Hypoxia-induced alveolar epithelial-mesenchymal transition requires mitochondrial ROS and hypoxia-inducible factor 1

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Zhou G, Dada LA, Wu M, Kelly A, Trejo H, Zhou Q, Varga J, Sznajder II. Hypoxia-induced alveolar epithelial-mesenchymal transition requires mitochondrial ROS and hypoxia-inducible factor 1. Am J Physiol Lung Cell Mol Physiol 297: L1120–L1130, 2009. First published October 2, 2009; doi:10.1152/ajplung.00007.2009—Patients with acute lung injury develop hypoxia, which may lead to lung dysfunction and aberrant tissue repair. Recent studies have suggested that epithelial-mesenchymal transition (EMT) contributes to pulmonary fibrosis. We sought to determine whether hypoxia induces EMT in alveolar epithelial cells (AEC). We found that hypoxia induced the expression of α-smooth muscle actin (α-SMA) and vimentin and decreased the expression of E-cadherin in transformed and primary human, rat, and mouse AEC, suggesting that hypoxia induces EMT in AEC. Both severe hypoxia and moderate hypoxia induced EMT. The reactive oxygen species (ROS) scavenger Euk-134 prevented hypoxia-induced EMT. Moreover, hypoxia-induced expression of α-SMA and vimentin was prevented in mitochondria-deficient ρ0 cells, which are incapable of ROS production during hypoxia. CoCl2 and dimethylxaloylglycine, two compounds that stabilize hypoxia-inducible factor (HIF)-α under normoxia, failed to induce α-SMA expression in AEC. Furthermore, overexpression of constitutively active HIF-1α did not induce α-SMA. However, loss of HIF-1α or HIF-2α abolished induction of α-SMA mRNA during hypoxia. Hypoxia increased the levels of transforming growth factor (TGF)-β1, and preincubation of AEC with SB431542, an inhibitor of the TGF-β1 type I receptor kinase, prevented the hypoxia-induced EMT, suggesting that the process was TGF-β1 dependent. Furthermore, both ROS and HIF-α were necessary for hypoxia-induced TGF-β1 upregulation. Accordingly, we have provided evidence that hypoxia induces EMT of AEC through mitochondrial ROS, HIF, and endogenous TGF-β1 signaling.

alveolar epithelial cells; pulmonary fibrosis; transforming growth factor-β1

EPITHELIAL-MESENCHYMAL TRANSITION (EMT) is a cellular process during which epithelial cells acquire mesenchymal properties while losing cell-cell interactions and apicobasal polarity (33, 44). EMT is characterized by changes in cell morphology and acquisition of mesenchymal markers such as α-smooth muscle actin (α-SMA) and vimentin as well as loss of epithelial markers, including E-cadherin (53). Transforming growth factor (TGF)-β1 is considered to be the prototypical cytokine for the induction of EMT (53). Active TGF-β1 binds to the transmembrane serine-threonine kinase receptor II and receptor I and activates Smad-mediated transcription of target genes, including α-SMA and vimentin, which leads to EMT (33, 53, 54).

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AEC perform many tasks necessary for normal alveolar functioning, including surfactant protein production and fluid and ion transport (17, 57). Recent evidence suggests that AEC may undergo EMT, contributing to the pathogenesis of pulmonary fibrosis (26, 49).

AEC are exposed to hypoxia in human lung diseases, including acute lung injury and pulmonary fibrosis (20, 41, 57). It has been described that during hypoxia, mitochondria increase the production of reactive oxygen species (ROS) at complex III (11), leading to inhibition of prolyl hydroxylase activity and subsequent stabilization of hypoxia-inducible factor (HIF)-α protein. HIF-α forms a heterodimeric transcription factor with HIF-β subunits (11, 22, 41) and induces transcription of genes participating in the cellular adaptation to hypoxia (35, 41). However, it is unknown whether hypoxia and HIF induce EMT in AEC.

We investigated the effect of hypoxia on the induction of EMT in AEC and examined the role of ROS, HIF, and TGF-β1 as mediators in this process. Our results suggest that hypoxia induces EMT in transformed human, rat, and mouse AEC lines and freshly isolated rat type II AEC and that this process requires the generation of mitochondrial ROS and stabilization of HIF. ROS and HIF participate in hypoxia-induced TGF-β1 production that results in EMT.

