Hypoxia alters biophysical properties of endothelial cells via p38 MAPK- and Rho kinase-dependent pathways

Steven S. An,1 Corin M. Pennella,2 Achuta Gonnabathula,2 Jianxin Chen,1 Ning Wang,1 Matthias Gaestel,3 Paul M. Hassoun,4 Jeffrey J. Fredberg,1 and Usamah S. Kayyali2

1Physiology Program, Department of Environmental Health, Harvard School of Public Health, Boston; 2Pulmonary and Critical Care Division, Department of Medicine/Tufts Research Institute, Tufts-New England Medical Center and Tufts University School of Medicine, Boston, Massachusetts; 3Institute of Biochemistry, Medical School Hannover, Hannover, Germany; and 4Division of Pulmonary and Critical Care Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland

Submitted 30 August 2004; accepted in final form 21 April 2005

HYPOXIC CONDITIONS AFFECT a wide variety of organs and are often associated with pathological conditions, including high-altitude pulmonary edema, acute respiratory distress syndrome, pulmonary hypertension, and ischemia-reperfusion injury (38, 54). Several studies have demonstrated that hypoxia disrupts the endothelial cell barrier and, consequently, increases endothelial permeability both in vitro (44, 46) and in vivo (53). Underlying biophysical mechanisms and key molecular processes that regulate endothelial permeability in hypoxic conditions remain to be elucidated, however.

We have recently shown in rat pulmonary microvascular endothelial cells (RPMEC) that hypoxia causes redistribution of the actin cytoskeleton (CSK) in a fashion that is dependent on p38 mitogen-activated protein kinase (MAPK) activation and leads to the phosphorylation of the small heat shock protein HSP27 (30, 31). HSP27 is a member of the stress-inducible small HSP family that is thought to act as a microfilament-capping protein in vitro (41) and to be constitutively expressed at high levels in the lung (33). During cellular stress and growth, HSP27 undergoes rapid phosphorylation, which in turn promotes actin polymerization and stress fiber formation (8, 26, 36). However, it remains unclear to what extent these structural changes contribute to functional changes in biophysical properties of the endothelium at cellular and subcellular levels.

Herein we report changes in biophysical properties of the CSK of RPMEC in response to hypoxia, as well as the modulatory effects of HSP27. To quantify cytoskeletal remodeling events, we measured spontaneous nanoscale movements of an individual microbead that was coated with a peptide containing Arg-Gly-Asp (RGD). Such beads bind avidly to cell surface integrin receptors (58) and form focal adhesions (40); they become well integrated into the CSK scaffolding (4, 20, 58) and display tight functional coupling to stress-bearing cytoskeletal structures and the contractile apparatus (3, 27, 59). We reasoned that the bead can move only if microstructures to which it is attached rearrange; accordingly, spontaneous nanoscale motions report cytoskeletal rearrangements in space and time. To quantify cell stiffness, we used magnetic twisting cytometry (20, 58), and to quantify traction forces exerted by the cell on its substrate, we used Fourier transform traction microscopy (11, 59). We present evidence indicating the existence of differentially regulated biochemical and biophysical events in response to hypoxia. On the one hand, hypoxia causes early Rho-kinase-mediated myosin phosphorylation, leading to the development of contractile stress within the endothelial cell and, on the other hand, it causes belated p38-mediated HSP27 phosphorylation, leading to stabilization of the actin CSK at or near focal adhesions. These findings help to explain how hypoxia alters endothelial cell barrier function.

MATERIALS AND METHODS

Materials and reagents. RPMI 1640, phosphate-free Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), dialyzed FBS, penicillin G potassium, streptomycin, Fungizone, and glutamine were purchased from Invitrogen (Carlsbad, CA). Ham’s F-12 nutrient medium and amphotericin B were purchased from

Address for reprint requests and other correspondence: U. S. Kayyali, Pulmonary & Critical Care Division, Tufts-New England Medical Center, 750 Washington St. #257, Boston, MA 02111 (e-mail: ukayyali@tufts-nemc.org).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
GIBCO (Grand Island, NY). The synthetic RGD peptide (Peptide 2000; Integra Life Sciences) was provided by Dr. Jürg Tschopp. All other reagents and drugs were obtained from Sigma (St. Louis, MO), with the exception of SB-203580-HCl (p38 MAPK inhibitor) and Y-27632 (Rho-kinase inhibitor), which were purchased from Calbiochem (La Jolla, CA) and Tocris Cookson (Ellisisville, MO), respectively. SB-203580-HCl and Y-27632 were reconstituted in sterile distilled water, whereas myosin light-chain kinase (MLCK) inhibitor ML-7 was prepared in DMSO. On the day of the experiments, all drugs were reconstituted in serum-free medium; ML-7 was diluted to final concentrations in serum-free medium yielding <0.1% DMSO in final volume.

