Rac1 and RhoA as regulators of endothelial phenotype and barrier function in hypoxia-induced neonatal pulmonary hypertension

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Wojciak-Stothard, Beata, Lillian Yen Fen Tsang, Ewa Paleolog, Susan M. Hall, and Sheila G. Haworth. Rac1 and RhoA as regulators of endothelial phenotype and barrier function in hypoxia-induced neonatal pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 290: L1173–L1182, 2006. First published January 20, 2006; doi:10.1152/ajplung.00309.2005.—Hypoxia is a common cause of persistant pulmonary hypertension in the newborn (PPHN), a condition associated with endothelial dysfunction and abnormal pulmonary vascular remodeling. The GTPase RhoA has been implicated in the pathogenesis of PPHN, but its contribution to endothelial remodeling and function is not known. We studied pulmonary artery endothelial cells (PAECs) taken from piglets with chronic hypoxia-induced pulmonary hypertension and from healthy animals and analyzed the roles of Rho GTPases in the regulation of the endothelial phenotype and function under basal normoxic conditions, acute hypoxia, and reoxygenation. The activities of RhoA, Rac1, and Cdc42 were correlated with changes in the endothelial cytoskeleton, adherent junctions, permeability, ROS production, VEGF levels, and activities of transcription factors hypoxia-inducible factor (HIF)-1α and NF-κB. Adenoviral gene transfer was used to express dominant-negative GTPases, kinase-dead p21-activated kinase (PAK)-1, and constitutively activated Rac1 in cells. PAECs from pulmonary hypertensive piglets had a stable abnormal phenotype with a sustained reduction in Rac1 activity and an increase in RhoA activity, which correlated with an increase in actin stress fiber formation, increased permeability, and a decrease in VEGF and ROS production. Cells from pulmonary hypertensive animals were still able to respond to acute hypoxia. They also showed high activities of HIF-1α and NF-κB, likely to result from changes in the activities of Rho GTPases. Activation of Rac1 and its effector PAK-1 as well as inhibition of RhoA restored the abnormal phenotype and permeability of hypertensive PAECs to normal.

permeability

PERSISTENT PULMONARY HYPERTENSION (PH) of the newborn (PPHN) is a leading cause of morbidity and death in the neonatal period, and abnormalities in pulmonary endothelial remodeling and endothelial dysfunction are characteristic features of this condition (7). Chronic hypoxia is a relatively common cause of PPHN. In newborn piglets, chronic hypoxia induces vascular abnormalities similar to those seen in infants with this condition (2, 34). In normal piglets at birth, pulmonary artery endothelial cells (PAECs) are squat, have a narrow base on the subendothelium, a low surface-to-volume ratio, and many interdigitated cell-cell contacts with associated microfilaments (20). Immediately after birth, as the arteries dilate, the cells increase their surface area, spread rapidly, and lose junction-associated microfilaments (20). However, in animals exposed to chronic hypoxia, PAECs fail to remodel and retain their fetal phenotype (2). Lack of structural remodeling is accompanied by endothelial dysfunction characterized by an increased release of vasoconstrictor agonists and a decreased release of vasorelaxants (23). Increased endothelial permeability and the expression of inflammatory mediators have also been associated with PPHN (7). Abnormalities in vascular remodeling in PH occur in both conduit and resistance arteries, with both showing an increase in wall thickness and evidence of endothelial dysfunction, shown by a lack of endothelium-dependent relaxation (6, 21, 25, 34).

The mechanisms of development of these endothelial abnormalities are uncertain. Recent studies have suggested that Rho GTPases, key regulators of the actin cytoskeleton, may play a role in the pathogenesis of chronic hypoxia-induced PH (28). The Rho GTPases RhoA, Rac1, and Cdc42 regulate the endothelial phenotype and permeability and are activated by several vasoactive agents (37, 41). Rac1 and RhoA appear to have antagonistic effects on endothelial barrier function (41). Rac1 is required for the assembly and maturation of endothelial junctions and its activity increases during junction formation (16, 27), whereas RhoA destabilizes endothelial junctions by increasing isometric tension at the cell margins due to increased actomyosin contractility (37). Another Rho GTPase, Cdc42, might be important, as it was shown to play a role in the restoration of endothelial barrier function after exposure to thrombin (26). Our recent study (40) has shown that acute hypoxia inhibits Rac1 and activates RhoA in normal adult PAECs, a change that leads to the breakdown of endothelial barrier function and is reversed by reoxygenation. We (40) also found that changes in Rho GTPase activity depend on NADPH oxidase-mediated ROS production.

