Regulation of vimentin intermediate filaments in endothelial cells by hypoxia

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Liu T, Guevara OE, Warburton RR, Hill NS, Gaestel M, Kayyali US. Regulation of vimentin intermediate filaments in endothelial cells by hypoxia. Am J Physiol Cell Physiol 299: C363–C373, 2010. First published April 28, 2010; doi:10.1152/ajpcell.00057.2010.—Hypoxia triggers responses in endothelial cells that play roles in many conditions including high-altitude pulmonary edema and tumor angiogenesis. Signaling pathways activated by hypoxia modify cytoskeletal and contractile proteins and alter the biomechanical properties of endothelial cells. Intermediate filaments are major components of the cytoskeleton whose contribution to endothelial physiology is not well understood. We have previously shown that hypoxia-activated signaling in endothelial cells alters their contractility and adhesiveness. We have also linked p38-MAP kinase signaling pathway leading to HSP27 phosphorylation and increased actin stress fiber formation to endothelial barrier augmentation. We now show that vimentin, a major intermediate filament protein in endothelial cells, is regulated by hypoxia. Our results indicate that exposure of endothelial cells to hypoxia causes vimentin filament networks to initially redistribute perinuclearly. However, by 1 hour hypoxia these networks reform and appear more continuous across cells than under normoxia. Hypoxia also causes transient changes in vimentin phosphorylation, and activation of PAK1, a kinase that regulates vimentin filament assembly. In addition, exposure to 1 hour hypoxia increases the ratio of insoluble/soluble vimentin. Overexpression of phosphomimicking mutant HSP27 (pm-HSP27) causes changes in vimentin distribution that are similar to those observed in hypoxic cells. Knocking-down HSP27 destroys the vimentin filamentous network, and disrupting vimentin filaments with acrylamide increases endothelial permeability. Both hypoxia- and pmHSP27 overexpression-induced changes are reversed by inhibition of phosphatase activity. In conclusion hypoxia causes redistribution of vimentin to a more insoluble and extensive filamentous network that could play a role in endothelial barrier stabilization. Vimentin redistribution appears to be mediated through altering the phosphorylation of the protein and its interaction with HSP27.

permeability; edema; pulmonary; vascular; cytoskeleton

Hypoxia has been shown to alter the structure and function of endothelial cells in a manner that contributes to a variety of diseases. Signaling pathways that are activated by hypoxia such as p38 mitogen-activate protein (MAP) kinase and Rho kinase pathways alter the actin cytoskeleton and contractile proteins leading to changes in biomechanical forces in these cells (3). These forces likely play an important role in endothelial cell migration and interaction with leukocytes as well as in regulation of endothelial barrier permeability which is altered in hypoxia (10, 14, 19). Intermediate filaments constitute one of the three major components of the cytoskeleton which also include actin microfilaments and tubulin microtubules.

These structures act in concert to control cell morphology and biomechanics. The role of intermediate filaments in regulation of endothelial function has not been sufficiently studied.

We have focused on the regulation of vimentin filaments in rat pulmonary microvascular endothelial cells (RPMEC) exposed to hypoxia. Vimentin and keratin are the major intermediate filament proteins in endothelial cells. Endothelial barrier-disrupting agents such as histamine induce vimentin phosphorylation in HUVEC, and cause its dissociation from adhesive complexes (18). Vimentin has also been shown to regulate focal adhesions in a bone marrow endothelial cell line (23). Moreover, vimentin-null HUVEC and lymphocytes exhibit abnormal homing of leukocytes and vimentin absence compromises the permeability barrier (13). Hypoxia has also been shown to upregulate vimentin in brain capillary endothelial cells (6). In RPMEC exposed to hypoxia we have previously shown that hypoxia causes an initial increase in cell contractility by 30 minutes, which is followed by increased cell adhesiveness by 1 h (3). The latter appears to be mediated by p38 signaling pathway leading to the phosphorylation of the small heat shock protein HSP27 by MK2 (3). Overexpressing phosphomimicking-HSP27 (pmHSP27) in RPMEC, increases actin stress fiber formation and, adhesive forces similar to 1 h hypoxia (3, 8). pmHSP27 overexpression also appears to augment the endothelial barrier by increasing actin stress fiber formation, rather than weaken it (10). In addition to its well-characterized role in regulation of the actin cytoskeleton, HSP27 is believed to stabilize the intermediate filament network and prevent gelling and aggregation (16). Mutations in HSP27 have been linked to peripheral neuropathies, which are characterized by disruption of intermediate filaments (1). Since intermediate filaments play an important role in many cell functions affecting their biomechanical properties, motility and adhesive structure, we have investigated the regulation of vimentin intermediate filaments in hypoxic endothelial cells.