Cell culture and exposure to hypoxia. Rat pulmonary microvascular endothelial cells (RPMEC) were a gift from Dr. Una Ryan (Avant Immunotherapeutics, Needham, MA) and have been well characterized by us and others (15). These cells exhibit typical endothelial “cobblestone” morphology and stain positively with antibodies against von Willebrand factor. RPMEC were grown in culture as previously described (15). Unless otherwise specified, 1 day before the experiments, cells were harvested with 0.25% trypsin-0.02% ethylenediaminetetraacetic acid (EDTA) solution, plated on tissue culture petri dishes at subconfluence (9.6 cm² growth area; Becton Dickinson), and maintained in serum-free media at 37°C in humidified air containing 5% CO₂.

For hypoxic exposure, cells were placed in humidified, airtight incubation chambers (Billups-Rothenberg, Del Mar, CA) and gassed with 3% O₂, 5% CO₂, and balanced N₂. The hypoxic chambers were kept at 37°C in a tissue culture incubator for the duration of 0.5, 1, or 4 h. For normoxic exposure (0.5, 1, or 4 h), cells were maintained at 37°C in humidified air containing 5% CO₂.

Transfection of endothelial cells. Phospho-mimicking mutant human HSP27 (HSP27-PM) construct was generated as previously described (19, 48). This construct was made in the pcDNA3 vector (Invitrogen), in which the cytomegalovirus promoter drives the eukaryotic expression of the corresponding protein. The vectors used for transfection were pcDNA3 alone and pcDNA3-HSP27-PM, in which the residues S15D, S78D, S82D were mutated to mimic phosphorylated HSP27. The vectors were introduced (5 μg) into endothelial cells by electroporation. Stable cell lines were obtained by selection with geneticin, and resistant colonies were isolated, expanded, and then screened for the level of human HSP27 expression. Unless otherwise noted, cells in passages 6 and 7 were used that typically represented a pure population of stable cell lines.

Characterization of spontaneous bead motion. The dynamics of the CSK network were measured as described previously (4). Using microscopic observation, we visualized spontaneous nanoscale movements of an individual RGD-coated microbead tightly anchored to the CSK of the endothelial cell (~50–100 beads/field of view) and recorded its positions every 83 ms. The trajectories of bead motions in two dimensions were then characterized by computing the mean square displacement of all beads as a function of time: [MSD(t)] (nm²)  

\[
\text{MSD}(t) = \frac{1}{N} \sum_{i=1}^{N} r_i(t)^2 
\]

where \(r_i(t)\) is the distance of the \(i\)th bead at time \(t\) relative to its position at time \(0\). The MSD was computed at intervals that were equally spaced in time (1.3 s). Bead motions were corrected for the confounding effects of microscope stage drift; the stage drift was estimated from changes of the mean position of all beads within a field of view. The limit of resolution in our system was on the order of ~10 nm, but by 10 s, most beads had displaced a much greater distance. Accordingly, we analyzed MSD data for times >10 s up to 300 s. As shown in results, the MSD of RGD-coated microbeads increased with time according to a power law relationship:

\[
\text{MSD}(t) = D^* (t/t_0)^{\alpha} 
\]

The coefficient \(D^*\) and the exponent \(\alpha\) of the bead motion were estimated from a least-squares fit of a power law relationship to the aggregate average of MSD data vs. time; we took \(t_0\) to be 1 s and expressed \(D^*\) in nm². For ordinary Brownian motion, the distribution of displacements would be expected to be Gaussian and the exponent \(\alpha\) to be unity, in which case the coefficient \(D^*\) is related to the familiar diffusion coefficient in two dimensions (5, 35). When a power law relationship is shown to exist but the exponent \(\alpha\) differs from unity, the random motions are said to reflect anomalous or fractional diffusion (21); when the exponent \(\alpha\) is smaller than unity, random motions are said to be subdiffusive; and when the exponent is larger than unity, random motions are said to be superdiffusive (21). In this present study, we evaluated bead motions both before and after each time exposure (0.5, 1, or 4 h) to hypoxia or to normoxia.

Magnetic twisting cytometry with optical detection. The CSK stiffness of each individual RPMEC was measured as described previously (20). Briefly, the RGD-coated ferrimagnetic microbeads bound to adherent cells were first magnetized horizontally (parallel to the surface on which cells were plated) with a brief 1,000-Gauss pulse and then twisted in a vertically aligned homogeneous magnetic field (40 Gauss) at a frequency of 0.75 Hz. This sinusoidal magnetic twisting magnetic field was too weak to remagnetize the beads and instead caused both a rotation and a pivoting displacement of the beads. As the beads move laterally to and fro, therefore, the cell resists their motion by developing internal stresses that depend on the mechanical properties of the cell (20, 27). Accordingly, lateral bead displacements in response to the resulting oscillatory torque were detected optically (in spatial resolution of ~10 nm), and the ratio of specific torque to lateral bead displacements was computed and expressed as the cell stiffness in units of Pa/μm. Cell stiffness was measured both before and after each exposure (0.5, 1, or 4 h) to hypoxia or normoxia.