It is generally accepted that sustained hypoxia in vivo can cause or contribute to PPHN, and PH can persist when the child is no longer hypoxic. In the present study, we hypothesized that endothelial cells isolated from piglets made PH by exposure to chronic hypobaric hypoxia in vivo would 1) maintain a stable, abnormal structural phenotype in vitro; 2) show increased permeability; and 3) not have the ability to remodel in response to changes in oxygenation, as do normal endothelial cells (40). We further hypothesized that these changes would be associated with a stable imbalance in activities of Rho GTPases and changes in ROS production. VEGF production was also studied because it is a well-known regu-
lator of vascular permeability, Rho GTPases, and ROS production. Low levels of this growth factor in the lungs of experimental animals have been associated with the development of PH (13, 18). The oxygen-sensitive transcription factors NF-κB and hypoxia-inducible factor (HIF)-1α (19) regulate the transcription of several genes whose products may affect both the endothelial phenotype and Rho GTPases, and they have been implicated in the pathogenesis of PH (8, 11, 24, 31, 42). Their activities were also studied.

MATERIALS AND METHODS

PH was induced in Large White piglets by an exposure to chronic hypobaric hypoxia (50.8 kPa) for 11 days, from 3 to 14 days of age (21). Animals treated in this manner develop PH with right ventricular hypertrophy and pulmonary arterial medial hypertrophy (21). Animals were killed immediately after their removal from the hypobaric chamber. Findings were compared with those in normal animals aged 14 days. These piglets were kept in room air and killed at 14 days of age. All animals received humane care in compliance with British Home Office Regulations and with the Principles of Laboratory Animal Care formulated by the National Society of Medical Research and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1985).

Cell Culture, Adenoviral Infection, and Use of Inhibitors

Porcine PAECs were isolated by collagenase digestion from conduct pulmonary arteries of all piglets and cultured under normoxic conditions. PAECs for the study were harvested from three normal piglets and three PH piglets from the same litter in two separate exposures to chronic hypoxia (total of 6 animals/group). For all experiments, cells from passages 2 and 3 were used because they retained the typical endothelial cobblestone morphology, a feature that was progressively lost with higher passage numbers. Cells were grown to confluence in 6-cm petri dishes, Thermanox plastic coverslips, and 96-well tissue culture microwell plates (Nunc; Roskilde, Denmark) or on polyester Transwell-Clear filters (3 μm pore size, Costar, High Wycombe, UK) under normoxic conditions. Cells were then either left under normoxic conditions (20% O2-5% CO2-75% N2) or exposed to acute hypoxia (3% O2-5% CO2-92% N2) for 15 min to 4 h. After 1 h, some hypoxic cultures were returned to normoxic conditions for a 1-h period of reoxygenation. Adenoviral gene transfer was used to express green fluorescent protein (GFP); dominant negative RhoA, Rac1, and Cdc42 (N19RhoA, N17Rac1, and N17Cdc42); kinase-dead (K298A) p21-activated kinase (PAK)-1; and constitutively active Rac1 (V12Rac1) proteins in PAECs (40). The adenoviral vector containing kinase-dead PAK-1 was a kind gift of Prof. Fiemu Nwariaku (Department of Surgery, Southwestern Medical Center, Dallas, TX). Sphingosine 1-phosphate (S1-P) was added to some cultures at a concentration of 0.5 μM as in Ref. 15.

Transendothelial Permeability

Transendothelial permeability was measured using Transwell-Clear chambers (3-μm pore size, 12-mm diameter, Costar Corning, Costar), as previously described (40).