EXPERIMENTAL PROCEDURES

Materials. TGF-β1 (Calbiochem, La Jolla, CA), dimethylxaloylglycine (DMOG) (Frontier Scientific, Logan, UT), Euk-134 (Cayman, Ann Arbor, MI), CoCl2, SB431542, U0126, and bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) were used in this study. The following antibodies were used in this study: α-tubulin, actin, vimentin (Sigma-Aldrich), α-SMA (R&D Systems, Minneapolis, MN), E-cadherin, and 14-3-3 (Santa Cruz Biotechnology, Santa Cruz, CA).

Cell culture. Human lung adenocarcinoma cells (A549), rat alveolar epithelial cells (RLE-6TN), and mouse alveolar epithelial cells (MLE 12) were obtained from the American Type Tissue Collection and grown in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. These cells exhibit the cuboidal cell morphology of type II alveolar epithelial cells (AEC). Cells were incubated in a humidified atmosphere of 5% CO2-95% air at 37°C. Cell cultures were routinely split when 85–90% confluent. A549-shHIF-1α and A549-shHIF-2α are stable cell lines with suppression of HIF-1α or HIF-2α by small hairpin RNAs (shRNAs), as previously described (56). A549 cells expressing shRNA of Drosophila melanogaster HIF (A549-shdHIF) were used as control. These cell lines were maintained in media containing 1 μg/ml puromycin. Hypoxic conditions (1.5 or 3% O2) were achieved in a humidified work station (In vivo2; Ruskin Technologies, Leeds, UK).
UK), which contained an oxygen sensor to continuously monitor the chamber’s oxygen tension.

Isolation of rat primary type II alveolar epithelial cells. Type II AEC were isolated from the lungs of Sprague-Dawley rats by the Pulmonary Division Cell Culture and Physiology Core B, as previously described (38). All animals were provided with food and water ad libitum, maintained on a 12:12-h light-dark cycle, and handled according to National Institutes of Health guidelines and the Institutional Animal Care and Use Committee-approved experimental protocols. The purity of initial isolation of rat type II AEC cells is consistently >95% as determined by staining of surfactant protein C. The fibroblast contamination is <5% as determined by staining of vimentin. Rat type II AEC were cultured in DMEM containing 10% FBS with 2 mM L-glutamine, 40 µg/ml gentamicin, 100 µg/ml penicillin, and 100 µg/ml streptomycin. These cells were plated on coverslips or Transwells (Corning, Corning, NY) overnight and exposed to normoxia or hypoxia for the indicated period of time.

Western blotting. Cultured cells were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed in 250 µl of lysis buffer [120 mM Tris-HCl, pH 7.4, 200 mM diethiothreitol, 20% glycerol, 4% sodium dodecyl sulfate (SDS), and protease inhibitors]. The cell lysates were cleared by centrifugation at 13,000 g for 5 min, and protein concentrations were determined using a DC protein assay (Bio-Rad, Hercules, CA). Typically, 25–50 µg of protein were then separated by SDS-polyacrylamide gel electrophoresis. The gel was transferred using a Semi-Dry transfer cell (Bio-Rad) to BA-S 85 nitrocellulose membrane (OPTITRAN, Middlesex, UK). Proteins were detected with Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer, Wellesley, MA). Gray density of Western blots was measured using ImageJ software (National Institutes of Health, Bethesda, MD).

Confocal immunofluorescence studies. Cells were grown on coverslips and cultured for the indicated time and conditions, followed by fixation with 2% formaldehyde (formalin) for 10 min. The coverslips were washed in a small plate with wash solution (PBS + 0.5% Tween 20) once and with PBS twice at room temperature. Cells were permeabilized by incubation for 30 min at 37°C with 0.1% Triton X-100 in PBS, followed by washing with wash solution and PBS. After incubation with the blocking solution (PBS + 6% normal goat serum + 2% BSA) for 30 min at 37°C, cells were incubated with primary antibody (rabbit anti-E-cadherin, 1:200; mouse anti-α-SMA, 1:500) diluted in the blocking solution for 30 min. After being washed, cells were incubated with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody and Alexa Fluor 568-conjugated goat anti-mouse secondary antibody and Alexa Fluor 647-conjugated goat anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA) at 1:200 dilution for an additional 30 min at 37°C. Nuclei were labeled with Hoechst 33342 (Invitrogen) before coverslips were mounted with aqueous mounting solution (INNOVEX, Parsippany, NJ).