Traction microscopy. A detailed description of this technique was provided by Butler and colleagues (11, 59). Using traction microscopy, we measured the distribution of contractile stresses arising at the interface between each adherent cell and its substrate (traction field). To obtain each traction field, RPMEC were plated sparsely on a polycarboxylamide elastic gel block coated with collagen type I (0.2 mg/ml) and allowed to spread and stabilize for 6 h. Cells were then exposed for 0.5 h with either normoxia or hypoxia. After each normoxic or hypoxic exposure, images of fluorescent microbeads (0.2 μm in diameter; Molecular Probes, Eugene, OR) embedded near the gel apical surface were taken both before and after the cell was completely detached from the substrate by trypsin. The fluorescent image of the same region of the gel after trypsin was used as the reference (traction free) image. The displacement field between a pair of images was then obtained by identifying the coordinates of the peak of the cross-correlation function (11, 59).

From the displacement field and known elastic properties of the gel (Young’s modulus of the gel was determined to be ~1,300 Pa, and a Poisson ratio was taken to be 0.48), the traction field was calculated using both constrained and unconstrained Fourier transform traction cytometry (FTTC) as described previously (11, 59). The computed traction field was then used to obtain 1) the contractile stress (pre-stress), defined as the net tensile force transmitted by the actin CSK across a cross-sectional area of the cell per unit area; and 2) the net contractile moment, defined as a scalar measure of the cell’s contractile “strength” (11, 59). In this study, prestress is expressed in pascals (Pa) and the net contractile moment is expressed in piconewton meters (pNm).

Statistical analysis. Data are presented as means ± SE, and \(n\) represents the number of cells. Statistical differences were determined using either Student’s t-test for comparison of two sample means or ANOVA for comparison of more than two sample means, followed by Bonferroni post hoc testing for multiple comparisons between two sample means. \(P < 0.05\) was considered statistically significant.
RESULTS

Spontaneous bead movements track motions of underlying cytoskeletal structure. Over the course of 5 min, the trajectory of each RGD-coated microbead (4.5 μm in diameter) anchored to the CSK of RPMEC displayed random motions that amounted to only a small fraction of the bead diameter (~0.6 μm) and an even smaller fraction of cell size. On the basis of these very small bead motions, and therefore, for all practical purposes, the cell would seem to be virtually infinitely lateral in extent. Accordingly, the expectations would be that for the duration of these experiments spontaneous bead motions would not be constrained by distant cell boundaries. Consistent with this interpretation, mean square displacement (MSD) was found to grow in an unbounded fashion with time (Fig. 1) and, more specifically, varied with time as a power law (Eq. 2). MSD increased with time as $t^{1.8}$, whereas an exponent of unity would be expected for a simple passive diffusion. Here and in all subsequent figures, the MSDs were computed at intervals that were equally spaced in time (1.3 s). For clarity, however, we have suppressed many of these data and left only data at approximately logarithmically spaced intervals. Data presented as means ± SE (n = 2,069 cells).

Hypoxia alters the dynamics of spontaneous bead motions. Previously, we have shown that hypoxia (3% O₂) causes structural reorganization of the actin CSK and, in particular, the formation of stress fibers in RPMEC (31). In the present study, we evaluated the relationship, if any, between reported structural changes in the actin CSK and the anomalous bead motions in response to hypoxia. Exposure of RPMEC to hypoxia (0.5, 1, or 4 h) caused time-dependent changes in spontaneous bead motions (Fig. 2). Cells exposed to hypoxia for 0.5 h showed no qualitative differences in the behavior of bead motions compared with cells exposed to normoxia for the same duration (Fig. 2A). Nevertheless, compared with cells exposed to normoxia (0.5 h), cells exposed to hypoxia exhibited bead motions that were appreciably different at small times, i.e., the coefficient D* was smaller by a factor of 2 and the superdiffusive exponent $\alpha$ was slightly bigger. However, the MSD evaluated at 300 s for these cells (hypoxic and normoxic) was not different (Fig. 2D).

