Role of 12-lipoxygenase in hypoxia-induced rat pulmonary artery smooth muscle cell proliferation

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Pulmonary hypertension occurs in animals and humans exposed to acute or sustained hypoxia (26), and this is a well-established in vivo experimental model of pulmonary hypertension. The initial event is acute hypoxic pulmonary vasoconstriction, followed by remodeling of small and medium-sized pulmonary arteries (46). Muscular intrapulmonary arteries become further muscularized, and distal, nonmuscular arteries become newly muscularized (26). Pulmonary artery smooth muscle cell (PASMC) proliferation is a key feature of pulmonary vascular remodeling. The mechanism of PASMC proliferation is thought to involve an imbalance between mediators of apoptosis and cell proliferation.

Local paracrine mediators induced by hypoxia may also participate in the proliferative response. Among these local mediators, metabolites of arachidonic acid derived via the lipoxygenase pathway are known to exert vascular effects. In particular, the leukocyte-type 12-lipoxygenase (12-LO) pathway has been shown to stimulate cell proliferation and participate in inflammatory reactions in aortic smooth muscle cells (SMCs) (4, 27, 29–32, 35, 36) and in migration and metastasis of various cancer cell lines (6, 11, 18, 23, 24, 28, 38, 39, 41, 42, 44, 45). In addition, the 12-LO product, 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), has a direct hypertrophic effect on systemic vascular SMCs (29) and mediates hypertrophic effects of angiotensin II (29) and chemotactic effects of platelet-derived growth factor (27).

The 12-LO pathway has also been implicated in the proliferative response of vascular SMCs in vivo. 12-LO gene expression is enhanced in SMCs of balloon-injured rat carotid arteries and 12-LO inhibition by ribozyme-mediated cleavage of the 12-LO mRNA significantly reduces neointimal proliferation in this model (17).

Despite the evidence that 12-LO and its products contribute to SMC proliferation in the systemic vasculature, no studies have yet addressed the role of 12-LO on SMC proliferation in the pulmonary circulation exposed to hypoxia. We hypothesized that hypoxia stimulates PASMC proliferation through a mechanism that involves the 12-LO pathway. We sought to study the effects of hypoxia on PASMCs obtained from adult rats and to determine if the 12-LO pathway participates in this process.

MATERIALS AND METHODS

Materials. Dulbecco’s modified Eagle’s medium (DMEM) was from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Antibodies to ERK and phospho-ERK were from Cell Signaling (Beverly, MA). 12(S)-HETE- and secondary horseradish peroxidase-conjugated rabbit anti-mouse antibodies were from Cell Signaling (Beverly, MA). 12(S)-HETE- and secondary horseradish peroxidase-conjugated rabbit anti-mouse antibodies were from Cell Signaling (Beverly, MA). [3H]thymidine was purchased from Perkin-Elmer (Boston, MA), and the PCR kit was purchased from Qiagen (Santa Clarita, CA).

Exposure of animals to chronic hypoxia. Animal protocol was approved by the Institutional Animal Care and Use Committee at Tufts-New England Medical Center. Adult male Sprague-Dawley rats were randomized to 3 wk of normoxia or hypobaric hypoxia (0.5 atm).

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Hypobaric chambers were briefly opened three times weekly for cleaning and to replace food and water. Twelve-fourteen light exposure cycles, standard rat chow, and water ad libitum were provided to all rats. Normoxic rats were kept in the same room adjacent to the hypobaric chamber. At the end of the 3-wk exposure period, rats were anesthetized, and hemodynamic measurements were obtained as previously described (22). After killing the rats by pentobarbital injection (120 mg/kg ip), we immediately opened the thorax and removed the heart and lungs. The hearts were dissected into right ventricle (RV) free wall and left ventricle plus septum (LV+S), and the ratio of RV/(LV+S) was used as an index of RV hypertrophy. The left lung was frozen in liquid nitrogen and stored at −80°C for further measurements. Double fixation of the right lung was achieved in the distended state by infusion of 4% aqueous buffered formalin into the trachea at 25 cmH2O pressure and into the pulmonary trunk at 5 cmH2O pressure. The right lung was then processed for paraffin embedding. Sections of 5 μm were immunostained with the leucocyte-type 12-LO antibody and counterstained with hematoxylin-eosin. Vessels with a layer of smooth muscle cells comprising ≥½ of the vessel perimeter were categorized as fully muscularized. Vessels with a smooth muscle layer comprising <½ of the vessel perimeter were categorized as partially muscularized, and vessels that had no identifiable smooth muscle cells were categorized as nonmuscularized vessels.