Generation of mitochondria-deficient cells. Mitochondria-deficient (p<sup>−/−</sup>) A549 cells were generated as previously described (12). Briefly, A549 cells were incubated in medium containing ethidium bromide (50 ng/ml), sodium pyruvate (1 mM), and uridine (50 µg/ml) for 4–6 wk. The p<sup>−/−</sup> status of cells was confirmed by the absence of cytochrome oxidase subunit II by PCR.

TGF-β1 measurements. TGF-β1 was measured by ELISA kit (R&D Systems) following the manufacturer’s protocol. Briefly, RLE-6TN cells were incubated under normoxic or hypoxic conditions with 2.5% FBS, as indicated, and the culture media were collected at the end of incubation. For active TGF-β1 measurements, a 96-well microplate was coated with capture antibody overnight at room temperature. After three washes with wash buffer (0.05% Tween 20 in PBS), the plates were blocked with the blocking buffer (5% Tween 20 in PBS with 0.05% Na<sub>2</sub>SO<sub>4</sub>) for a minimum of 1 h. Aliquots of samples or standards were added to each well, covered with an adhesive strip, and incubated for 2 h at room temperature. After three washes, the detection antibody was added and incubated for 2 h. The working dilution of streptavidin was added to each well and incubated for 20 min at room temperature in the dark. After three washes, the substrate solution was added and incubated for 20 min. For the addition of stop solution, the optical density of each well was determined immediately with the use of a microplate reader set to 450 nm with wavelength correction set to 540 nm. The total TGF-β1 was measured after the culture media were preactivated by addition of acid.

Quantitative real-time RT-PCR. Total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia CA). Complementary DNA (cDNA) was synthesized from 0.5 µg of total RNA using the iScript cDNA synthesis kit (Bio-Rad) with a mixture of oligo(dT) and random hexamer primers. PCR were carried out with iQ SYBR green supermix (Bio-Rad) on the iCycler iQ multicolor real-time PCR detection system (Bio-Rad). Cycle threshold values were normalized to amplification of the mitochondrial ribosomal protein L19 (RPL19). Sequences of primers used for quantitative real-time RT-PCR (qRT-PCR) are as follows: human α-SMA, AAGAGTTAGACCTGCTGAT (sense) and TGATGCTGTAGTGTTAGTGTTT (anti-sense); human RPL19, ATCATCGCAAGCTGTCG (sense) and TGACCTCCTCTGGGCTTCC (antisense); and human TGF-β1, GCAACATTCTCCTGGCATACC (sense) and CTCCAGGCT-CAACC (antisense).

Establishment of a stable cell line with constitutively active HIF. A HIF-1α mutant containing double mutation of proline to alanine at residues 402 and 564 (HIF-DPA) was constructed into the pLxin retroviral vector (Clontech, Mountain View, CA). Because HIF-DPA lacks proline hydroxylation sites, it is constitutively stabilized under normoxic conditions (8, 13). HIF-DPA construct and vector were transfected into pT67 packaging cells (Clontech). The culture medium was collected as crude viral particles to infect A549 cells, which were subjected to selection with geneticin G418 (400 µg/ml; Invitrogen). The selected clones were transfected with a luciferase reporter construct driven by phosphoglyceric kinase promoter containing HIF response elements (HREs) (37). Luciferase assay (Promega, Madison, WI) was employed to screen for colonies expressing active HIF.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software, La Jolla, CA) and Microsoft Excel when applicable. Data are means ± SE. One-way ANOVA with the Dunnet post test was used in multiple comparisons, and a t-test was performed between two groups. P < 0.05 and P < 0.01 were selected as significance levels.