By contrast, cells exposed to hypoxia for 1 h displayed a substantial decrease in bead motions compared with cells exposed to normoxia for the same duration; changes of both the coefficient D* and the exponent $\alpha$ contributed strongly to these changes (Fig. 2B). Consequently, the MSD evaluated at 300 s decreased dramatically after a 1-h exposure to hypoxia (Fig. 2D). Interestingly, however, cells exposed to hypoxia for 4 h showed no qualitative or quantitative differences in their bead motions compared with cells exposed to normoxia for the same duration (Fig. 2, C and D). Taken together, these observations indicate that hypoxia causes dynamic changes in spontaneous bead motions that recapitulate remarkably in time the associated structural changes in the actin CSK (31). Accordingly, these findings imply that anomalous movements of RGD-coated microbeads reflect ongoinganoscale remodeling events in the cytoskeletal structure and that hypoxia-induced decrease in spontaneous bead motions is probably due to stabilization of the actin CSK. Consistent with these interpretations, the more polymerized the network of filamentous (F) actin, the more tethered the beads are to that cytoskeletal scaffolding and the less able they are to move as a result.

Hypoxia tethers spontaneous bead motions via the activation of p38 MAPK. The formation of F-actin in response to hypoxia is thought to be mediated via the activation of p38 MAPK (30, 31). Once activated, p38 phosphorylates and activates its downstream effector MAP kinase-activated protein kinase 2/3 (MK2), which in turn phosphorylates HSP27 and, in doing so, prevents it from binding to actin monomers and causes F-actin polymerization (26, 49). To assess the role of p38 and its downstream signals on spontaneous bead motions, RPMEC were treated with 0.5 h with or without a p38 MAPK inhibitor SB-203580 before 1-h exposure to hypoxia. Under baseline conditions, cells treated with 3 μM SB-203580 exhibited an increase in bead motions as evaluated by MSD at 300 s (Fig. 3A) and, more important, displayed no further decrease in bead motions in response to hypoxia (1 h). Cells treated with a Rho-kinase inhibitor (Y-27632; 3 μM) also exhibited an increase in bead motions and showed no appreciable decrease in bead motions in response to hypoxia. Such hypoxic exposure in the case of untreated cells caused a significant decrease in bead motions (Fig. 3A).

To assess the specific contribution of HSP27 to spontaneous bead motions, RPMEC were stably transfected with HSP27-PM, in which phosphorylatable amino acids (Ser15, Ser78, and Ser82) were replaced by negatively charged aspartate as described previously (48). In normoxic cells, overexpression of the HSP27-PM mutant caused an increase in the distribution of stress fibers (Fig. 3B, inset) that mimicked and resembled the structural changes in response to hypoxia (31). Under baseline conditions, cells overexpressing HSP27-PM exhibited a signif-
Hypoxia causes rapid (0.5 h) increases in cell stiffness compared with their respective normoxic controls (Fig. 4). Thus hypoxia caused a rapid (0.5 h) but transient increase in endothelial cell stiffness.

Cell stiffening response to hypoxia is independent of p38 activation. We next focused on the mechanisms by which the rapid stiffening response develops and, in particular, on the biochemical signaling during the early exposure to hypoxia (0.5 h). Because cell stiffness varied from cell to cell, well to well, and day to day, we included appropriate controls for each experimental protocol (Figs. 5 and 6). We first evaluated the role of p38 and its downstream signals on hypoxia-induced increase in cell stiffness. Under baseline conditions, RPFMEC treated with SB-203580 showed no appreciable changes in cell stiffness (1.10 \pm 0.06 Pa/nm) compared with untreated cells (1.10 \pm 0.05 Pa/nm). In response to hypoxia (0.5 h), both cells treated with or without SB-203580 exhibited increases in cell stiffness (Fig. 5A). Hypoxia-induced increase in cell stiffness was similar in these cells (2.08 \pm 0.10 Pa/nm for SB-203580-treated and 2.13 \pm 0.08 Pa/nm for untreated cells), indicating that inhibiting p38 did not block hypoxia-induced increase in cell stiffness. Consistent with these findings, overexpression of the HSP27-PM mutant in RPFMEC did not cause noticeable changes in cell stiffness compared with cells transfected with empty vectors (Fig. 5B). Therefore, whereas p38-mediated phosphorylation of HSP27 played an important role in F-actin polymerization (31) and bead tethering (Fig. 3), this particular signaling pathway did not contribute to the increase in cell stiffness in response to hypoxia.

Cell stiffening response to hypoxia is dependent on Rho kinase. Exposure of RPFMEC to hypoxia caused a time-dependent increase in the level of MLC phosphorylation. MLC phosphorylation increased quickly and appeared to be most intense after 0.5 h but decreased to basal levels by 4-h exposure to hypoxia (data not shown). MLC phosphorylation is thought
HYPOXIA ALTERS BIOMECHANICAL PROPERTIES OF THE ENDOTHELIAL CELL

