Introduction

Pulmonary Hypertension (PH) is a progressive disorder characterized by endothelial dysfunction and proliferation. Hypoxia induces PH by increasing vascular remodeling. A potential mediator in hypoxia-induced PH development is arachidonate 5-Lipoxygenase (ALOX5). While ALOX5 metabolites have been shown to promote pulmonary vasoconstriction and endothelial cell proliferation, the contribution of ALOX5 to hypoxia-induced proliferation remains unknown. We hypothesize that hypoxia exposure stimulates HPAEC proliferation by increasing ALOX5 expression and activity. To test this, human pulmonary artery endothelial cells (HPAEC) were cultured under normoxic (21% O2) or hypoxic (1% O2) conditions for 24-, 48-, or 72 hours. In a subset of cells, the ALOX5 inhibitor, zileuton, or the 5-lipoxygenase activating protein inhibitor, MK-886, was administered during hypoxia exposure. ALOX5 expression was measured by qRT-PCR and western blot and HPAEC proliferation was assessed. Our results demonstrate that 24 and 48 hours of hypoxia exposure have no effect on HPAEC proliferation or ALOX5 expression. Seventy two hours of hypoxia significantly increases HPAEC ALOX5 expression, hydrogen peroxide (H2O2) release, and HPAEC proliferation. We also demonstrate that targeted ALOX5 gene silencing or inhibition of the ALOX5 pathway by pharmacological blockade attenuates hypoxia-induced HPAEC proliferation. Furthermore, our findings indicate that hypoxia-induced increases in cell proliferation and ALOX5 expression are dependent on H2O2 production, as administration of the antioxidant PEG-catalase blocks these effects and addition of H2O2 to HPAEC promotes proliferation. Overall, these studies indicate that hypoxia exposure induces HPAEC proliferation by activating the ALOX5 pathway via the generation of H2O2.

Abstract

Pulmonary Hypertension (PH) is a progressive disorder characterized by endothelial dysfunction and proliferation. Hypoxia induces PH by increasing vascular remodeling. A potential mediator in hypoxia-induced PH development is arachidonate 5-Lipoxygenase (ALOX5). While ALOX5 metabolites have been shown to promote pulmonary vasoconstriction and endothelial cell proliferation, the contribution of ALOX5 to hypoxia-induced proliferation remains unknown. We hypothesize that hypoxia exposure stimulates HPAEC proliferation by increasing ALOX5 expression and activity. To test this, human pulmonary artery endothelial cells (HPAEC) were cultured under normoxic (21% O2) or hypoxic (1% O2) conditions for 24-, 48-, or 72 hours. In a subset of cells, the ALOX5 inhibitor, zileuton, or the 5-lipoxygenase activating protein inhibitor, MK-886, was administered during hypoxia exposure. ALOX5 expression was measured by qRT-PCR and western blot and HPAEC proliferation was assessed. Our results demonstrate that 24 and 48 hours of hypoxia exposure have no effect on HPAEC proliferation or ALOX5 expression. Seventy two hours of hypoxia significantly increases HPAEC ALOX5 expression, hydrogen peroxide (H2O2) release, and HPAEC proliferation. We also demonstrate that targeted ALOX5 gene silencing or inhibition of the ALOX5 pathway by pharmacological blockade attenuates hypoxia-induced HPAEC proliferation. Furthermore, our findings indicate that hypoxia-induced increases in cell proliferation and ALOX5 expression are dependent on H2O2 production, as administration of the antioxidant PEG-catalase blocks these effects and addition of H2O2 to HPAEC promotes proliferation. Overall, these studies indicate that hypoxia exposure induces HPAEC proliferation by activating the ALOX5 pathway via the generation of H2O2.

Materials and Methods

Reagents

Trypan blue, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), PEG-Catalase, and gelatin were obtained from Sigma-
Aldrich (St. Louis, MO). Zileuton was obtained from Patheon Pharmaceuticals (Cincinnati, OH). MK-886 was purchased from Calbiochem (San Diego, CA). Scrambled and silencing RNA (siRNA) for ALOX5 was obtained from Qiagen (Valencia, CA).

Cell Culture
Human pulmonary artery endothelial cells (HPAEC) were obtained from Lonza Clonetics (Walkersville, MD). HPAEC were grown in EGM-2 medium (Lonza), which contains basic growth medium (EBM-2), fetal bovine serum (FBS), and antibiotics, ascorbic acid, vascular endothelial growth factor (VEGF), human fibroblast growth factor (hFGF-B), hydrocortisone, human epidermal growth factor (hEGF), R3-IGF-1 (insulin-like growth factor), GA-1000 (gentamicin, amphotericin B), and heparin. Unless otherwise stated, cells were maintained in a 37°C incubator at 5% CO2.