RESULTS

Hypoxia induces EMT in transformed and primary AEC. To determine whether hypoxia induces EMT in transformed AEC, we incubated RLE-6TN cells in normoxic (21% O<sub>2</sub>) or hypoxic conditions (1.5% O<sub>2</sub>) for 4 days. As shown in Fig. 1A, left blot, hypoxia increased α-SMA and vimentin expression in RLE-6TN cells. To investigate whether this process also occurs in mouse and human AEC, we incubated MLE 12 and A549 cells in hypoxic conditions. As shown in Fig. 1A, middle and right blots, hypoxia increased α-SMA and vimentin expression, whereas it decreased E-Cadherin levels in MLE 12 and A549 cells after 8 days of exposure. α-SMA can form filament bundles, contributing to contractile activity and spreading of fibroblasts (47). We assessed the formation of α-SMA fibers using confocal immunofluorescence microscopy, and as shown in Fig. 1B, hypoxia increased the staining of α-SMA and the formation of α-SMA fibers in RLE-6TN cells. These cells displayed an elongated and flattened morphology. These results suggest that hypoxia induces EMT in transformed human, rat, and mouse AEC.
To investigate whether moderate hypoxia also induces EMT, we exposed RLE-6TN cells to severe hypoxia (1.5% O2) and moderate hypoxia (3% O2) for 4, 6, and 8 days, respectively, and measured the expression levels of α-SMA. As shown in Fig. 1, severe and moderate hypoxia induced the expression of α-SMA as early as 4 days of exposure, suggesting that moderate hypoxia also induces EMT.

To determine whether hypoxia induces EMT in primary AEC, we isolated rat primary type II AEC and exposed them to normoxia (21% O2) or hypoxia (1.5% O2) for 0, 4, and 8 days. As shown in Fig. 2A, rat primary type II AEC displayed typical type II AEC phenotype featuring cuboidal cell shape and membrane staining of E-cadherin. Rat primary type II AEC incubated in hypoxic condition for 4 days started expressing α-SMA, whereas the levels of E-cadherin remained unchanged. Although rat primary type II AEC incubated in the normoxic condition for 8 days displayed some levels of α-SMA expression, those cells incubated in the hypoxic condition for 8 days lost the expression of E-cadherin and showed a stronger expression of α-SMA. Figure 2B confirms that exposure to hypoxia for 8 days decreased the expression of E-cadherin, as shown by Western blot analysis. Together, these results suggest that hypoxia induces EMT in primary AEC in a time-dependent manner.

**Hypoxia-induced EMT requires mitochondrial ROS.** We set out to determine whether ROS are required for hypoxia-induced EMT. In RLE-6TN cells, treatment of the ROS scavenger Euk-134 decreased hypoxia-induced upregulation of α-SMA and vimentin expression (Fig. 3A). It has been reported that mitochondria are the main source of ROS production during hypoxia (10, 27). To determine the role of mitochondrial ROS in EMT during hypoxia, we generated mitochondria-deficient p53-A549 cells and exposed them to normoxic or hypoxic (1.5% O2) conditions for 8 days. As shown in Fig. 3B, hypoxia decreased α-SMA and vimentin expression in p53-A549 cells, suggesting that the hypoxia-induced EMT is dependent on mitochondrial ROS.
Hypoxia and Alveolar Epithelial-Mesenchymal Transition

HIF is necessary but not sufficient for hypoxia-induced EMT. Under hypoxic conditions, the production of mitochondrial ROS leads to stabilization of HIF-1α (10, 41). Hypoxia-mediated stabilization of HIF-1α in AEC is transient (Fig. 4A). Chemicals such as CoCl₂ or DMOG stabilize HIF in normoxic conditions (4, 11) and induced HRE reporter luciferase activity in RLE-6TN cells (Fig. 4B). To investigate whether HIF-α is sufficient to induce EMT, we treated RLE-6TN cells with CoCl₂ or DMOG. As shown in Fig. 4, C and D, neither CoCl₂ nor DMOG induced α-SMA under normoxia. To corroborate these results, we established a cell line expressing constitutively active HIF-1α by transfecting A549 cells with DPA-HIF. HIF-DPA cells displayed constant HIF activity as measured by HRE luciferase activity (Fig. 4E, bottom); however, these cells did not have elevated α-SMA levels (Fig. 4E, top).
Fig. 3. Mitochondrial reactive oxygen species (ROS) are necessary for hypoxia-induced EMT. 