C525

Fig. 3. The decrease in spontaneous bead motions in response to hypoxia is dependent on p38 and heat shock protein 27 (HSP27) phosphorylation. A: RPMEC were treated for 0.5 h with or without 3 μM SB-203580 or 3 μM Y-27632, and subsequently bead motions were measured both before and after 1-h exposure to hypoxia. Open bars represent bead MSD evaluated at 300 s for cells treated with or without SB-203580 or Y-27632. Closed bars represent MSD evaluated at 300 s for these cells after 1-h exposure to hypoxia (SB-203580 or Y-27632 was present in the medium throughout hypoxic exposure). *Significant difference from MSD evaluated 300 s before hypoxic exposure. B: RPMEC were stably transfected with a phospho-mimicking mutant HSP27-PM. In control experiments, cells were transfected with empty vectors only. Under baseline conditions, spontaneous bead motions were measured. MSD for the control experiments (●) and cells overexpressing HSP27-PM (○) are shown. The coefficient D*a and the exponent α of the bead motion were estimated from a least-squares fit of a power law relationship to the aggregated average of the MSD data vs. time. Inset: representative rhodamine-conjugated phalloidin staining of filamentous (F) actin. Data are means ± SE (n = 325–451 cells for A; n = 566–671 for B).

to be regulated by both MLCK and Rho-kinase (ROCK) (22, 24, 32, 42). To assess the role of these kinases, RPMEC were treated for 0.5 h with or without either 10 μM ML-7 (a MLCK inhibitor) or 10 μM Y-27632 (a ROCK inhibitor); these doses and durations are sufficient to inhibit MLC phosphorylation as reported by others (2, 6, 32, 47, 50, 52). Under baseline conditions, cells treated with ML-7 or Y-27632 showed marked decreases in cell stiffness compared with untreated cells (Fig. 6). However, in response to hypoxia, cells treated with ML-7 showed an increase in cell stiffness (2.35 ± 0.09 Pa/nm), which was not different from the control (2.53 ± 0.10 Pa/nm). By contrast, cells treated with Y-27632 exhibited a significant reduction in hypoxia-induced increase in cell stiffness (1.54 ± 0.08 Pa/nm). Taken together, these findings suggest that hypoxia-induced increase in cell stiffness is largely attributable to the activation of ROCK-associated but not MLCK-associated signaling pathways, leading to the activation of actomyosin motors.

Hypoxia causes increases in cell traction, tensile stress, and contractile moment. Using traction microscopy, we next quantified the level of tensile stress within individual RPMEC (prestress), their contractile moments (contractile “strength”), and their changes in response to hypoxia. Figure 7 shows phase-contrast images of RPMEC (normoxic and hypoxic) cultured on a flexible polyacrylamide gel and their respective traction fields computed from the corresponding bead displacement fields using constrained FTTC (see MATERIALS AND METHODS). The arrows in Fig. 7 show the relative magnitudes and directions of the tractions, and the colors show the absolute magnitude of the traction vector. The greatest traction, in general, occurred at the cell periphery and was directed centripetally; the root mean square traction averaged across the entire cell-projected area increased appreciably with hypoxic exposure. Accordingly, compared with cells exposed to normoxia (1.274 ± 0.204 Pa), cells exposed to hypoxia (0.5 h) developed significantly greater mean prestress levels (2.077 ± 0.234 Pa) (Fig. 8A). Nonetheless, to avoid any systematic errors associated with the estimation of the prestress, we separately computed net contractile moment (a scalar measure of the contraction intensity) directly from the traction field without invoking assumptions concerning the cell cross-sectional area (11, 59). Compared with cells exposed to normoxia (5.49 ± 0.99 pNm), cells exposed to hypoxia (10.20 ± 2.23 pNm) generated significantly higher net contractile moment (Fig. 8B). These findings provide, for the first time, direct mechan-
We have characterized changes in biophysical properties of rat pulmonary microvascular endothelial cells in response to hypoxia, as well as the underlying signaling pathways. Spontaneous nanoscale motions of microbeads anchored to the CSK of the endothelial cell were random, unbounded, and superdiffusive. In response to hypoxia, activation of p38 MAPK and the resulting phosphorylation of HSP27 appeared to mediate decreases in spontaneous bead motions; these functional changes corresponded closely in time to structural changes in the actin CSK that were reported previously (31). Moreover, hypoxic conditions caused transient increases in cell stiffness and traction forces by a mechanism that was dependent on the activation of Rho kinase leading to the activation of actomyosin motors.

Current thinking on endothelial cell barrier function holds that vascular permeability occurs mainly through paracellular pathways and is tightly regulated by a balance of physical forces (18, 23). On the one hand, myosin-based contractile forces are developed within the endothelial cell, and, on the other, those internal forces must be taken up at the cell boundary by adhesive tethering forces at cell-cell contacts and cellular-extracellular matrix (ECM) interactions (9, 14, 38, 43, 55, 62). In the endothelial cell, myosin exerts its mechanical effects by interacting with actin within an integrated scaffolding comprising scores of cytoskeletal and signaling molecules (18, 24, 25, 58). It is now known that this scaffolding is dynamic and in a continuous state of remodeling; the actin lattice, the myosin filament, and the focal adhesion complex are all considered to be evanescent structures that can be virtually demolished in some circumstances and then reconfigured and stabilized in others (4, 7). Accordingly, disruption of these molecular events may modulate the balance of physical forces that regulate endothelial cell barrier function and thereby contribute to the formation of intercellular gaps and altered endothelial cell permeability. We begin the discussion by addressing methodological issues and then elaborate on the findings of this report and their implications.