Immunofluorescence and Localization of F-Actin

F-actin, Myc-tagged proteins, and vascular endothelial (VE)-cadherin (Santa Cruz Biotechnology) were visualized by immuno- and affinity-fluorescence methods and analyzed by confocal microscopy as described in Ref. 40. To determine changes in F-actin levels, fluorescent emissions from TRITC-phalloidin bound to F-actin were quantified from the basal aspect of cells at the same level of laser intensity for all the studied samples. Data were pooled from 3 separate experiments of which 10 fields were examined on 3 separate coverslips from both normal and PH cells.

Rho, Rac, and Cdc42 Pulldown Assays

RhoA activity was measured with recombinant GST-RBD bound to glutathione beads (Upstate Biotechnology). Rac1 activity with GST-PAK1-PBD (Upstate Biotechnology), and Cdc42 activity with GST-WASP-PBD (40). Affinity-precipitated RhoA, Rac1, and Cdc42 proteins were resolved by SDS-PAGE and detected by Western blot analysis.

ROS Generation

ROS generation in PAECs was assessed using 2′,7′-dichlorodihydrofluorescein diacetate (5 μM, Calbiochem) (40).

Transfection and Reporter Gene Assays

Transfections of 90% confluent PAECs with a HIF-1α luciferase (Luc) reporter driven by a trimer of murine Epo 3′-enhancer and the glucose transporter 1 (Glut1) promoter (pEpo3′-Glut1-Luc, a gift of Dr. P. Schumacker, Department of Medicine, Chicago University, Chicago, IL) were carried out using Metafectene (Bionet; Munich, Germany). AdNFκB-Luc, which contains the Luc gene driven by four tandem copies of the NF-κB consensus sequence, was fused to a TATA-like promoter from the Herpes simplex virus thymidine kinase gene. Luc assays were performed using a Luciferase Assay System (Promega).

VEGF production was measured in PAECs plated in 96-well plates at a density of 1 × 10^4 cells/well and incubated overnight under normoxic or hypoxic conditions with or without inhibitors. ELISA assays were carried out on fixed and permeabilized cells (39). Mouse monoclonal anti-VEGF165 (Abcam; Cambridge, UK) and rabbit anti-mouse horseradish peroxidase-linked IgG (DAKO; Glostrup, Denmark; 1:1,000) were used, and the color was developed with 3,3′,5,5′-tetramethylbenzidine (Sigma). The reaction was stopped with 2 N H2SO4 and read at 450 nm in an ELISA plate reader.

Statistical Analysis

All the experiments were performed in triplicate for PAECs from each animal (n = 18 per group). Data are presented as means (SD). Comparisons between two groups were carried out with Student’s t-test. When more than two conditions were compared, a one-way ANOVA test followed by Dunnett’s posttest was used. Statistical significance was accepted for P < 0.05, and all tests were performed with GraphPad Prism version 3.0.

RESULTS

Endothelial Cells From PH Animals Are Different From Those of Healthy Animals Under Basal (Normoxic) Conditions

Cell phenotype. Confluent PAECs from normal newborn animals showed a cobblestone morphology with strong cortical localization of F-actin and junctional localization of the integral cell-cell adherens protein VE-cadherin (Fig. 1, A and B). Transmission electron microscopy (TEM) micrographs (Fig. 1, E and G) showed well-spread cells with well-developed adherens junctions in vitro and in vivo. In contrast, cells from PH animals had discontinuous, jagged staining of VE-cadherin (Fig. 1, C and D) and formed numerous actin stress fibers, a feature reflected by the higher F-actin content in those cells (Fig. 1, C and J). The cells were less spread and generally lacked mature adherens junctions with TEM (Fig. 1, F and H).
The PH cell phenotype was stable and persisted during the time chosen for the study.

**Barrier function.** Cells from PH animals showed a higher basal permeability than normal cells, 10.2 (SD 0.7) compared with 7.8 (SD 0.5) [difference of 31% (SD 7), \( P < 0.01; \) Fig. 1I].