In this report we show that hypoxia causes changes in the localization of the vimentin intermediate filament network that are accompanied by changes in vimentin phosphorylation and distribution between the soluble and insoluble pools. The mechanism of this response to hypoxia in relation to PAK1 and HSP27 is further investigated. Our findings indicate that intermediate filaments respond to hypoxia through redistribution of vimentin into stable structures that appear continuous across cells, and hence might contribute to previously described endothelial adhesiveness and barrier augmentation. Elucidating the role and mechanism of vimentin redistribution in the endothelial response to hypoxia might contribute to developing treatments for conditions such as endothelial barrier disruption in pulmonary edema.
**EXPERIMENTAL PROCEDURES**

**Materials and reagents.** Media and supplements (RPMI), fetal bovine serum (FBS), penicillin G potassium, streptomycin, fungizone, and glutamine were purchased from Invitrogen (Carlsbad, CA). PAK18 and Okadaic acid were purchased from Calbiochem (Gibbstown, NJ). Rhodamine phalloidin was obtained from Molecular Probes (Eugene, OR).

**Cell culture.** Rat pulmonary microvascular endothelial cells (RPMEC) were cultured in RPMI containing 10% FBS, penicillin, streptomycin, fungizone, and glutamine were purchased from Invitrogen (Carlsbad, CA). Probes (Eugene, OR). Town, NJ). Rhodamine phalloidin was obtained from Molecular

**Stable transfectants were made as we described earlier (8). For hypoxic exposure, cells were placed in humidified airtight incubation chambers (Billups-Rothenberg, Del Mar, CA) and gassed with 3% O2, 5% CO2, balanced N2. The hypoxic chambers were kept at 37 °C in humidified air containing 5% CO2.

**SDS-PAGE and immunoblotting.** Cells were lysed in RIPA or Triton buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, 5 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 1% Triton X-100, 50 mM NaF plus 1% Calbiochem Protease Inhibitor Cocktail) and lysates were assayed for protein using the Bradford protein assay and then diluted with 2x Laemmli loading buffer for SDS-PAGE. Stable transfectants were made as we described earlier (8). For hypoxic exposure, cells were placed in humidified airtight incubation chambers (Billups-Rothenberg, Del Mar, CA) and gassed with 3% O2, 5% CO2, balanced N2. The hypoxic chambers were kept at 37 °C in a tissue culture incubator. For normoxic exposure, cells were maintained at 37 °C in humidified air containing 5% CO2.

**Immunofluorescence microscopy.** After treatment, endothelial cells grown on collagen-coated coverslips were rinsed with PBS, fixed in 4% formaldehyde for 10 min, and permeabilized twice with 0.4% Triton X-100 for 5 min. After being washed twice with PBS, cells were incubated with 5 U/ml of Rhodamine-phalloidin (Molecular Probes, Eugene, OR) for 20 min. Following 3 washes with PBS, coverslips were incubated with 15 μg/ml goat serum in 1% BSA-PBS solution. Then coverslips were incubated with anti-Vimentin antibody (1:50) in 1 % BSA-PBS solution for overnight at 4°C. Next day coverslips were incubated with Alexa 488-conjugated anti-rabbit secondary antibody for 1 hr at room temperature. After being washed twice with PBS, coverslips were mounted on slides with Citifluor mounting medium (TED PELLA, Redding, CA) and analyzed with Zeiss Fluorescence Microscope and imaging system at 400× magnification.

**RESULTS**

**Hypoxia alters vimentin intermediate filament distribution.** Vimentin is an important component of the endothelial intermediate filament network, which has been implicated in the effects of permeability-inducing agents such as histamine (12). To study what role vimentin might play in the regulation of endothelial physiology in hypoxia we began by characterizing its distribution in hypoxic endothelial cells. RPMEC were grown on coverslips, and then exposed to hypoxia (3% oxygen) for 30 min, and 1 h. Next the coverslips were fixed and stained with an antivimentin antibody. The coverslips were next incubated with Alexa 488-conjugated secondary antibody to visualize vimentin. At normoxic conditions vimentin labeling revealed a filamentous network in the cytoplasm that was close to the plasma membrane but rarely extended all the way to that membrane (Figure 1). As shown in Figure 1, hypoxia appeared to cause the vimentin filament staining to switch from a spread filamentous network appearance to a mostly perinuclear localization by 30 min. After 1 hr of exposure to hypoxia the vimentin intermediate filament network appeared to reform with strengthening of filamentous structures that extended to the cell membrane and appeared to be continuous across the cells. The observed perinuclear vimentin redistribution coincides with increased endothelial cell contractility at 30 min, while the formation of the continuous filament network coincides with increased adhesiveness at 1 hr of exposure to hypoxic conditions. We noticed that this change in vimentin distribution coincides with increased endothelial cell contractility at 30 min, while the formation of the continuous filament network coincides with increased adhesiveness at 1 hr of exposure to hypoxic conditions.
hypoxia that we previously described (3). Thus redistribution of vimentin in response to hypoxia is consistent with a role in or a response to different forces in hypoxic endothelial cells, such that collapse of vimentin around the nucleus should allow endothelial cells to contract more easily. On the other hand formation of networks that appear to be connected across cells might contribute to strengthening adhesive forces.