Spontaneous bead motions, CSK remodeling dynamics, and cellular-ECM tethering interactions. The anomalous bead motions that we observed are inconsistent with simple Brownian motion. We suggest instead that they track rearrangements of underlying structures to which they are firmly attached. This is in keeping with the reports of others, but with some notable differences. A microbead coated with fibronectin, when placed briefly on the surface of lamellipodia of migrating fibroblasts (Fig. 5). The increase in cell stiffness in response to hypoxia is independent of p38 and HSP27 phosphorylation. A: RPMEC were treated for 0.5 h with or without 3 μM SB-203580, and subsequently cell stiffness was measured both before and after 0.5-h exposure to hypoxia. Open bars represent baseline stiffness for cells treated with or without SB-203580. Closed bars represent cell stiffness for these cells after a 0.5-h exposure to hypoxia. (SB-203580 was present in the medium throughout the hypoxic exposure.) B: RPMEC were stably transfected with HSP27-PM. For control, endothelial cells were transfected with empty vectors only. Cell stiffness was measured both before (open bars) and after (closed bars) a 0.5-h exposure to hypoxia. ∗Significant difference from respective baseline cell stiffness. Data are means ± SE (n = 285–494 cells for A; n = 399–550 cells for B).

Fig. 6. Increase in cell stiffness in response to hypoxia is dependent on Rho-kinase. RPMEC were treated for 0.5 h with or without 10 μM myosin light-chain kinase (MLCK) inhibitor ML-7 or 10 μM Y-27632. Subsequently, cell stiffness was measured both before and after 0.5-h exposure to hypoxia. Open bars represent baseline stiffness for cells treated with or without ML-7 or Y-27632. Closed bars represent cell stiffness for these cells after 0.5-h exposure to hypoxia. ∗Significant difference from baseline cell stiffness (control), and #significant difference from the respective baseline cell stiffness (control, ML-7, or Y-27632) before hypoxic exposure. Data are means ± SE (n = 282–503 cells).
moves a large distance (>4 μm) and preferentially rearward toward the nucleus (13, 51). Such directed motions are thought to be driven by the rearward flow of cortical actin CSK to which the bead is attached (16, 17, 51). In our experiments with RGD-coated microbeads on the surface of the endothelial cell, we observed no directed motions. Unlike motions of beads on locomoting fibroblasts, spontaneous motions of beads on the RPMEC were random and were smaller by 100- to 1,000-fold; they were nanoscalar, spanned only a small fraction of the bead diameter, and were certainly much less than the lateral extent of the cell boundaries. A number of factors could account for these differences.

First, the RPMEC was not visibly motile; cells seemed to be firmly adherent to the substrate and displayed no characteristic polar (asymmetric) morphology that is commonly observed in locomoting cells (13, 51). Experiments conducted on micropatterned substrates on which the cell could adhere but not crawl (45) showed similar bead motions (data not shown). Second, whereas actin filaments in motile cells are mainly distributed as a network in the cellular cortex (17), actin filaments of RPMEC are bundled with other proteins into contractile stress fibers (18, 23, 31, 55). The strong attachment of the bead to these stress-bearing cytoskeletal structures by RGD-integrin binding is well established (3, 27, 59), and the development of focal adhesions and complex interconnections to the actin CSK are readily observed at the bead locations (20, 40, 58). Taken together, therefore, the differences in the nature of bead behavior on migrating fibroblasts vs. rather stationary RPMECs are likely attributable to differences in cell biophysics as well as in the mode of bead anchorage to underlying cytoskeletal structures.

Exposure of endothelial cells to hypoxia caused time-dependent changes in spontaneous bead motions, whereas exposure to normoxia caused no appreciable changes in bead motions for the indicated times (Fig. 2). Interestingly, changes in bead motions in response to hypoxia recapitulated remarkably in

---

**Fig. 7.** Exposure of endothelial cells to hypoxia causes increases in the traction fields. RPMEC were cultured on the polyacrylamide gel coated with collagen type I. A: cells were exposed for 0.5 h to normoxia. B: cells were exposed for 0.5 h to hypoxia. The traction field was computed from the displacement field using constrained Fourier transform traction cytometry (FTTC); the cell boundary is indicated by the white line. Colors show the magnitude of the tractions in Pa (see color scale). Arrows indicate the direction and relative magnitude of traction. Young’s modulus of the gel was 1,300 Pa. Insets: respective phase-contrast images of RPMEC. Scale bars, 50 μm.