A: RLE-6TN cells were pretreated with 20 μM Euk-134 (Euk) for 1 h and then exposed to normoxia or hypoxia for 4 days. Cells were lysed, and aliquots containing the same amount of proteins were subjected to SDS-PAGE, followed by Western blot analysis for detection of α-SMA and vimentin protein levels. 14-3-3 protein was used as control for equal loading. Relative amounts of α-SMA and vimentin were quantified with ImageJ. Data are means ± SE (n = 3). Representative Western blots of α-SMA, vimentin, and 14-3-3 are shown (right).

B: mitochondria-deficient p53-A549 cells were exposed to hypoxia for 8 days. Cells were lysed, and aliquots containing the same amount of proteins were subjected to SDS-PAGE, followed by Western blot analysis for detection of α-SMA and vimentin protein levels. Tubulin was used as control for equal loading. Relative amounts of α-SMA and vimentin were quantified with ImageJ. Data are means ± SE (n = 5). **P < 0.01. Representative Western blots of α-SMA, vimentin, and tubulin are shown (right).

C: rat primary type II AEC were grown on coverslips overnight and treated with DMSO or 20 μM Euk-134 before exposure to normoxia or hypoxia (1.5% O2) for 8 days. Cells were fixed and stained with α-SMA and E-cadherin antibodies, followed by incubation with fluorescent dye-tagged secondary antibodies and Hoechst 33342 for nuclei labeling. α-SMA, E-cadherin, and nuclei were visualized using confocal fluorescence microscopy.
Fig. 4. Hypoxia-inducible factor (HIF) is required but not sufficient to induce EMT during hypoxia. A: A549 cells were exposed to hypoxia (1.5% O₂) for 6, 10, and 24 h and lysed. Aliquots containing the same amount of proteins were subjected to SDS-PAGE, followed by Western blot analysis for detection of HIF-1α and actin protein levels. B: RLE-6TN cells were transfected with a luciferase reporter construct driven by a phosphoglyceric kinase promoter containing HIF response element (HRE) and exposed to hypoxia (1.5% O₂) or treated with CoCl₂ (250 µM) or dimethylxaloylglycine (DMOG; 500 µM) for 24 h. Relative luciferase activity was measured as described in EXPERIMENTAL PROCEDURES. C: RLE-6TN cells were exposed to normoxia or hypoxia (1.5% O₂) or treated with 250 µM CoCl₂ for 4 days. Cells were lysed, and aliquots containing the same amount of proteins were subjected to SDS-PAGE, followed by Western blot analysis for detection of α-SMA protein levels. 14-3-3 protein was used as control for equal loading. The relative amount of α-SMA was quantified with ImageJ and normalized to 14-3-3 protein (top). Data are means ± SE (n = 5). Representative Western blots of α-SMA and 14-3-3 are shown (bottom). D: RLE-6TN cells were exposed to normoxia or hypoxia or treated with 500 µM DMOG for 4 days. Cells were lysed, and aliquots containing the same amount of proteins were subjected to SDS-PAGE, followed by Western blot analysis for detection of α-SMA protein levels. Tubulin was used as control for equal loading. The relative amount of α-SMA was quantified with ImageJ (top). Data are means ± SE (n = 3). Representative Western blots of α-SMA and tubulin are shown (bottom). E: A549 cells were infected with a HIF-1α mutant containing double mutation of proline to alanine at residues 402 and 564 (HIF-DPA) or control (Lxin) viruses. Positive colonies were selected with the geneticin G418, and HIF activity was screened with a luciferase activity assay (bottom). Data are means ± SE (n = 3). A549-Lxin and A549-HIF-DPA cells were lysed, and aliquots containing the same amount of proteins were subjected to SDS-PAGE, followed by Western blot analysis for detection of α-SMA protein levels. Tubulin protein was used as control for equal loading. F: A549 cells alone, A549 cells expressing small hairpin RNAs of Drosophila melanogaster HIF (A549-shdHIF), and A549 cells suppressing HIF-1α or HIF-2α shRNAs (A549-shHIF-1α and A549-shHIF-2α) were exposed to normoxia or hypoxia for 2 days, and the mRNA was extracted. The amount of α-SMA mRNA was determined with quantitative real-time RT-PCR (qRT-PCR). The RPL19 gene was used as an internal control, and the induction of α-SMA mRNA was presented as relative change after normalization to RPL19. Data are means ± SE (n ≥ 3). *P < 0.05; **P < 0.01.
suggested that stabilization of HIF-α is not sufficient to induce EMT.