Hypoxia causes a transient change in the phosphorylation of vimentin. Redistribution of vimentin filaments has been proposed to be triggered by changes in the phosphorylation of vimentin. Permeability inducing agents such as histamine induce vimentin phosphorylation, and cause its dissociation from adhesive complexes (18). To test whether the changes in vimentin distribution we observed were related to its phosphorylation we probed cell lysates from hypoxic RPMEC exposed to different periods of hypoxia for vimentin and phosphoryvimentin. Using an antibody against phosphorylated ser-56, we showed that hypoxia triggers a change in the phosphorylation state of vimentin. As shown in Figure 2A hypoxia caused the level of phospho-vimentin to decrease at 15–60 minutes and then return to baseline afterwards. As phosphorylation of vimentin at ser-56 is believed to regulate filament assembly and rearrangement we believe the transient changes in vimentin phosphorylation to reflect hypoxia-induced intermediate filament rearrangement we observed. However, based on published reports vimentin phosphorylation would have been expected to increase at 30 minutes of hypoxia when the cells are contracting and filamentous network appeared to collapse around the nucleus (9). Furthermore, vimentin phosphorylation would have been expected to decrease further at 60 minutes when extended filamentous structures associated with adhesive structures are observed and increased adhesiveness is observed. Nevertheless our results were drawn from cell lysates which include only detergent-soluble vimentin. This pool might not reflect the initial increased phosphorylation at 30 minutes that is expected to disassemble the filaments because the latter structures might not be part of the soluble cell lysates (see Figure 3 below).

Since PAK 1 has been proposed to regulate vimentin filament formation through phosphorylation of ser-56 (9, 21) and since other groups implicated it in mediating endothelial responses to hypoxia (24), we tested if exposure to hypoxia alters its activation. We probed cell lysates with antibodies against phosho-thr-423-PAK1 (active) and total PAK1. As shown in Figure 2, B and C, hypoxia induced a decrease in PAK1 activation that was most significant at 60 minutes, and which returned to normal levels by 120–240 minutes. Thus PAK1 activity was lowest at a time point in hypoxia when vimentin filament structures reformed extensively (Figure 1). These data suggest that changes in PAK1 activity might play a role in the observed changes in vimentin filament structure in response to hypoxia.

Hypoxia increases the ratio of insoluble/soluble vimentin. We previously demonstrated that 1 hr of hypoxia increased actin stress fiber formation (8) and adhesiveness (3) which we correlated with augmentation of endothelial permeability barrier (10). In this report we also showed that 1 hr hypoxia increases vimentin filamentous networks (Figure 1). We next tested if 1 hr hypoxia also biochemically alters the distribution of vimentin between detergent-soluble and insoluble pools. RPMEC left in normoxia or exposed to hypoxia (1hr) were lysed in Triton X-100-containing buffer. The soluble fraction and the pellet which was solubilized in loading buffer were then analyzed by SDS-PAGE and immunoblotting with antibimentin antibodies. As shown in Figure 3, 1 h hypoxia significantly increases the percentage of vimentin in the insoluble pool. These data are consistent with the formation of stable vimentin filament networks in endothelial cells after 1 h of hypoxia which might play a role in strengthening adhesion and augmenting the permeability barrier.

pmHSP27 overexpression causes changes in vimentin that are similar to those observed in hypoxic cells. Since our previous results have demonstrated a role for HSP27 phosphorylation in hypoxia mediated responses in RPMEC including increased actin stress fiber formation and increased adhesiveness, we tested whether overexpression of phosphomimicking mutant (pmHSP27) also affects intermediate vimentin filament distribution. As shown in Figure 4, compared to wild type RPMEC in which vimentin forms a filamentous network that rarely extends all the way to the cell membrane or beyond it, pmHSP27-overexpressing cells exhibited a filamentous vimentin network that appears to extend throughout the cytoplasm to the cell membrane and even appeared continuous with networks in other cells. These structures are similar to those observed in response to 1 hr hypoxia (Figure 1) but appear to be more prominent in pmHSP27-overexpressing cells.