**Fig. 8.** Exposure of endothelial cells to hypoxia causes increases in tensile stress and contractile moment. A: calculated mean prestress from the mean traction as described previously (11, 59). B: calculated net contractile moment directly from the mean traction. No assumptions are made regarding the shape of the cell. *Significant difference from the normoxic cells. Data are means ± SE (n = 11 cells for control; n = 9 cells for hypoxia).
time structural changes in the actin CSK that were reported previously by us (31). In that study, we observed the greatest increase in F-actin polymerization after a 1-h exposure to hypoxia, which corresponds closely to the greatest decrease in spontaneous bead motions (Fig. 2B). Conversely, spontaneous bead motions increase dramatically when the actin CSK is disrupted by cytochalasin D or latrunculin A (4). Consistent with these observations, cells treated with Rho-kinase inhibitor Y-27632 exhibited an increase in spontaneous bead motions that persisted even after a 1-h exposure to hypoxia (Fig. 3A): Rho-kinase inhibition has been reported to disrupt the actin CSK (1, 39). Taken together, these findings imply that the more polymerized network of F-actin, the more tethered are the beads to the cytoskeletal scaffolding and the less they move. We suggest that the decrease in spontaneous bead motions in response to hypoxia is probably due to stabilization of the actin CSK, perhaps reflecting an increase in the cellular-ECM tethering forces at or near focal adhesions.

In that connection, numerous investigators have inferred that the small heat shock protein HSP27 is likely to play an important role in stabilization of the actin CSK (26, 37), but these findings have been based exclusively on structural evidence. In rat pulmonary microvascular endothelial cells, we recently demonstrated that hypoxia reversibly triggers p38-mediated activation of MK2 that in turn results in HSP27 phosphorylation and F-actin formation (30, 31). Indeed, compared with cells stimulated with hypoxia, endothelial cells overexpressing constitutively active MK2 or HSP27-PM exhibit similar increases in F-actin formation (31). By contrast, cells overexpressing dominant-negative MK2 not only show reduced F-actin content but also exhibit no further increases in F-actin formation in response to hypoxia (31). Consistent with this structural evidence, the decrease in spontaneous bead motions in response to hypoxia (i.e., beads tethering to, and perhaps being incorporated into, the newly formed cytoskeletal scaffolding at the site of focal adhesions) was attributable largely to the activation of p38 MAPK, leading to the phosphorylation of HSP27 (Fig. 3). Under baseline conditions, cells treated with a p38 MAPK inhibitor SB-203580 exhibited the expected increase in spontaneous bead motions and, most important, displayed no decrease in bead motions in response to hypoxia (Fig. 3A). By contrast, cells overexpressing HSP27-PM demonstrated a significant decrease in bead motions compared with cells transfected with the empty vector alone (Fig. 3B). These findings provide direct functional evidence that HSP27 phosphorylation indeed stabilizes the actin CSK, thus confirming inferences of others that were based solely on structural findings (26, 31, 37).

At this time, however, we are not certain how these changes in spontaneous bead motions relate to cadherin-mediated cell-cell adhesions. We speculate that local remodeling and stabilization at sites of cellular-ECM adhesions may be accompanied by local destabilization at the cell periphery that tethers cell-cell adhesions (9, 14). In support of this idea, studies have shown that hypoxia causes disassembly of the peripheral actin bands delineating the margins of the cells and, at the same time, causes assembly of stress fibers within the cell body and subsequently the appearance of intercellular gaps (31, 46). These findings have a particular importance to the understanding of endothelial cell barrier function, because the adhesive tethering forces of cell-cell and cellular-ECM adhesions are intimately linked to the actin-based CSK (18, 23, 55), which defines not only endothelial cell morphology (58) but also its ability to generate contractile force (18, 25, 63).

Cell stiffness, tensile stress, and the brisk biomechanical response of the endothelial cell. Direct quantitative indices of the physical forces within the endothelial cell have been difficult to measure (10, 25, 63). As a result, studies have often relied on the structural changes in the F-actin polymerization and stress fiber formation as the surrogates for changes in the contractile behavior. In the airway smooth muscle, we have demonstrated that agonist-evoked cell stiffening is attributable mostly to myosin activation, whereas polymerization of the actin lattice is necessary but not sufficient to account for the observed increase in cell stiffness (3). Similarly, in the RMEC, the time course of hypoxia-induced changes in cell stiffness (Fig. 4), as well as tensile stresses developed within the CSK of individual endothelial cells (Fig. 8), did not correspond to the temporal dynamics of spontaneous bead motions (Fig. 2) or to the reported time course of structural changes in the actin CSK (31). Indeed, both cell stiffness and tensile stress quickly increased after a 0.5-h exposure to hypoxia, at which time we found no qualitative changes in spontaneous bead motions or quantitative changes in F-actin content (31). More interestingly, the increase in cell stiffness in response to hypoxia was not blocked by SB-203580 or observed in cells overexpressing HSP27-PM (Fig. 5). These findings provide, for the first time, direct, quantitative, mechanical evidence demonstrating the contractile status, the amount of physical force within the endothelial cell, and their changes in response to hypoxia. Moreover, they draw a consistent picture in which hypoxia-activated biochemical events leading to F-actin polymerization (i.e., p38-mediated HSP27 phosphorylation) are not sufficient to account for the increase in the contractile state of RMEC.