To determine whether HIF-α is required for α-SMA induction during hypoxia, we utilized stable A549-shHIF-1α and A549-shHIF-2α cells, where HIF-1α and HIF-2α were silenced by shRNAs, respectively, as described previously (56). We exposed these cells to normoxia or hypoxia, and the mRNA levels of α-SMA were determined by performing qRT-PCR with RPL19 as internal control. As shown in Fig. 4F, exposure to hypoxia for 2 days induced α-SMA mRNA expression in nontransfected A549 and A549-shdHIF cells (used as a transfection control) but decreased α-SMA mRNA levels in A549-shHIF-1α and A549-shHIF-2α cells, suggesting that HIF-1α and HIF-2α are necessary for hypoxia-induced EMT.

Hypoxia-induced EMT is TGF-β1 dependent. To examine the role of TGF-β1 signaling in hypoxia-induced EMT, we first measured levels of TGF-β1 mRNA by performing qRT-PCR in A549 cells exposed to hypoxia (1.5% O2). As shown in Fig. 5A, hypoxia increased TGF-β1 gene expression in A549 cells up to 8 days, with the maximal induction at 48 h. Hypoxia also resulted in increased levels of active and total TGF-β1 in RLE-6TN cells (Fig. 5, B and C). To further investigate whether TGF-β1 signaling is involved in the hypoxia-induced EMT, we preincubated RLE-6TN cells with SB431542, an inhibitor of ALK5, a TGF-β1 type I receptor kinase. As shown in Fig. 6A, treatment with SB431542 decreased the basal levels of α-SMA and prevented the hypoxia-induced upregulation of α-SMA. Hypoxia and exogenous TGF-β1 treatment induced similar expression of α-SMA and vimentin (Fig. 6, A and B). Moreover, in rat primary type II AEC, treatment with SB431542 prevented the induction of α-SMA and the loss of E-cadherin during hypoxia (Fig. 6C and Fig. 7). As expected, treatment with Euk-134 also prevented the loss of E-cadherin (Fig. 7). Together, these results suggest that TGF-β1 signaling is required for hypoxia-induced EMT.

Hypoxia-induced TGF-β1 production requires ROS and HIF-α. To determine whether ROS and HIF are required for hypoxia-induced upregulation of TGF-β1 expression, we pre-treated A549 cells with Euk-134 for 2 h and exposed them to hypoxia. TGF-β1 mRNA levels were measured using qRT-PCR. As shown in Fig. 8A, Euk-134 prevented the upregulation of TGF-β1 mRNA expression during hypoxia, suggesting that ROS are necessary for TGF-β1 induction during hypoxia. To investigate whether the upregulation of TGF-β1 during hypoxia is HIF dependent, we exposed A549-shdHIF, A549-shHIF-1α, and A549-shHIF-2α cells to hypoxia (1.5% O2) for 2 days and assessed the TGF-β1 mRNA levels by performing qRT-PCR. As shown in Fig. 8B, suppression of HIF-1α and -2α prevented hypoxia-induced TGF-β1 mRNA expression, suggesting that HIF is necessary for TGF-β1 induction during hypoxia.

DISCUSSION

Recently, it was reported that type II AEC can undergo EMT in response to stimuli such as TGF-β1 and endothelin-1 (3, 23, 25, 26, 49, 50). In the present study, we have provided evidence that hypoxia induces EMT in primary and transformed AEC, as determined by the upregulation of α-SMA and vimentin, the formation of α-SMA fibers, and the downregulation of E-cadherin. Hypoxia-induced EMT requires the mitochondrial production of ROS and the stabilization of HIF and is TGF-β1 dependent. Both the generation of mitochondrial ROS and the presence of HIF-α are necessary for hypoxia-induced TGF-β1 production. Together, these results provide evidence that hypoxia stimulates generation of mitochondrial ROS and leads to stabilization of HIF-α, resulting in endogenous TGF-β1 production and EMT in AEC.