Hypoxia Exposure
HPAEC, passages 3-8, were exposed to hypoxia in a Biospherix exposure chamber (Lacona, NY) as previously described [18,19]. For normoxic conditions, HPAEC were placed into a standard incubator maintained at 37°C and 5% CO2 levels. For hypoxic conditions, HPAEC were placed in a hypoxia chamber maintained at 37°C, 1% oxygen, and 5% CO2 levels. Human pulmonary artery endothelial cells (HPAEC) were cultured in normoxic or hypoxic conditions for 24-, 48-, or 72 hours. To investigate the contribution of ROS levels, PEG-Catalase (Life Technologies) was administered during the final 24 hours of the 72 hours of hypoxia exposure. Hydrogen Peroxide (H2O2) release was quantified using the Amplex Red Assay. Cells were incubated in a solution containing the Amplex Red reagent (Molecular Probes), horseradish peroxidase (HRP, EMD Chemicals, Gibco), 10 μM H2O2, and 100 μg/mL PEG-Catalase. H2O2 was normalized to cellular protein concentration. Overall statistical analysis was performed using one-way ANOVA with Tukey’s posttest was used for the comparison of multiple groups. All experiments using cell cultures were repeated at least twice on different cell lines, and samples were run in duplicate or triplicate. Statistical significance was defined as P < 0.05, and all graphs are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism software (La Jolla, CA).

Results
Prolonged Hypoxia Exposure Promotes Endothelial Cell Proliferation
Hypoxia is associated with significant endothelial alterations which are thought to contribute to PH development and progression [3,20]. Pulmonary arteries [8] and lung sections of PH patients demonstrate abnormal endothelial cell growth [5,6]. To determine whether hypoxia alters HPAEC function in vivo, we...
Figure 1. Prolonged hypoxia exposure increases endothelial cell proliferation. Seventy two hours of hypoxia exposure significantly stimulates endothelial cell proliferation when compared to all other groups. Human pulmonary artery endothelial cells (HPAEC) were exposed to normoxic or hypoxic (1% O2) conditions for 24-, 48-, or 72 hours (n = 4). Following exposure, cell proliferation was assessed by MTT (Figure 1A) assay and Trypan Blue Dye Exclusion Assay (Figure 1B). ∗ p < 0.0001.

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Figure 2. Chronic hypoxia exposure increases endothelial ALOX5 expression. Seventy two hours of hypoxia exposure significantly stimulates endothelial ALOX5 expression when compared to all other groups. HPAEC were exposed to normoxic or hypoxic conditions for 24-, 48-, or 72 hours. Following exposure, cells were collected, and total RNA and protein were isolated for expression analyses via quantitative real time PCR and Western blot respectively. Results indicate that ALOX5 mRNA levels are significantly increased following hypoxia exposure (A, n = 5). Chronic hypoxia exposure also causes a 3-fold elevation in ALOX5 protein expression levels (B, n = 4). Endothelial FLAP expression is also increased when compared to all other groups (C, n = 5–7). Values are expressed as percent of control. ∗p < 0.001 when compared to all other groups.

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assessed HPAEC proliferation following 24, 48, and 72 hours of hypoxia exposure. While 24 and 48 hours of hypoxia exposure had no effect on cellular proliferation, 72 hours of hypoxia increases cellular proliferation when measured by MTT assay (Figure 1A) and Trypan Blue Dye Exclusion Assay (Figure 1B).

Chronic Hypoxia Exposure Stimulates HPAEC 5-Lipoxygenase Expression

In vivo studies demonstrate that both hypoxia exposure and MCT administration upregulate 5-lipoxygenase (ALOX5) [9,21]. To specifically investigate the effect of hypoxia on pulmonary artery endothelial cell ALOX5, HPAEC were exposed to hypoxia for 24-, 48-, or 72-hours. Our results indicate that 72 hours of hypoxia exposure significantly increases HPAEC ALOX5 mRNA (Figure 2A) when analyzed by qRT-PCR and ALOX5 protein expression (Figure 2B) as measured by western blot. Prolonged hypoxia exposure also stimulates a significant increase in the expression of the ALOX5 required cofactor, FLAP when measured by qRT-PCR (Figure 2C).

ALOX5 Gene Silencing Reduces Pulmonary Endothelial Proliferation Following Prolonged Hypoxia Exposure

Recent studies demonstrate that ALOX5 contributes to HPAEC proliferation [11]. Additionally, concomitant increases in ALOX5 and HPAEC proliferation following hypoxia exposure suggest a potential association between ALOX5 activity and hypoxia-induced endothelial proliferation. To determine whether ALOX5 mediates hypoxia-induced endothelial proliferation, human pulmonary artery endothelial cells (HPAEC) were transfected with control (scrambled) or ALOX5 silencing RNA. Transfection with ALOX5 siRNA reduces ALOX5 protein expression when compared to normoxic and hypoxic controls (Figure 3A). Densitometry reveals that ALOX5 siRNA transfection decreases hypoxia-induced ALOX5 protein levels by greater than 50% when compared to untreated hypoxic groups (Figure 3A). Following transfection, control- and siALOX5 human pulmonary artery endothelial cells were placed in normoxic or hypoxic conditions for 72 hours then collected for cell proliferation analyses. Transfection with ALOX5 siRNA significantly decreases hypoxia-induced HPAEC proliferation when compared to hypoxia scrambled-controls as measured by trypan blue dye exclusion assay (Figure 3B). These results indicate that ALOX5 is a significant contributor to hypoxia-induced increases in endothelial cell proliferation.