Immunohistochemical detection of leucocyte-type 12-LO in rat lungs. The immunohistochemical methods were carried out by the technique described previously (31). In brief, 5-μm paraffin-embedded tissue sections were mounted on Probe-on slides (Biotek Solutions) and dried for 1 h in a 56°C oven and overnight at 45–50°C, deparaffinized in xylene, and rehydrated in graduated alcohol to distilled water. The slides were loaded into a Techmate Slide holder and placed into 0.1 mol/l citrate buffer solution for heat-induced epitope retrieval. The slides were steamed in 0.1 M citrate buffer for 10 min and then placed into 0.1 mol/l citrate buffer solution for heat-induced epitope retrieval. The slides were steamed in 0.1 M citrate buffer for 20 min and then allowed to cool for 5 min. After treatment with the first and second antibody, slides were stained by a modified avidin-biotin complex (ABC) technique using 3,3′-diaminobenzidine tetrahydrochloride as the chromogen and counterstained with Mayer’s hematoxylin. Staining was performed using a Birotek Techmate 1000 Immunostainer (Techmate, Santa Barbara, CA) with Biotek solutions and an ABC detection system (Techmate). We used the 12-LO antibody at 1:500 concentration. Parallel controls were run without primary antibody.

Isolation and culture of PASMCS. For isolation of PASMCS, freshly excised lobar pulmonary arteries obtained from adult male Sprague-Dawley rats were stripped of adventitia. Vascular segments were then cut open, and endothelium was removed by gentle scraping of the luminal surface of the vessel. Rat PASMCS were cultured from explants as previously described (1, 9). SMC phenotype was assessed by the hill-and-valley morphology (1). Cells were grown in DMEM supplemented with 10% FBS, penicillin (100 units/ml), streptomycin (100 units/ml), and fungizone (1.25 μg/ml) and were passaged every 1–2 wk at a 1:3 ratio with trypsin. Passage 3–8 cells at 80% confluence were used in all reported experiments. Before exposure to hypoxia or treatment with different agents, cells were starved in 0.1% FBS DMEM with antibiotics for 72 h. Cells were exposed to hypoxia or treated with different agents in 0.1% FBS DMEM with antibiotics, unless otherwise specified. Medium was changed every 2–3 days.

Exposure of PASMCS to hypoxia. PASMCS were exposed to 3% hypoxia in a humidified modular incubator chamber (Billups-Rothenberg, Del Mar, CA) that was maintained at 37°C. The incubator chamber was sealed and purged with 3% O2, 5% CO2, balance N2 for 15 min. Normoxic control PASMCS were exposed to 95% ambient air-5% CO2 for the entire incubation period. A portable gas analyzer (Hudson Ventronics Division) was used to ensure that the O2 concentration inside the chamber was 3%. Hypoxia had no effect on the pH of the medium, which was maintained at 7.36 ± 0.3.

[^3H]thymidine incorporation. PASMCS were plated in complete growth media containing 10% FBS in 96-well plates at a density of 4 × 10^4 cells/cm². After plates were 80% confluent, cells were starved in 0.1% FBS for 72 h. Cells were then either exposed to hypoxia or normoxia or treated with different agents 30 min before the beginning of the hypoxia exposure. Twenty-four hours before the end of the experiment, [^3H]thymidine (0.1 mCi/ml) was added. We stopped incorporation of labeled thymidine by aspirating the medium and trypsinizing the cells for 15 min at 37°C, after which cells were transferred onto a filter membrane with a cell harvester. Radioactivity was counted in a microplate liquid scintillation counter (Perkin-Elmer) after 20 μl of scintillation liquid were added to each well.