Exposure of transformed human, rat, and mouse AEC or rat primary AEC to hypoxia induced the expression of α-SMA and vimentin and suppressed the expression of E-cadherin, suggesting EMT of AEC during hypoxia. Although we detected...
expression of basal levels of α-SMA and vimentin in A549 and RLE-6TN cells, as reported previously (3, 25, 49), we did not detect the basal levels of α-SMA and vimentin in MLE 12 cells, a mouse AEC transformed with SV40 (Fig. 1), suggesting that A549 and RLE-6TN cells may be in a certain stage of transition from epithelial cell phenotype to mesenchymal cell phenotype. This is not surprising, since A549 is a cell line derived from lung adenocarcinoma tissue and RLE-6TN cells are rat AEC immortalized with SV40 transfection. RLE-6TN cell expressed relatively higher basal levels of α-SMA than A549 cells, and the E-cadherin was hardly detectable (Fig. 1), suggesting that RLE-6TN cells have more transitional phenotypes than A549 or MLE 12 and are more readily to be induced to mesenchymal cell phenotypes. Accordingly, in RLE-6TN cells, severe hypoxia (1.5% O₂) induced α-SMA as early as 4 days after exposure, but it took 8 days to obtain EMT phenotype in MLE 12 and A549 cells (Figs. 1 and 2).

Our data suggest that hypoxia also induces EMT in primary rat type II AEC. Although few cells gained expression of α-SMA after 4 days of incubation in hypoxic environment, it took 8 days to have both decreased expression of E-cadherin and increased expression of α-SMA in primary rat type II AEC (Fig. 2). Willis et al. (49) reported that 12 days were required for induction of EMT by exogenous TGF-β1 in freshly isolated primary rat alveolar epithelial type II cells. However, it is known that primary alveolar epithelial type II cells gradually gain type I cell markers in culture within days, and there is a certain degree of fibroblast contamination, making data interpretation challenging (1, 7, 15, 28). Since hypoxia induced EMT in A549, MLE 12, and rat primary type II AEC within the same time frame, we have reasoned that the mechanisms involved in EMT are likely conserved among primary and transformed AEC, suggesting that these immortalized alveolar epithelial cell lines are a useful tool for the study of mechanisms underlining EMT in vitro.
It has been shown previously that hypoxia increases mitochondrial ROS production (10, 27). We explored whether ROS are involved in the hypoxia-mediated EMT. In our study, we treated RLE-6TN cells and primary type II AEC with the ROS scavenger Euk-134 and observed that Euk-134 treatment prevented hypoxia-induced EMT (Figs. 3 and 7). Furthermore, p0-A549 cells, which do not produce ROS during hypoxia (12), failed to exhibit EMT during hypoxia (Fig. 3), suggesting that mitochondrial ROS participate in the hypoxia-induced EMT. Our results are consistent with previous reports in other cell types, supporting the hypothesis that ROS contribute to EMT (36, 55).

Although ROS are known to stabilize HIF during hypoxia (10, 27), our results show that stabilization of HIF-α during normoxia failed to induce α-SMA (Fig. 4), suggesting that HIF alone is not sufficient to induce EMT in AEC. However, silencing of HIF-1α or -2α prevented hypoxia-mediated induction of α-SMA gene expression (Fig. 4F), suggesting that HIF is necessary for hypoxia-induced EMT. It has been shown that HIF is sufficient to induce EMT in other cell types (14, 39, 51). Recently, two articles (14, 39) reported that HIF induces gene expression of lysyl oxidase in human breast and ovarian cancer cells, which enhances Snail transcriptional activity to suppress E-cadherin, facilitating EMT. Thus these results suggest a cell-specific effect of HIF on EMT.

Previously, it has been reported that exogenous TGF-β1 induces EMT in AEC (25, 26, 49). We investigated whether TGF-β1 signaling participated in the hypoxia-induced EMT. We showed that hypoxia induced TGF-β1 gene expression in a time-dependent manner and increased levels of the active and total TGF-β1 in culture media (Fig. 5). Moreover, treatment of the TGF-β1 type I receptor kinase inhibitor SB431542 prevented hypoxia-induced EMT (Figs. 6 and 7), suggesting that hypoxia-induced EMT is dependent on TGF-β1 signaling.