Fig. 1. Hypoxia causes redistribution of vimentin filaments in pulmonary endothelial cells. RPMEC were grown on collagen-coated coverslips for 24 hr prior to hypoxia treatment (3% Oxygen). Exposure to 30 min hypoxia caused vimentin labeling to be more prominent in perinuclear region. Exposure to 1 hr hypoxia caused more prominent labeling on structures that appeared continuous across cells. Vimentin filaments were visualized through immunofluorescence with anti-vimentin antibody as described in “Experimental Procedures”. The pictures shown are representative of three different experiments.
Next, we tested if overexpressing pmHSP27 affects vimentin phosphorylation. As shown in Figure 5A, no phospho-vimentin was detected in pmHSP27-overexpressing cell lysates. Compared to wild type cells (Figure 2), hypoxia (15–240 min) did not affect the phosphorylation of soluble vimentin which remained undetectable. Since phosphorylation of vimentin is an indicator for vimentin filament disassembly, these data are consistent with increased filament stability and the extensive network of vimentin filaments observed in pmHSP27-overexpressing cells. Examination of the level of vimentin (total) in cell lysates also showed a significant drop at 60 and 120 minutes. These results suggest that pmHSP27 overexpression increases the ratio of insoluble/soluble vimentin. These findings are consistent with the reduced phosphorylation of vimentin observed in Figure 5A. Exposure of pmHSP27-overexpressing RPMEC to 1 hr hypoxia in some experiments increased the ratio of insoluble/soluble vimentin, which is consistent with the reduction of total vimentin by 1 hour hypoxia described in Figure 5A. However, this increase was not consistently observed suggesting that there might be an upper limit on the ratio of insoluble/soluble vimentin. These findings suggest that HSP27 phosphorylation might mediate some of the effects of vimentin redistribution observed in hypoxic endothelial cells.

Since we observed that activation of PAK1 is altered by hypoxia in wild-type cells, we first tested if pmHSP27 overexpression reduces PAK1 activation. As shown in Figure 5, D and E, pmHSP27-overexpressing cells did not express less phospho-PAK1 than wild type cells. Indeed, phospho-PAK1 was more easily detected in pmHSP27 overexpressing cells than wild type cells. Yet, the level of phospho-PAK1 decreased in hypoxic pmHSP27-overexpressing cells (Figure 5, D and E). These findings might explain the additional reduction in soluble vimentin in hypoxic pmHSP27 cells (Figure 5A).
PAK1, PAK18 (10 μM) inhibition is expected to increase vimentin filament stability through inhibiting its phosphorylation. To examine its role in RPMEC we incubated cells with a peptide inhibitor of PAK1, PAK18 (10 μM for 1 h), and then exposed to 1 hr hypoxia. As shown in Figure 6, inhibition of PAK1 increased the ratio of insoluble/soluble significantly in wild type RPMEC. The effect of inhibition of PAK1 was greater than the effect of hypoxia on wild-type RPMEC but not greater than the effect of pmHSP27 overexpression on insoluble/soluble vimentin ratio. Furthermore, inhibiting PAK1 did not increase the insoluble/soluble vimentin ratio in pmHSP27-overexpressing cells (Figure 6). These data are consistent with the possibility we mentioned above of there being an upper limit on the ratio of insoluble/soluble vimentin we can assess.

**Hypoxia or pmHSP27 overexpression increase the interaction of HSP27 with vimentin.** Since both wild-type and pmHSP27-overexpressing cells exhibited similar responses in terms of PAK1 deactivation, we examined other mechanisms by which pmHSP27 overexpression can alter vimentin assembly and distribution. First, we examined if direct interaction between vimentin and HSP27 plays a role. HSP27 has been reported to stabilize the intermediate filament network (16) and mutations in that protein have been linked to familial peripheral neuropathies, which are characterized by disruption of the intermediate filament network (1). Hence, we tested whether interaction of HSP27 and vimentin is altered in 1 hr hypoxia or pmHSP27 overexpressing cells by co-immunoprecipitation. As shown in Figure 7A, when HSP27 was immunoprecipitated with an anti-total HSP27 antibody vimentin co-immunoprecipitated with HSP27. The amount of vimentin co-immunoprecipitating with HSP27 appeared less in hypoxic or pmHSP27 overexpressing cells than normoxic wild type cells. However, as shown in Figure 7B, these cells contain less vimentin in the whole lysates before immunoprecipitation. Indeed, when the amount of co-immunoprecipitating vimentin was normalized to the amount of vimentin in the lysates it became apparent that relatively more vimentin bound to HSP27 in hypoxic or pmHSP27 overexpressing cells than wild-type cells (Figure 7C). The combination of pmHSP27 overexpression and hypoxia was synergistic. Thus 1 h hypoxia or phosphorylation of HSP27 appear to increase the interaction of vimentin with HSP27, suggesting that phosphorylated HSP27 could mediate the stabilizing effect of 1 hr hypoxia on the vimentin filament structure.