Cell proliferation. PASMCS were plated at 2 × 10^4 cells/well in six-well plates in DMEM and 10% FBS with antibiotics, growth arrested for 72 h, and then exposed to normoxia or hypoxia for various times. Cells were removed from the wells by trypsin digestion. Cells were counted by a Fischer hemocytometer (Pittsburgh, PA). In each of the three experiments, the results from three wells were averaged to obtain a single cell count (± SD) for each time point.

Western blot analysis. Protein determinations from both lung homogenates and cell lysates were made by the Bradford method (3). To prepare lysates, cells were washed in phosphate-buffered saline and solubilized with 50 mM HEPES solution (pH 7.4) containing 1% (vol/vol) Triton X-100, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM tetrasodium pyrophosphate, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Cell lysates (20 μg of protein) were electrophoresed through 12% SDS-polyacrylamide gel and electroblotted onto a polyvinylidene difluoride membrane. The membrane was blocked and incubated with primary antibodies for phospho-specific ERK1/2, ERK, phospho-specific p38 MAPK, p38 MAPK, or 12-LO. The polyclonal antibody against amino acids 646–662 peptide sequence of porcine leukocyte 12-LO protein was raised in rabbits and has been shown to have excellent cross-reactivity with rat 12-LO (49). The levels of proteins and phosphoproteins were detected with horseradish peroxide-linked secondary antibodies and the ECL System (Amer sham Biosciences).

RT-PCR for 12-LO. Lungs were homogenized and total RNA was extracted from the lung tissues by using the RNeasy Total RNA Isolation Kit (Qiagen, Valencia, CA). The PCR method is specific for the leucocyte-type 12-LO and does not cross-react with platelet-type 12-LO (17), which is the product of a separate, linked gene. The primers for the rat 12-LO gene were: sense, 5′-TGG GCC AAC TGG AAG G-3′, located at the 324–339 position of rat 12-LO gene, and antisense, 5′-AGA GCG CTT CAG CAC CAT-3′ (2), located at the 718–735 position. PCR reaction was carried out under the following conditions: 38 cycles of denaturation at 94°C for 45 s, annealing at 68°C for 60 s, and extension at 72°C for 60 s. A final extension was performed at 72°C for 10 min. PCR products were separated by 3% polyacrylamide gel electrophoresis in Tri-borate-EDTA buffer. For internal control, we performed RT-PCR, then PCR for tubulin.

Statistical analysis. Data are expressed as means ± SD. Statistical analysis was performed by the unpaired Student’s t-test or analysis of variance, as indicated. Differences were considered to be significant when P < 0.05.

RESULTS

12-LO mRNA and protein expression is increased in hypoxic rat lungs. Adult rats exposed to 3 wk of hypobaric hypoxia (0.5 atm) developed pulmonary hypertension, as demonstrated by an increase in systolic pulmonary artery pressures (27.5 ± 0.8 mmHg in normoxic rats and 42 ± 2 mmHg in hypoxic rats, P < 0.05) and RV hypertrophy (RV/LV+S was 0.28 ± 0.005 in normoxic rats and 0.46 ± 0.02 in hypoxic rats, P < 0.05, n = 5 animals per group). Lung homogenates from these
animals showed increases in both 12-LO mRNA (Fig. 1A) and protein (Fig. 1, B and C) compared with homogenates from normoxic controls. Histological examination (hematoxylin-eosin staining) revealed thickening of the small pulmonary arterioles and no evidence of inflammation (Fig. 2).