**Activities of Rho GTPases RhoA, Rac1, and Cdc42.** In cells from PH animals, RhoA activity levels were increased by 61% (SD 15) (\( P < 0.01 \)) and Rac1 activity levels were decreased by 28% (SD 12) (\( P < 0.05 \)) compared with basal values in normal cells, whereas the activity levels of Cdc42 were normal (Fig. 1, I and J). Protein expression levels of Rho GTPases in PH cells were similar to normal (Fig. 1J).

**ROS production.** Endothelial cells from PH animals showed a 30% (SD 8) decrease in ROS levels (Fig. 1I).

**VEGF production.** VEGF is known to induce morphological changes in endothelial cells, increase ROS production, and induce endothelial permeability (10). To determine whether the morphological changes seen in cells from PH animals could result from changes in VEGF production, we measured the levels of VEGF189 secretion by normal and PH PAECs. VEGF189 is a cell- and extracellular matrix-associated splice variant of VEGF (10) that predominates in lung tissue from rats (>50% total VEGF) (4) and is important in lung capillary development (14). In fact, PH cells produced 28% (SD 14) less VEGF189 than normal cells under normoxic conditions (\( P < 0.01; \) Fig. 1I).

**Activities of transcription factors HIF-1\( \alpha \) and NF-\( \kappa B \).** Luciferase reporter gene assays showed an increase in the activity of HIF-1\( \alpha \) by 410% (SD 215) and an increase in NF-\( \kappa B \) activity by 280% (SD 90) in PH cells compared with normal cells under normoxic conditions.

**Endothelial Cells From PH Animals Respond Abnormally to Acute Hypoxia and Reoxygenation**

**Cell phenotype.** Normal PAECs in vitro (Fig. 2, A and B) responded to short-term hypoxia (1 h) by forming stress fibers
and losing junctional VE-cadherin (Fig. 2, E and F), changes largely reversed by 1 h of reoxygenation (Fig. 2, J and K). PH cells (Fig. 2, C and D) responded to acute hypoxia in a similar manner (Fig. 2, G and H), but reoxygenation only partially restored the junctional localization of F-actin (Fig. 2, K and L). Extending the reoxygenation time to 4 h did not restore the phenotype of PH cells to normal (data not shown).

**Barrier function.** Acute hypoxia induced an increase in endothelial permeability in both normal and PH cells, reaching a maximum at 1 h of 147% (SD 7) and 151% (SD 8) of basal normoxic values, respectively. During the next 3 h of hypoxic exposure, the permeability returned to normoxic basal values in normal cells but not in PH cells (Fig. 2M). When cells were subjected to 1 h of reoxygenation after a 1-h exposure to hypoxia, endothelial permeability returned to normoxic basal levels in normal PAECs but not in PH cells (Fig. 2M).

**Activity of Rac1, RhoA, and Cdc42.** Acute hypoxia induced a gradual decrease in Rac1 activity in both normal and PH cells, with both reaching a nadir at 1 h of hypoxic exposure at 52% (SD 6) and 54% (SD 8) of basal normoxic levels, respectively (Fig. 2N). After 4 h of hypoxic exposure, Rac1 activity increased in both groups to reach the basal values, and therefore Rac1 activity remained significantly reduced in PH cells compared with normal cells. Hypertensive and normal cells responded differently to reoxygenation. Rac1 activity increased by 140% (SD 25) of the basal level in normal cells but only by 29% (SD 7) in PH cells (Fig. 2N).

The activity of RhoA in both normal and PH cells increased significantly during 1 h of acute hypoxic exposure and then gradually decreased, returning to basal levels at 4 h of hypoxic exposure (Fig. 2N). After 1 h of hypoxia, reoxygenation reduced RhoA activity to the basal level in both normal and PH cells, and therefore the reduction was significantly greater in normal cells (Fig. 2N). Cdc42 was transiently activated by hypoxia and inhibited by reoxygenation in both normal and PH cells (Fig. 2N). Protein expression levels of Rho GTPases RhoA, Rac1, and Cdc42 in PH cells remained normal (Fig. 2N).