Our results also suggest that hypoxia and exogenous TGF-β1 treatment induced comparable levels of α-SMA and vimentin (Fig. 6). However, the concentrations of active and total TGF-β1 in the media collected from cells exposed to hypoxia were <100 pg/ml (data not shown). This is much lower than the concentration of exogenous active TGF-β1 (5 ng/ml) required to induce EMT (25, 49), suggesting that additional intracellular signaling pathways may be involved in the cellular response to hypoxia.

ROS can stimulate the expression of TGF-β1 and its downstream genes in mammary and lens epithelial cells (16, 36). Moreover, ROS can oxidize latent associated peptide (LAP) and induce a conformation change, leading to the release of bioactive TGF-β1 from a latent complex with LAP (5, 16, 30, 34). Consistently, we found that hypoxia upregulated TGF-β1 and increased bioactive TGF-β1 in culture media (Fig. 5). Moreover, the ROS scavenger prevented the hypoxia-induced upregulation of TGF-β1 (Fig. 8). These results suggest that ROS is a key regulator of TGF-β1 gene expression and activation for hypoxia-induced EMT.

AEC express both HIF-1α and HIF-2α (46). We have shown that hypoxia-induced TGF-β1 production requires the presence of HIF-1α or -2α, since silencing of HIF-1α or -2α prevented the induction of TGF-β1 mRNA in AEC during hypoxia (Fig. 8). These results are consistent with previous reports showing that HIF regulates TGF-β1 (24, 42). Moreover, TGF-β3 promoter contains a HRE for HIF-1 association to increase transactivation of TGF-β3 gene (9, 32). Thus it is likely that HIF may directly upregulate TGF-β1 gene through a consensus HRE site.
Although our data indicate that ROS signaling and HIF are upstream of TGF-β1 production and that both ROS and HIF are required for the hypoxia-induced production of TGF-β1, accumulating evidence suggests that TGF-β1 also can increase ROS production and HIF stabilization. TGF-β1 can either upregulate gene expression of Nox4 NADPH oxidase (21, 43) or directly activate NADPH oxidase (31) to generate ROS. TGF-β1 decreases mitochondrial complex IV activity, resulting in disruption of mitochondrial membrane potential and ROS production (52). Moreover, TGF-β1 induces cytochrome P-450 1A1, generating the microsomer-derived ROS (2). Recently, TGF-β1 was reported to stabilize HIF-α through ROS generation (18) and by decreasing PHD2, a HIF-α prolyl hydroxylase, to reduce HIF-α prolyl hydroxylation (29). Thus TGF-β1 and ROS/HIF may form a feedback loop to maintain a prolonged signaling cascade initiated by either ROS/HIF or TGF-β1. We reason that during hypoxia, mitochondria generate relatively low-level ROS at the early onset of hypoxia to stabilize HIF, inducing TGF-β1 gene expression at a later stage. Elevated TGF-β1 levels sustain the ROS production, maintaining a prolonged ROS/HIF/TGF-β1 signaling.

EMT plays a fundamental role in tissue development and in pathological conditions such as cancer and tissue fibrosis (6, 19, 26, 33). Recent studies suggest that EMT may generate fibroblasts from the epithelial cells, thus contributing to the initiation and/or progression of fibrosis (19, 26). Although hypoxia promotes development of renal fibrosis partly by EMT (19), it is unknown whether hypoxia-induced EMT contributes to pulmonary fibrosis. Our findings suggest a functional significance of hypoxia-induced epithelial plasticity in fibrogenesis in the lung. Moreover, our studies highlight the functional importance of ROS and HIF in hypoxia-induced EMT, suggesting that ROS and HIF are critical in development of pulmonary fibrosis. A recent study indicated that the hyperplastic epithelium of fibrotic lungs overexpressed HIF-1α and its target genes, p53 and VEGF (45). Thus our study suggests that ROS and/or HIF may be promising targets for therapeutic agents to treat pulmonary fibrosis.

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REFERENCES