**Evidence of increased 12-LO expression in hypoxic rat lung.** In normoxic rats, medium-size and small pulmonary arteries were nonmuscularized (Fig. 2C). After 3 wk of exposure to hypoxia, many of these arteries became either partially or fully muscularized (Fig. 2F). Immunohistochemistry of rat lungs showed intense 12-LO staining of bronchial epithelial cells and alveolar macrophages that remained unchanged with hypoxia (Fig. 2, A, C, D, and F). In normoxic lungs, there was no 12-LO staining of the pulmonary vasculature (Fig. 2, B and C). In contrast, there was intense 12-LO immunostaining of endothelial cells of large pulmonary arteries (Fig. 2, D and E), SMCs (and possibly endothelial cells) of small pulmonary arterioles, and alveolar pneumocytes of hypoxic lungs (Fig. 2F).

**Hypoxia stimulates PASMC proliferation.** To provide an in vitro correlate for the hypoxia-induced medial hypertrophy of pulmonary arteries seen in vivo, we determined whether cultured SMCs proliferate during exposure to hypoxia. PASMCs were grown arrested in 0.1% FBS before being exposed to hypoxia or normoxia with or without 10% serum. Hypoxia stimulated DNA synthesis, as assessed by incorporation of [3H]thymidine into cells in both 0.1 and 10% serum (Fig. 3, A and B). The proliferative effect of hypoxia was validated by demonstrating an increase in cell number in hypoxic compared with control SMCs (see Fig. 3C for representative experiment).

**12-LO is upregulated in hypoxic rat PASMCs in culture.** Because PASMCs are believed to participate in the pulmonary vascular response to hypoxia, we next evaluated the influence of hypoxia on 12-LO protein expression in vitro. Hypoxia upregulated 12-LO protein expression in PASMCs at 2 h, and this was maintained at 48 h (Fig. 4, A and B).

**12(S)-HETE stimulates PASMC proliferation through activation of ERK.** Having established that hypoxia enhances 12-LO expression and stimulates SMC proliferation, we next sought to evaluate the effects of the 12-LO product, 12(S)-HETE, on SMC proliferation. Figure 5A shows that 12(S)-HETE directly stimulates PASMC proliferation at concentrations as low as $10^{-5}$ μM. In contrast, 15-HETE, a product resulting from arachidonic acid metabolism by 15-LO, did not have a proliferative effect. In addition, 12(S)-HETE-induced PASMC proliferation was completely blocked by PD-98059, a specific MEK inhibitor (an upstream factor of ERK) (Fig. 5B). These results suggest that ERK and 12-LO are linked in the proliferative pathway and that 12-LO upregulation and 12(S)-HETE formation precede activation of ERK.

**12(S)-HETE stimulates ERK activation.** To establish whether ERK is downstream of 12(S)-HETE, we measured ERK activation in PASMCs stimulated with 12(S)-HETE. Figure 6, A and B, demonstrates that 12(S)-HETE directly activates the ERK pathway by inducing phosphorylation of ERK1/ERK2. This is a biphasic action with peaks at 10 and 90 min. Another signaling pathway that has been shown to be activated during cell proliferation is p38 MAPK (10, 13). We found that 12(S)-HETE did not induce p38 MAPK phosphorylation (Fig. 6, C and D).

**Hypoxia-induced PASMC proliferation is blocked by the MEK-1 inhibitor PD-98059.** To determine the cellular pathways that may be activated during cell exposure to hypoxia, we evaluated the MAPK signaling pathway, in particular ERK1/2 MAPK and p38 MAPK activation. PASMCs were exposed to 72 h of hypoxia (3% O2) in the presence or absence of a specific MEK inhibitor, U0126, as well as in the presence or absence of a specific p38 MAPK inhibitor, SB-20390. Figure 7 demonstrates inhibition of hypoxia-stimulated cell proliferation by the MEK inhibitor and lack of effect by the p38 MAPK inhibitor.