**ROS production.** After 1 h of acute hypoxia, ROS levels decreased in normal and PH cells by 50% (SD 5) and 22% (SD 6) of the basal levels (Fig. 3A). Reoxygenation restored the levels of ROS in both normal and PH cells to their basal normoxic values (Fig. 3A); the ROS levels in PH cells remained significantly less than normal at 68% (SD 8) of the basal value in normal cells.

**VEGF production.** Hypoxia (24 h) induced a small but significant increase in VEGF189 production in normal cells by 17% (SD 8) and a larger increase in PH cells by 30% (SD 7) (Fig. 3B). 

**Activities of transcription factors HIF-1α and NF-κB.** Lucifer reporter gene assays showed that PH cells responded to acute hypoxia with a significantly stronger activation of HIF-1α (Fig. 3C) and NF-κB (Fig. 3D) than normal cells.

**Effects of RhoA, Rac1, and Cdc42 Inhibitors and PAK-1 on Hypoxia-Induced Endothelial Remodeling of PAECs From PH Animals**

Rac1 activation and RhoA inhibition restore the PH PAEC phenotype to normal. The RhoA inhibitor N19RhoA reduced the number of stress fibers and increased junctional localization.

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Fig. 2. Hypoxia and reoxygenation-induced changes in endothelial cell phenotype, permeability, and activity of Rho GTPases in normal and PH PAECs. A–L: F-actin and VE-cadherin immunostaining. A, E, and I: F-actin in normal cells in normoxia (A), hypoxia (E), and on reoxygenation (reox; I); B, F, and J: distribution of VE-cadherin on corresponding images. C, G, and K: F-actin in PH cells in normoxia (C), hypoxia (G), and on reoxygenation (K); D, H, and L: VE-cadherin distribution in corresponding images. Bar = 20 μm. M: changes in permeability in normal and PH cells subjected to hypoxia for 15 min to 4 h and following reoxygenation after 1 h of hypoxia. N: activities of Rac1, RhoA, and Cdc42 and the representative Western blots. *P < 0.05 and **P < 0.01, comparisons with normoxic basal values in normal PAECs; #P < 0.05 and ##P < 0.01, comparisons between PH cells with their normal counterparts at the same time points under the same condition.
of VE-cadherin in PH cells cultured under normoxic conditions (Fig. 4E) and in acute hypoxia (data not shown). Activation of Rac1 with V12Rac1 had a protective effect on intercellular junctions in hypertensive PAECs in normoxia (Fig. 4F) and acute hypoxia (Fig. 4K). In contrast, inhibition of Rac1 with N17Rac1 led to increased stress fiber formation and loss of VE-cadherin from intercellular junctions (Fig. 4J). Expression of GFP (controls; Fig. 4, A–C) or the inhibitor of Cdc42 N17Cdc42 had no effect on endothelial phenotype (data not shown).

We (40) recently found that downregulation of Rac1 by acute hypoxia contributes to RhoA activation in normal PAECs. Other investigators showed that PAK-1, a downstream target of Rac1, downregulates RhoA activity in HEK-293 cells (1). To find out whether inhibition of PAK-1 in PAECs would activate RhoA in normal PAECs and induce phenotype changes resembling those seen in cells from PH animals, we expressed kinase-dead PAK-1 via adenoviral gene transfer in normal PAECs. This inhibition of PAK-1 induced the formation of stress fibers and a dispersion of VE-cadherin from endothelial junctions (Fig. 4G). In contrast, the Rac1/PAK-1 activator S1-P (15) completely restored an apparently normal phenotype to PH cells under normoxic (Fig. 4H) and hypoxic (Fig. 4L) conditions, an effect attenuated by PAK-1 (K298A) (Fig. 4I). Activation of Rac1 by S1-P in PAECs from PH animals and activation of RhoA by PAK-1 (K298A) in normal PAECs are shown in Fig. 4M. N17Cdc42 had no effect on changes in endothelial phenotype (data not shown).

