches we demonstrated that IH increases ROS generation in lung microvascular endothelial cells. MnTMPyP, a membrane-permeable ROS scavenger, prevented ROS generation and blocked IH-induced endothelial barrier dysfunction. In a previous study, we reported that a comparable cumulative duration of continuous hypoxia had no effect on ROS generation in cell cultures (29). The central role for ROS in mediating endothelial barrier dysfunction by IH is further supported by a previous study showing that H2O2, an oxidant, also produces similar barrier dysfunction (11). Therefore, it is likely that ROS generation by IH makes it a more potent stimulus than a given duration of continuous hypoxia.

How might ROS contribute to IH-induced endothelial barrier dysfunction? MAP kinase-mediated phosphorylation of scaffold and junction proteins of the endothelial cells has been implicated in endothelial barrier dysfunction (25, 26). IH-activated ERK1/2 and JNK and MnTMPyP prevented this...
Effect suggesting that MAP kinase activation is mediated by ROS. Quantitative immunofluorescence analysis demonstrated increased interaction between actin and cortactin, and actin polymerization resulting in stress fiber formation. The contribution of cortactin redistribution to endothelial barrier function is complex. Previous studies reported redistribution of cortactin correlates with increased (9) and in certain conditions to decreased (24) endothelial barrier function. Our results with IH.

Fig. 5. Effect of IH on junction proteins in human lung microvascular endothelial cells. A: immunofluorescence analysis of the distribution of zona occludens-1 (ZO-1) and vascular endothelial (VE)-cadherins in control (left), IH (left middle), 1 h post-IH (right middle), and IH + MnTMPyP-treated cells (right). Nuclei were stained with DAPI. The yellow arrows indicate sites of membrane ruffling as well as cytoplasmic redistribution of VE-cadherins and ZO-1. Bar in the control at top = 20 μm. B and C: quantitative analyses of the distribution of ZO-1 (B) and VE-cadherins (C) are shown. Control (n = 17 cells), IH (n = 16 cells), 1 h post-IH (n = 15), and IH + MnTMPyP (n = 16 cells). Data (means ± SE) are expressed as percentage of control. **P < 0.01, compared with vehicle-treated control.

Fig. 6. IH increases ERK and JNK phosphorylation. Representative immunoblots (top) and average data of densitometric analysis (bottom) of ERK1/2 (A) and JNK (B) phosphorylation in control and in cells exposed to 30 cycles of IH with and without MnTMPyP (50 μM) treatment, and in the cells subjected to reoxygenation after IH exposure (1 h post-IH) are presented. Average data are presented as a ratio of phosphorylated and unphosphorylated ERK1/2 and JNK protein. Data are presented as means ± SE from 5 independent experiments. *#P < 0.01 and **##P < 0.001.
not only affects pulmonary gas exchange but also leads to fluid exchange in the lung, and disruption of barrier function cytosekeletal and junction proteins. Activation of MAP kinases, which initiates reorganization of induced barrier dysfunction is mediated by ROS-dependent exposure cells. These results taken together suggest that IH-concomitant restoration of endothelial barrier function in IH-reorganization of cytoskeleton and junction proteins with a scavenger. Furthermore, inhibitors of ERK1/2 or JNK blocked pretreatment with MnTMPyP, a membrane-permeable ROS lation of ERK1/2 and JNK and these effects were prevented by cortactin, ZO-1, and VE-cadherins in cells showed that cortactin redistribution was associated endothelial barrier dysfunction. However, besides cortactin, endothelial barrier function is also regulated by a complex interplay of other molecules including junction proteins. Indeed, we observed translocation of VE-cadherins and ZO-1 to the cytosol in IH-exposed cells, resulting in formation of intercellular gaps and membrane ruffling, which are characteristic of endothelial barrier dysfunction. Remarkably, the reorganization of cytoskeletal and junction proteins was associated with phosphorylation of ERK1/2 and JNK and these effects were prevented by pretreatment with MnTMPyP, a membrane-permeable ROS scavenger. Furthermore, inhibitors of ERK1/2 or JNK blocked reorganization of cytoskeleton and junction proteins with a concomitant restoration of endothelial barrier function in IH-exposed cells. These results taken together suggest that IH-induced barrier dysfunction is mediated by ROS-dependent activation of MAP kinases, which initiates reorganization of cytoskeletal and junction proteins.

The Microvascular endothelial barrier is critical for gas and fluid exchange in the lung, and disruption of barrier function not only affects pulmonary gas exchange but also leads to pulmonary edema. Previous studies reported the occurrence of pulmonary edema in OSA patients (4–6) and also in experimental models of OSA (10). Although several other pathologies are associated with OSA, the current study suggests that IH, which is a hallmark manifestation of OSA, is a potent stimulus for disrupting endothelial barrier function, which might account for the development of pulmonary edema. Recent studies showed that increased transcription and activation of the prooxidant enzyme Nox2 and decreased transcription and activity of the antioxidant enzyme (e.g., Sod2) contribute to increased ROS levels by IH (15, 16). Analysis of the molecular mechanisms revealed that activation of HIF-1 mediates the increased Nox2 transcription (17, 30). On the other hand, IH decreases HIF-2 activity, which in turn results in insufficient transcription of the antioxidant enzyme Sod2 (15). Thus the imbalance between HIF-1 and HIF-2 and resulting changes in pro- and antioxidant enzyme expressions contribute to IH-induced oxidative stress. However, further studies are needed to demonstrate that IH-induced imbalance in HIF-1 and HIF-2 contributes to endothelial barrier dysfunction via ROS.
GRANTS
This work was supported by grants from National Institutes of Health, National Heart, Lung and Blood Institute RO1-HL-76537 and PO1-HL-90554 (to N. R. Prabhakar) and PO1-HL-58064 (to V. Natarajan).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
N.R.P., conception and design of research; V.V.M., P.V.U., G.Y., M.M.L., J.N., performed experiments; V.V.M., M.M.L., J.N., V.N. analyzed data; V.V.M., N.R.P. interpreted results of experiments; V.V.M. prepared figures; V.V.M., G.K.K., drafted manuscript; G.K.K., N.R.P. edited and revised manuscript; N.R.P. approved final version of manuscript.

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