**Knocking-down HSP27 inhibits vimentin polymerization.** Since overexpressing pmHSP27 increases the interaction of HSP27 and vimentin and appears to stabilize vimentin intermediate filaments, we knocked-down pmHSP27 with siRNA to further test the relationship of pmHSP27 overexpression to vimentin distribution. We have used an siRNA that we have recently demonstrated to be effective in knocking down exogenous pmHSP27 (10). After siRNA transfection, immunofluorescence staining for vimentin was performed. As shown in Figure 8, knocking-down HSP27 significantly inhibited vimentin filamentous network formation and caused the labeling to become predominantly perinuclear rather than extending to neighboring cells (Figure 8). These data support the idea that overexpressing pmHSP27 plays a role in promoting vimentin polymerization.

**Inhibition of vimentin polymerization increases endothelial permeability.** We have recently shown that pmHSP27 overexpression augments the endothelial permeability barrier partly through altering the actin cytoskeleton (10). To test if vimentin intermediate filaments play a role in that effect on permeability we disrupted intermediate filament formation using the intermediate filament disrupter acrylamide at a level that we determined not to be toxic (3 mM). We treated RPMEC monolayer with acrylamide for 1 hr, then we assayed permeability using fluorescent-dextran flux assay. The results shown in Figure 9 demonstrate that acrylamide pretreatment increased the permeability compared to control in both normoxic and hypoxic wild-type, as well as pmHSP27-overexpressing RPMEC where the effect was more drastic (Figure 9). These data suggest that vimentin polymerization plays critical role in enhancing endothelial barrier in pmHSP27-overexpressing RPMEC.

**Inhibition of phosphatase activity reverses vimentin filament formation in response to hypoxia or pmHSP27 overexpression.** Another mechanism by which vimentin filaments can be stabilized is through increased dephosphorylation of vimentin. Protein phosphatase 1 (PP1) has been implicated in regulation of vimentin filament formation through dephosphorylating the protein (7). Our previous studies demonstrated that cells that overexpress pmHSP27 exhibit decreased MLC-2 phosphorylation probably through increased activation of MLC phosphatase (10). We based that conclusion on the observation that pmHSP27 overexpression blocked hypoxia- or TGF-β-induced phosphorylation of MYPT1, the regulatory subunit of myosin phosphatase, since phosphorylated MYPT1 inhibits the activity of myosin phosphatase. Since myosin phosphatase is a PP1
type phosphatase, and since other reports suggested that HSP27 interacts with MYPT1 (15), HSP27 might regulate intermediate filament assembly by altering its phosphorylation by phosphatase 1. At this point we do not know the identity of the regulatory subunit that would target PP1 to vimentin. Nevertheless, to assess the potential involvement of phosphatase activity in vimentin redistribution we tested whether we can reverse the stabilization of vimentin in hypoxic or pmHSP27 overexpressing cells by treating the cells with okadaic acid at a concentration that inhibits phosphatase 1 (1 μM). As shown in Figure 10, okadaic acid decreased the ratio of insoluble to soluble vimentin. Okadaic acid reversed both the effects of hypoxia and overexpression of pmHSP27. Since the phosphomimicking activity of mutated pmHSP27 is not affected by okadaic acid, the data suggest that phosphorylation of vimentin or some other protein but not HSP27 is the target of phosphatase inhibition. While okadaic acid treatment increased vimentin phosphorylation at S56 in both wild type and pmHSP27-overexpressing RPMEC, the levels did not become comparable, suggesting that other phosphorylation sites in vimentin might also be important for vimentin interaction with HSP27 and/or filament formation. These data are consistent with a role for phosphatase activation in stabilization of hypoxic and/or pmHSP27 overexpressing RPMEC.