Rac1 activators and RhoA inhibitors restore endothelial barrier function in PH cells. Under normoxic conditions, N19RhoA and V12Rac1 had no effect on endothelial permeability in normal cells but decreased the permeability of PH cells (Fig. 4N). In acute hypoxia, N19RhoA and V12Rac1 significantly reduced endothelial permeability in both normal and PH cells (Fig. 4N). The Rac1 inhibitor N17Rac1 and the PAK-1 inhibitor PAK-1 (K298A) significantly increased endothelial permeability in both normal and PH cells in normoxia and hypoxia (Fig. 4N). S1-P was the most effective agent in...
preventing endothelial leakage and restored normal basal permeability values in PH cells under normoxic and hypoxic conditions (Fig. 4N). N17Cdc42 had no effect on changes in endothelial permeability (Fig. 4N).

ROS levels are increased by S1-P and V12Rac1 in PH cells but not in normal PAECs. N19RhoA and N17Cdc42 did not have a significant effect on ROS production in normal or PH cells under normoxic conditions. The Rac1 activators V12Rac1 and S1-P did not affect ROS levels in normal cells but significantly increased ROS production in PH cells (Fig. 4N). In contrast, the Rac1 inhibitor N17Rac1 reduced ROS levels in both normal and PH cells to 58% (SD 7) and 56% (SD 9) of basal values in normal cells, respectively. Kinase-dead PAK-1 had no effect on ROS production in PH PAECs but significantly decreased ROS production in normal PAECs ($P < 0.01$ compared with the basal value in normal cells; Fig. 5A).

Effects of Rac1, RhoA, Cdc42, and PAK-1 on VEGF production in normal and PH cells. In normal cells cultured under normoxic conditions, VEGF$_{189}$ production was significantly reduced by the inhibitors of Rac1/PAK-1 and Cdc42 ($P < 0.01$) and, to a lesser extent, by activated Rac1 (V12Rac1) and N19RhoA ($P < 0.05$), whereas in PH cells it was reduced by the inhibitors of Rac1/PAK-1 and Cdc42 as well as V12Rac1 but not by dominant-negative RhoA (N19RhoA; Fig. 5B). After cells were exposed to hypoxia for 24 h, the increase in VEGF$_{189}$ production was significantly reduced in all treated groups except for GFP-expressing controls (Fig. 5B), with N17Rac1, N17Cdc42, and PAK-1 (K298A) having the strongest effects.

Effects of Rac1, RhoA, Cdc42, and PAK-1 on the activation of HIF-1$\alpha$ and NF-kB. In normoxia, the basal HIF-1$\alpha$ activity in normal cells was not significantly affected by any of the tested inhibitors (Fig. 5C). In PH cells under normoxic conditions, V12Rac1 reduced HIF-1$\alpha$ activity to normal, whereas other agents did not have a significant effect (Fig. 5C). After cells were exposed to hypoxic conditions for 4 h, activation of HIF-1$\alpha$ in normal and PH cells was significantly reduced by V12Rac1 ($P < 0.01$) and, to a lesser extent, by N17Rac1 and kinase-dead PAK-1 ($P < 0.05$). N19RhoA and N17Cdc42 had no effect (Fig. 5C).

NF-kB activity in normal cells in normoxia was not affected by inhibitory proteins or V12Rac1, but in PH cells it was significantly reduced by N17Rac1, N17Cdc42, and PAK-1 (K298A) (Fig. 5D), whereas V12Rac1 and N19RhoA had no effect. NF-kB activation under hypoxic conditions in normal and PH cells was significantly reduced by N17Rac1, N17Cdc42, and kinase-dead PAK-1 ($P < 0.01$ compared with values in untreated cells). V12Rac1 reduced NF-kB activity under hypoxic conditions in PH cells ($P < 0.05$) but not in normal cells. N19RhoA had no effect (Fig. 5D).