**Hypoxia-induced PASMC proliferation is blocked by 12-LO inhibition.** A partial blockade of hypoxia-stimulated SMC proliferation was obtained with the 12-LO inhibitor, baicalein (Fig. 8). Studies using trypan blue exclusion demonstrated that concentrations of baicalein from 0.5 to 10 μM had no effect on cell viability (data not shown).

**DISCUSSION**

It is now generally accepted that hypoxia induces pulmonary hypertension that is associated with pulmonary vascular remodeling and, in particular, SMC hyperplasia and hypertrophy. A large number of intermediate cell signaling pathways by which this occurs have been proposed (1, 7–9, 14, 15, 19, 20, 22). The present study provides evidence that the 12-LO pathway via its metabolite, 12(S)-HETE, contributes to hypoxia-induced PASMC remodeling. We found upregulation of 12-LO mRNA and protein expression in lung homogenates of rats exposed to chronic hypoxia and that 12(S)-HETE, but not 15-HETE, stimulates SMC proliferation in vitro. We also
found increased 12-LO immunostaining of pulmonary arteries and pneumocytes of hypoxic, but not normoxic, rat lungs. Furthermore, we substantiated the contribution of the 12-LO pathway to hypoxia-induced pulmonary vascular SMC proliferation by demonstrating that inhibition of 12-LO blunts the proliferative response.

The lipoxygenases comprise a family of nonheme iron-containing dioxygenases that catalyze the stereospecific oxygenation of the 5-, 12-, or 15-carbon atoms of arachidonic acid. There are four functionally distinct 12-LOs that vary in cell and tissue distribution and catalytic activity, and each is a product of linked, but separate genes. These consist of platelet-type 12-LO, leukocyte-type 12-LO, epidermal-type 12-LO (25), and 12(R)-LO. The 12-LO isoforms and their metabolite 12(S)-HETE have been described in a variety of rat, mouse, and human tissues (37). Although macrophages have the capacity to synthesize the leukocyte-type 12-LO in rat lungs (5), we do not believe that macrophages are the source of the increased 12-LO in our in vivo rat model because histological studies did not reveal an inflammatory pattern (see Fig. 2), although this does not exclude the possibility.

12-LO products such as 12(S)-HETE have been implicated in tumor cell proliferation, particularly that of prostatic, pancreatic, and breast cancers (11, 12, 23, 28). At least some of these effects may be due to inhibition of apoptosis (41). No cellular receptor for 12(S)-HETE has yet been identified, but some potential pathways by which it may stimulate cellular activities have been explored. For example, the mechanisms implicated in tumor invasion and metastasis that are due, at least in part, to generation of 12(S)-HETE, are believed to involve activation of MAPK signaling pathways, in particular the ERK pathway (11, 39, 40, 44). In addition, ERK activation has been demonstrated in the normal growth of mesangial cells in association with the stimulation of the 12-LO pathway (34) and in systemic vascular SMCs stimulated by arachidonic acid and its metabolites (33).

Although we believe that ours is the first study exploring the role of 12-LO and 12(S)-HETE in pulmonary vascular SMC proliferation, previous studies have explored the influence of 12-LO and its product, 12(S)-HETE, on systemic vascular cells. 12(S)-HETE is a known mitogenic factor for microvascular endothelial cells (43) and systemic SMCs (29). In addition, 12-LO participates in angiotensin II-induced vascular SMC hypertrophy (29, 30), and 15-LO has been shown to activate MAPK in systemic vascular SMCs (33). The previously reported proliferative effects of 12(S)-HETE on systemic SMCs are consistent with our results demonstrating that 12(S)-HETE has a direct stimulatory effect on PASMC growth. This
effect, similar to the hypoxic stimulus, seems to involve ERK MAPK activation, as evidenced by the increased phosphorylation of ERK that we observed during 12(S)-HETE treatment. What seems to be unique for the PASMCs is the biphasic activation of ERK, which has not been reported in other cell systems. This possibly leads to a positive feedback mechanism involving activation of the ERK pathway at multiple steps. Furthermore, the proliferative effect of both hypoxia and...
12(S)-HETE on PASMCs was completely blocked by the ERK inhibitor, PD-98059, suggesting that the ERK pathway is critical in 12(S)-HETE-induced PASMC proliferation.