**DISCUSSION**

In this manuscript we have shown that hypoxia exerts a clear effect on the vimentin intermediate filament cytoskeleton and suggested mechanisms by which it can elicit vimentin redistribution. Hypoxia is a physiological stimulus that occurs in tissues in different conditions including ischemia, anemia, tumor growth, as well as in high altitude and lung disease. In addition to altering systemic responses in the organism, hypoxia exerts direct effects on individual cells. We have focused on the acute effects of hypoxia on the structure and function of endothelial cells. After previously demonstrating how hypoxia-activated signaling alters contractile and adhesive forces in parallel with redistribution of the actin cytoskeleton (2, 8, 10), we now provide data on its effects on the intermediate filament component of the endothelial cytoskeleton.

Reduced oxygen levels are associated with lung diseases such as high altitude pulmonary edema, acute respiratory distress syndrome, pulmonary hypertension, and ischemia-reperfusion injury. The vascular endothelium is one target of hypoxia that contributes significantly to many conditions such as lung injury since the lung is a heavily vascularized organ. Hypoxia has also been reported to alter the thrombogenic properties of endothelial cells, as well as, endothelial cell growth, homeostasis, and the ability to modulate blood vessel tone (for review, see Ref. 20). Several studies have reported increased permeability in the lung or in cultured endothelial monolayers in response to hypoxia (3, 10, 15, 16, 41, 43, 54, 63–66, 70, 80, 91). Despite the many studies linking hypoxia to permeability, the mechanism through which hypoxia exerts its effects is not well understood. Since the responses to hypoxia can be part of a physiological or pathological process, understanding the signaling pathways leading to specific responses is important for possible therapeutic intervention in diseases associated with hypoxia. The complexity of the response of endothelial cells to hypoxia is illustrated by our...
finding that hypoxia elicits an increase in contractile forces mediated by Rho kinase and myosin light chain phosphorylation, followed by an increase in adhesive forces mediated by HSP27 phosphorylation and actin stress fiber formation (3). We have also linked HSP27 phosphorylation, actin stress fiber formation, and increased adhesive forces to endothelial barrier protection rather than disruption (10). Since the biomechanical properties of cells are strongly influenced by the structure of intermediate filaments we were interested in testing if and how hypoxia affects endothelial intermediate filament structures.

Intermediate filaments, which constitute one of the three major cytoskeleton structures along with microfilaments and microtubules, have been implicated in endothelial permeability regulation, yet their role remains poorly understood (for review see Ref. 11). The major intermediate filament proteins in endothelial cells are vimentin and keratin. Our results demonstrate that in confluent RPMEC vimentin filaments appear as an elaborate network that forms close to but only rarely extends all the way to the cell membrane (Figure 1). In response to hypoxia, vimentin filaments initially appear to collapse around the nucleus (30 min), and then reform a network that appears to be continuous across cells (1 h). The perinuclear localization of vimentin coincides with the increased contractility at 30 min of hypoxia we described earlier (3). On the other hand the formation of connected vimentin filament network coincides with the increased endothelial adhesiveness we described at 1 h of hypoxia. We postulate that the redistribution of vimentin to perinuclear regions allows endothelial cells to contract while formation of extensive filament network contributes to adhesive forces we described earlier. Other researchers reported that permeability-inducing agents such as histamine induce vimentin phosphorylation in HUVEC, and cause its dissociation from adhesive complexes containing VE-cadherin and catenins (18). Moreover, vimentin has been shown to regulate focal adhesions in a bone marrow endothelial cell line where it localized with integrin upon exposure of these to shear stress, and vimentin siRNA reduced focal adhesion and cell size (23). Recent studies using vimentin-null HUVEC and lymphocytes...
indicate that vimentin is important for homing of leukocytes and that its absence compromises the permeability barrier (13). These studies suggest that the lack of vimentin or disruption of its filaments by phosphorylation appear to compromise endothelial adhesion and barrier function, which is consistent with our finding that in 1 hr hypoxia vimentin filament stability coincides with increased adhesiveness. The effect of 1 hr hypoxia on continuous vimentin filament network formation is mirrored by the biochemical experiments looking at vimentin distribution between soluble and insoluble pools (Figure 3). Hypoxia (1 hr) increased the ratio of insoluble/soluble vimentin reflecting more stable filament formation (Figure 3). Thus our results suggest that endothelial vimentin intermediate filaments respond dynamically to exposure to hypoxia.