![Fig. 5.](http://ajplung.physiology.org/) Effects of the inhibitors of Rho GTPases V12Rac1 and kinase-dead PAK-1 on ROS levels under normoxic conditions (A), VEGF production after a 24-h hypoxic exposure (B), and activation of HIF-1$\alpha$ (C) and NF-kB (D) after a 4-h hypoxic exposure in normal and PH PAECs. The duration of hypoxic exposure was varied to maximize responses to the inhibitors and activators of Rho GTPases. * $P < 0.05$ and ** $P < 0.01$, comparisons with the normoxic basal level in normal PAECs; # $P < 0.05$ and ## $P < 0.01$, comparisons between hypertensive PAECs and their normal counterparts at the same time point under the same condition; & $P < 0.05$ and && $P < 0.01$, comparisons between PH cells treated with inhibitors and activators of Rho GTPases in normoxia and hypoxia and their untreated controls.
DISCUSSION

The present investigation shows for the first time that PAECs isolated from animals with neonatal PH have an abnormal, stable phenotype in vitro. These cells showed an increase in stress fibers, increased permeability, and alterations in Rho GTPase activities (Fig. 6) when cultured under normoxic conditions. A similar phenotype was observed in normal PAECs exposed to acute hypoxia. It appears that chronic hypoxia-induced PH leads to the development of stable, abnormal features in both PAECs and smooth muscle cells (5). It is probably relevant that adult rats that have been exposed to chronic hypoxia in early life have an exaggerated response when reexposed to hypoxia (33), suggesting the preservation of stable, abnormal phenotypes in vivo. To find out whether PAECs from PH animals also respond abnormally to acute changes in oxygenation, we subjected normal and PH cells to acute hypoxia followed by reoxygenation. We observed that PH cells exposed to acute hypoxia showed the same pattern of changes in cytoskeletal and junctional remodeling, endothelial permeability, activity of Rho GTPases, ROS production, VEGF production, and activation of NF-κB and HIF-1α as seen in normal cells, but the reaction was often less marked because the basal state was already modified.

Changes in the appearance of PH PAECs and the increase in permeability appeared to result from a sustained inhibition of Rac1 and activation of RhoA. The increase in permeability could not be directly attributed to VEGF because PAECs from PH animals produced less VEGF than normal. They also produced less ROS and showed higher activity of the transcription factors HIF-1α and NF-κB, likely to be caused by changes in the activity of Rho GTPases (Fig. 6).

Changes in RhoA and Rac1 activity in PAECs from animals with PH were not caused by changes in protein expression, unlike in smooth muscle cells in systemic hypertension, where increased RhoA activation was associated with an increase in RhoA protein expression (32). The mechanism of RhoA activation in the pulmonary arteries of PH animals appears to be cell-type specific. In the same porcine model of neonatal PH, we (5) previously showed that activation of RhoA in smooth muscle cells could not be explained by changes in either Rac1 activity or RhoA protein expression. Rac1 is important for the maintenance of endothelial intercellular adherens junctions and the assembly of an active NAD(P)H oxidase complex (17). Its inhibition by acute hypoxia in normal adult PAECs leads to RhoA activation (40). In the present study, we demonstrated that inhibition of the Rac1 effector PAK-1 activated RhoA, which led to an increase in the formation of stress fibers, dispersion of adherens junctions, increased permeability, and reduced ROS formation in normal PAECs. These features resembled those described in PAECs from our PH animals studied under normoxic conditions. The mechanism by which PAK-1 regulates RhoA activity in PAECs is uncertain, but it is likely to involve serine phosphorylation of the RhoA-specific guanine nucleotide exchange factor NET1 (1). Conversely, activation of the Rac1/PAK-1 pathway by S1-P in cells from animals with neonatal PH produced an apparently normal phenotype with normal permeability, similar to the effects of S1-P in thrombin-treated cells (15). The work of Garcia et al. (15) on barrier-enhancing properties of S1-P was fundamental, and our study shows that this platelet-derived sphingolipid may have therapeutic potential to protect and preserve endothelial integrity in PH induced by chronic hypoxia.