Regarding the regulation of contractile force in the endothelium, phosphorylation of the 20-kDa MLC plays an important role (22, 28). When phosphorylated by Ca\(^{2+}\)/calmodulin-dependent MLCK (52), MLCK activates actomyosin motor activity and tension development within the endothelial cell (25, 34, 56). We have observed that hypoxia increases phosphorylation of MLC in pulmonary endothelial cells (data not shown), an effect that recently has been described in pulmonary arterial smooth muscle cells (60, 61). Indeed, several models of agonist-induced barrier dysfunction, such as the effects of thrombin or histamine, have implicated MLC phosphorylation for generation of contractile force and endothelial cell retraction (10, 25, 52, 56, 63). In human umbilical vein endothelial cells, Sheldon et al. (52) demonstrated that an inhibition of MLCK with ML-9 not only reduced basal MLC phosphorylation but also prevented histamine-induced increase in MLC phosphorylation and subsequently endothelial cell retraction. Consistent with their findings, we found that ML-7 decreased baseline stiffness of RMEC; ML-7 is a derivative of ML-9 that is thought to be an equally selective and potent inhibitor of MLCK (6, 50). Nevertheless, treatments of cells with ML-7 were not sufficient to prevent the increase in cell stiffness in response to hypoxia (Fig. 6).

More recently, studies have emphasized the increasing importance of Rho/Rho-kinase signaling pathways for the regulation of endothelial cell barrier function (12, 24). In particular, activation of Rho and its downstream effector ROCK have
been shown to phosphorylate and inhibit MLC phosphatase, which subsequently leads to increases in MLC phosphorylation, actomyosin interactions, focal adhesion, and stress fiber formation (2, 32, 47, 57). In this connection, Katoh et al. (29) reported two different classes of stress fibers in cultured FS-133 cells, which are differently regulated by MLCK and ROCK. Whereas peripheral stress fibers are dependent on the activity of MLCK, central stress fibers are dependent on the activity of ROCK (29). In contrast to the effects of ML-7, we found that cells treated with a ROCK inhibitor Y-27632 exhibited not only a decrease in baseline cell stiffness but also a marked reduction in hypoxia-induced increase in cell stiffness. These findings are in agreement with the notion that MLC phosphorylation may be differentially regulated by hypoxia (29, 42, 57, 60, 61) and also extend that idea by providing direct mechanical evidence. Currently, we are in the midst of localizing the temporospatial evolution of myosin phosphorylation, physical forces exerted by the endothelial cell, and their contributions to endothelial cell permeability in response to hypoxia. Taken together, our findings demonstrate that the major part of hypoxia-induced contractile response seems to be attributable largely to actomyosin motor activation through the Rho/Rho-kinase signaling pathway, whereas hypoxia-induced stabilization of the actin CSK at or near focal adhesions requires p38-mediated HSP27 phosphorylation.

Regulation of pulmonary vascular permeability is a complex process involving several mechanisms. The current model of endothelial cell barrier function holds that vascular permeability is tightly regulated through a balance between contractile forces within the endothelial cell and adhesive forces that connect endothelial cells to each other or to the ECM. Using a variety of novel tools in combination with various pharmacological and genetic manipulations, we have shown that hypoxia differentially regulates early contractile events within the endothelial cell that are followed in time by local changes in cellular-ECM adhesive interactions. The appreciable increase in cell stiffness and tensile stress in response to hypoxia were largely attributable to Rho-mediated activation of actomyosin motors. Spontaneous nanoscale motions of microbeads anchored to the CSK were superdiffusive, and activation of p38 leading to the phosphorylation of HSP27 was found to tether bead motions, thereby providing direct functional evidence that activation of HSP27 indeed stabilizes the actin CSK at or near focal adhesions. Taken together, these findings provide direct mechanical evidence that hypoxia alters a balance of physical forces that regulate endothelial cell barrier function.

REFERENCES


GRANTS

This study was supported by National Heart, Lung, and Blood Institute Grants HL-59682, HL-33009, HL/A1-65960, and HL-79320; a Tufts-New England Medical Center Research Grant; and an American Lung Association Grant.
C530

HYPOXIA ALTERS BIOMECHANICAL PROPERTIES OF THE ENDOTHELIAL CELL