Since overexpressing pmHSP27 produced changes in actin stress fibers and adhesive forces similar to those observed at 1 hr hypoxia, we tested if it exerts similar effects on vimentin distribution. Indeed pmHSP27 overexpression caused extensive filament networks to form that appeared to be continuous between cells (Figure 4). Consistent with that finding pmHSP27 overexpression significantly increased the ratio of insoluble/soluble vimentin (Figure 5B). These findings demonstrate that pmHSP27 overexpression produces changes in vimentin that mimic those observed in response to exposure to 1 hr hypoxia.

In considering the mechanism by which hypoxia causes the redistribution of vimentin in endothelial cells we focused on vimentin phosphorylation and interaction of vimentin with

Fig. 6. PAK inhibition causes an increase in the percentage of insoluble vimentin in both normoxic and hypoxic wild type RPMEC. (A) Wild type and pmHSP27-overexpressing RPMEC were exposed to 1 hr hypoxia in the absence or presence of the PAK1 inhibitor PAK18 (10 μM). Both insoluble and soluble fractions of cell lysates were analyzed by Western blotting. (B) Intensity of insoluble vimentin bands was normalized to that of the sum of both soluble and insoluble vimentin bands. Data are presented as means ± standard deviation (n = 3 samples). *Statistically significant difference from normoxic wild type control mean, # statistically significant difference from hypoxic wild type control mean. P <0.05 in ANOVA and Holm-Sidak post hoc analysis.

Fig. 7. Vimentin co-immunoprecipitates with HSP27 and the co-immunoprecipitation is increased by hypoxia and/or pmHSP27 overexpression. (A) After exposure to 1 hr hypoxia, cell lysates of both wild type and pmHSP27-overexpressing RPMEC were immunoprecipitated with anti-HSP27 antibody, and then the precipitates were immunoblotted with anti-vimentin antibody. (B) Whole cell lysates were analyzed with Western blotting at the same time. (C) Intensity of vimentin bands from immunoprecipitates was normalized to that from the whole cell lysates. *Statistically significant difference from wild type normoxic mean, # statistically significant difference from normoxic pmHSP27-RPMEC mean P <0.05 in ANOVA and Holm-Sidak post hoc analysis.
Vimentin phosphorylation at serine 56 has been associated with filament network disassembly and redistribution of vimentin from the insoluble to the soluble pool (9). As our results show hypoxia causes a transient change in vimentin phosphorylation (Figure 2B). Since hypoxia causes redistribution of vimentin to the perinuclear region by 30 minutes, and since Rho kinase (7) and MK2 (4) have been reported to phosphorylate vimentin, we would have expected vimentin phosphorylation to increase at 30 min of hypoxia. However our data show that this is not the case (Figure 2). It is possible that vimentin becomes transiently phosphorylated at 30 min hypoxia but the phosphorylation occurs on specific vimentin pools, e.g., insoluble filaments that we could not isolate. Alternatively, the initial vimentin perinuclear redistribution at 30 min hypoxia could represent a passive response to cell contraction since the vimentin filaments in normoxic wild type cells do not appear to extend to cell junctions and might not resist compression. Regardless of the role of phosphorylation in the 30 min hypoxia perinuclear redistribution, vimentin dephosphorylation appears to be important for stabilizing the filamentous network. For example overexpressing pmHSP27 completely abolishes vimentin phosphorylation (Figure 5B) and the levels of phospho-vimentin remain undetectable in soluble lysates even after exposure to different duration of hypoxia (Figure 5B). The lack of detectable phospho-vimentin is consistent with vimentin filaments being more stable in pmHSP27 overexpressing cells. The link between pmHSP27 and vimentin filament stabilization is further supported by reversing its effect by siRNA knock-down (Figure 8). In addition, disrupting vimentin intermediate filaments with acrylamide weakened the endothelial permeability barrier in pmHSP27-overexpressing RPMEC more than in wild-type RPMEC (Figure 9). This finding supports the idea of vimentin filament stabilization contributing to

Fig. 8. Transfection of HSP27-siRNA inhibits its polymerization of vimentin. pmHSP27-overexpressing RPMECs were grown on collagen-coated coverslips for 24 hr prior to transfection with HSP27 siRNA (3% Oxygen). Vimentin filaments were visualized through immunofluorescence with anti-vimentin antibody as described in “Experimental Procedures”.