Although 15-HETE and 12(S)-HETE have similar effects in tumor cells, stimulating tumor invasion and metastasis, we found that, unlike 12(S)-HETE, 15-HETE exerts no proliferative effects in PASMCs. This indicates that some actions of the various lipoxygenases are cell specific.

Prior studies have observed activation of lipoxygenases in the lungs of animals exposed to hypoxia, but these focused on pulmonary vasoconstriction and examined gene regulation only to a limited extent. For example, Zhu et al. (52) found activation of 15-LO in the lungs of neonatal rabbits exposed to hypoxia and enhanced constriction of pulmonary vascular rings from these animals to 15-HETE. These data suggest that different lipoxygenases may play different roles in various species.

The effects of hypoxia on proliferation of cultured PASMCs has been less extensively studied than those on pulmonary vessels in intact experimental models, and results have been less consistent. Whereas fibroblasts from pulmonary arteries consistently proliferate in response to hypoxia (48), SMCs behave differently. For example, bovine pulmonary arteries have two distinct populations of cells, only one of which proliferates in hypoxia (16). On the other hand, hypoxia stimulates proliferation of cultured human PASMCs, even in the absence of serum (50). In addition, when combined with serum, moderate hypoxia (5% O2) synergistically stimulates human PASMC growth (9). Consistent with human PASMCs, the rat PASMCs isolated from lobar pulmonary arteries in our study proliferated in response to hypoxic exposure (3% O2) and manifested synergy in response to the combination of hypoxia and serum.

Prior studies on pulmonary arteries from chronically hypoxic rats have shown increases in JNK, ERK, and p38 MAPK activities, indicating involvement of MAPKs in hypoxia-induced remodeling (19). Hypoxia also increases phosphorylated...
MAPK immunostaining in both large and small intrapulmonary arteries (19). Our finding that an MEK inhibitor blocks hypoxia-stimulated PASMC growth supports the idea that MAPKs are involved in the response. On the other hand, p38 MAPK does not appear to be involved, as suggested by the lack of a change in expression of phosphorylated p38 and lack of effect of a specific p38 MAPK inhibitor on PASMC proliferation. In contrast, p38 MAPK is activated in hypoxic cultured pulmonary artery endothelial cells (21), suggesting that signaling pathways responding to hypoxic exposure are cell specific.

Limitations of our study include the fact that our focus was on the role of 12-LO in the pulmonary vasculature during hypoxia, and we did not examine the roles of other lipoxygenases, such as 5-LO or 15-LO. The 5-LO pathway appears to participate in the development of hypoxia-induced pulmonary hypertension, because inhibition of 5-LO-activating protein (FLAP) reduces pulmonary vascular reactivity and pulmonary hypertension in hypoxic rats (47), with the most intense FLAP immunoreactivity in pulmonary arterial endothelial cells. Also, chronic hypoxia activates 15-LO in neonatal rabbit lungs, with increased 15-LO being found in both pulmonary arterial endothelial cells and SMCs (52). Thus it is possible that an array of lipoxygenases is increased by hypoxia and each of them has different cellular effects on different cell types. It is also possible that the type of lipoxygenase that participates in hypoxia is species specific, since rabbit 15-LO has 70% homology with rat 12-LO (51).

We conclude that the 12-LO pathway and its metabolic product, 12(S)-HETE, participate in hypoxia-induced PASMC proliferation in vitro, most likely via the ERK1/ERK2 MAPK pathway and apparently without the involvement of p38 MAPK. We speculate that the 12-LO pathway also contributes to the development of hypoxia-induced pulmonary hypertension in vivo, but further studies will be necessary to substantiate this.

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REFERENCES
12-LO AND PULMONARY HYPERTENSION