A Reduction in Rac1 Activity Is Associated With a Decrease in VEGF Production and ROS Levels in PAECs From Hypertensive Animals

VEGF is known to increase endothelial permeability (44), activate Rac1 (9) and RhoA (36), and increase NADPH oxidase-mediated ROS production in endothelial cells (35). We found that in PAECs from PH animals, VEGF189 levels were significantly lower than in normal PAECs in vitro, in accord with the in vivo observations of other investigators (13, 18), where low levels of VEGF expression in the lungs of experimental animals were associated with the development of PH. A decrease in VEGF production coincided with low Rac1 activity and ROS production in PH cells, which may suggest that VEGF was a mediator of some of the cell responses. However, we also observed that changes in the activities of all three Rho GTPases studied reduced VEGF189 production in PAECs, which indicates that an imbalance in Rho GTPases signaling in PH cells may also play a role in the reduction in VEGF levels. Future studies will need to address whether Rho GTPases act upstream or downstream of VEGF expression and signaling in PAECs from animals with PH.

PAECs From PH Animals Show Increased Activities of Transcription Factors HIF-1 and NF-κB, Which May Result From Changes in Rho GTPase Activity

We studied the activation of HIF-1α and NF-κB because they are redox-sensitive transcription factors (19) and their gene products have been implicated in the pathogenesis of neonatal PH (6). Activated Rac1 restored normal HIF-1α activity in PH cells in normoxia, indicating that low levels of

![Fig. 6. Proposed pathway of Rho signaling in PAECs from PH animals. The PH phenotype is stable in normoxia, and the changes shown are similar to those seen in the normal phenotype exposed to acute hypoxia. Chronic hypoxia in vivo downregulates the activity of Rac1 and its effector PAK-1, resulting in activation of RhoA. Sustained changes in the activities of Rac1 and RhoA are characterized by the formation of stress fibers, junctional instability, and increased permeability. An imbalance in Rac1 and RhoA signaling affects the activities of transcription factors NF-κB and HIF-1α and VEGF production. Responses to acute changes in oxygenation follow the same pattern as seen in normal cells but are less pronounced. ATII, angiotensin II; ET, endothelin.](image-url)
this GTPase may play a role in the regulation of HIF-1α activity in these cells. In cells exposed to acute hypoxia, HIF-1α activity was reduced by both dominant-negative and constitutively active forms of Rac1, indicating that dynamic changes in Rac1 activity as well as a certain optimal level of activity may be required.

The activation of NF-κB in PH PAECs in normoxia depended on the activity of Rac1 and Cdc42 as well as PKA-1 but not RhoA. The individual contributions of Rho GTPases to NF-κB and HIF-1α activation appear to depend on the type of stimulating agent and cell type (3, 12, 24, 30). Rac1/PK-1 were important for the activation of both transcription factors in normal and PH PAECs, but the magnitude of the response to their inhibition or activation was higher in PH cells, possibly resulting from the higher basal activity levels of transcription factors in those cells.

Our results show that Rho GTPases RhoA and Rac1 play a key role in the regulation of endothelial permeability in PH cells and suggest that these GTPases may also be important in the regulation of transcription factors NF-κB and HIF-1α, as well as VEGF production in these cells (Fig. 6). However, the issue is as to whether Rho GTPases act upstream or downstream of HIF-1α and NF-κB, in complex, and resolving it is beyond the scope of this investigation. The activities of these transcription factors can be affected by their own gene products (8, 29), such as VEGF, angiotensin II, and endothelin-1, which in turn regulate the activities of Rho GTPases (9, 11, 31, 42).

In summary, we have shown that cultured PAECs from animals with neonatal PH have a stable, abnormal phenotype, manifested by increased formation of stress fibers and increased permeability under normoxic conditions. These cells are able to respond to exposure to acute hypoxia. These changes are likely to result from a sustained inhibition of Rac1 and its downstream effector PKA-1 followed by a sustained activation of RhoA. Our in vitro study indicates for the first time that leakage of the endothelial barrier is an early change in the presence of sustained PH, at a time when pulmonary arterial medial hypertrophy is present (21, 22). Future studies should explore the clinical potential of activators of Rac1, such as S1-P, to restore endothelial barrier function in PH.

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