Fig. 9. Acrylamide treatment increases permeability of RPMEC monolayer. Cells were grown on filter inserts and exposed to normoxia or hypoxia (3% O₂) with or without acrylamide. The transfer of Alexa Fluor-dextran (3 kDa) through the monolayer was measured over time, and the fold change in fluorescence after 32 minutes over corresponding control (no acrylamide) is shown. Data are presented as means ± standard deviation (n =3 samples). *Statistically significant difference from normoxic wild type buffer control mean, #statistically significant difference from hypoxic wild type buffer control mean, ^statistically significant difference from hypoxic pmHSP27-overexpressor buffer control mean. P <0.05 in ANOVA and Holm-Sidak post hoc analysis.

Fig. 10 Phosphatase inhibition attenuates the increase of ratio of insoluble vimentin induced by hypoxia or overexpression of pmHSP27. (A) Wild type and pmHSP27-overexpressing RPMEC were exposed to 1 hr hypoxia in the absence or presence of okadaic acid. Both insoluble and soluble fractions of cell lysates were analyzed by Western blotting. (B) Intensity of insoluble vimentin bands was normalized to that of the sum of both soluble and insoluble vimentin bands. Data are presented as means ± standard deviation (n =3 samples). *Statistically significant difference from normoxic wild type buffer control mean, #statistically significant difference from hypoxic wild type buffer control mean, ^statistically significant difference from hypoxic pmHSP27-overexpressor buffer control mean. P <0.05 in ANOVA and Holm-Sidak post hoc analysis.
the barrier augmenting effect observed in pmHSP27-overexpressing cells.

Since PAK1 has been described to play an important role in vimentin phosphorylation and control of vimentin filament dynamics, we also examined its activation in hypoxia. We observed that PAK1 activation is reduced at 1 hr hypoxia in both wild type and pmHSP27-overexpressing cells suggesting that it might play a role in the hypoxic response (Figure 2B and SD). Indeed inhibiting PAK1 increased the ratio of insoluble/soluble vimentin in wild-type but not pmHSP27-overexpressing RPMEC (Figure 6). Since the level of PAK1 or its activation were not reduced in normoxic pmHSP27 overexpressing cells, we postulated that HSP27 might be affecting vimentin through other mechanisms as well.

It has been previously reported that HSP27 stabilizes the intermediate filament network and prevents gelling and aggregation (16). The first mechanism we investigated is direct interaction between vimentin and HSP27. Both hypoxia and pmHSP27 overexpression appeared to increase the amount of vimentin coimmunoprecipitating with HSP27, and the combination of the two appeared synergistic (Figure 7). These findings are consistent with increased interaction between phosphoHSP27 and vimentin. Another mechanism we considered is the role of phosphatases in vimentin regulation in hypoxic or pmHSP27-overexpressing endothelial cells. Indeed inhibiting phosphatase activity by okadaic acid reversed the effect of both hypoxia and pmHSP27 overexpression on vimentin redistribution (Figure 10). Identifying the specific phosphatase involved is beyond the scope of this report. Vimentin can be dephosphorylated by PP1 type phosphatase whose specificity is generally determined by the specific regulatory subunit of the enzyme complex. While we cannot be certain about specific subunits at this point, it is possible that HSP27 might target that regulatory subunit to vimentin. For example, MYPT1 the regulatory subunit that targets PP1 to MLCK has been reported to interact with HSP27 (15). In addition, vimentin might itself bind the catalytic PP1 as in the case of the neurofilament-L intermediate filament protein (22).

Our findings suggest that vimentin filaments undergo a dynamic response to hypoxia that coincides with previously observed changes in biomechanical properties of hypoxic endothelial cells. The hypoxia-induced changes in vimentin are likely to be related to altered phosphorylation of vimentin and interaction with HSP27. Based on findings in this paper and others recently reported we postulate that HSP27 phosphorylation is important for strengthening the adhesive properties and barrier integrity of endothelial cells in hypoxia and other possible conditions partly through its effect on vimentin. Vimentin networks that appear to be continuous through cells, which are induced by 1 hr hypoxia and/or pmHSP27 overexpression likely represent an adaptive response that strengthens the endothelial tissue and protects it against injury. Hypoxia has been reported to upregulate vimentin in brain capillary endothelial cells (6). While we have not observed total vimentin upregulation by 1 hr hypoxia, pmHSP27 overexpression increased the level of total vimentin in endothelial cells (data not shown). It is worth pointing out that mutations in HSP27 have been linked to peripheral neuropathies, which are characterized by disruption of intermediate filaments (1). Whether mutations in HSP27 and similar proteins contribute to endothelial injury and vascular and lung disease remains to be seen.

Modulation of vimentin structure and expression through HSP27 might prove to be a new area worth exploring in developing therapeutics for acute lung injury and other diseases involving the endothelium.

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DISCLOSURES
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