Inhibition of ALOX5 Activity Attenuates Hypoxia-Induced Endothelial Proliferation

We next sought to determine whether alterations in ALOX5 activity contribute to hypoxia-induced endothelial proliferation. To confirm that ALOX5 promotes hypoxia-induced HPAEC proliferation, we measured cellular proliferation in response to hypoxia in the presence or absence of the ALOX5 inhibitor, zileuton. Inhibition of ALOX5 enzyme activity by zileuton administration during the final 24 hours of hypoxia exposure attenuates hypoxia-induced increases in endothelial proliferation (Figure 4A). Similarly, inhibition of the ALOX5 cofactor, FLAP by MK-886 reduces endothelial cell proliferation following hypoxia exposure (Figure 4B). Moreover, pre-treatment with the cysteinyl leukotriene receptor antagonist, montelukast prevents increases in endothelial proliferation during chronic hypoxia exposure (Figure 4C). These data demonstrate that ALOX5 and its LT metabolites mediate hypoxia-induced endothelial proliferation. Additionally, alterations in endothelial cell proliferation caused by hypoxia exposure are in part dependent on activation of CysLT receptors.

Hypoxia Promotes Endothelial ROS Release

Previous studies demonstrate that hypoxia promotes ROS generation in PH models [22–25]. To investigate whether hypoxia exposure increases endothelial ROS release, ROS release was
analyzed following 24, 48, and 72-hours of hypoxia exposure. DCF staining indicates that chronic hypoxia exposure increases ROS production in endothelial cells (Figure 5A). This increase in ROS release was attenuated by the administration of PEG-catalase and superoxide dismutase (Figure 5B). Furthermore, Amplex Red assay analysis of hydrogen peroxide (H₂O₂) release also demonstrates that 72 hours of hypoxia exposure is required for increased H₂O₂ (Figure 5C). These data suggest that chronic exposure to hypoxic conditions increases endothelial H₂O₂.

H₂O₂ Mediates Hypoxia-Induced Increases in HPAEC ALOX5 Expression and Cell Proliferation

Since elevated ROS production is associated with increased ALOX5 activity [14,15,17], we next sought to determine whether H₂O₂ mediates hypoxia-induced increases in endothelial ALOX5. HPAECs exposed to 0–200 μM concentrations of H₂O₂ for 24 hours revealed that 100 μM and 200 μM H₂O₂ concentrations increase endothelial ALOX5 mRNA levels (Figure 6A). Similarly, H₂O₂ exposure stimulates ALOX5 protein expression when assessed by Western blot (Figure 6B). These data indicate that ALOX5 expression levels are altered by increased ROS. To determine whether hypoxia-induced ROS mediate alterations in HPAEC ALOX5 levels and cell proliferation, we administered the antioxidant, PEG-catalase during the final 24 hours of the 72-hour hypoxia exposure period and determined ALOX5 mRNA levels by qRT-PCR and cell proliferation by MTT Assay. Results demonstrate that catalase administration significantly reduces hypoxia-induced elevations in endothelial ALOX5 gene expression (Figure 6C). Catalase treatment during the final 24 hours of the 72-hour hypoxia exposure also prevents HPAEC proliferation (Figure 6D) in a concentration-dependent manner when compared to untreated hypoxic groups.

Discussion

Our studies indicate that hypoxia exposure promotes pulmonary artery endothelial proliferation by stimulating key catalytic molecules in the LT pathway, ALOX5 and its required co-factor, FLAP. We also demonstrate that hypoxia mediates these events by increasing ROS particularly H₂O₂. Overall, these studies suggest that ALOX5 contributes to hypoxia-induced endothelial proliferation in a ROS-dependent manner.
In this study, we demonstrate that prolonged hypoxia exposure significantly increases ALOX5 expression when compared to normoxic controls. Although ALOX5 was originally believed to be expressed only in myeloid cells, previous studies confirm ALOX5 expression in pulmonary artery endothelial cells [26]. Additionally, our data demonstrating hypoxia-induced elevations in HPAEC ALOX5 expression correspond with previous studies performed using rat lung homogenates and pulmonary arteries [27]. Furthermore, endothelial cells are shown to produce leukotrienes in nitric oxide-deficient and hypertensive conditions [28,29]. These data demonstrate that endothelial cells express ALOX5 and are able to produce leukotrienes in environments of excessive oxidative stress and vascular injury. The results from our study provide similar evidence as pre-treatment with the cysteinyll LT receptor antagonist, montelukast prevents hypoxia-induced increases in HPAEC proliferation. Our data also suggest that endothelial ALOX5 expression is redox-sensitive as ALOX5 expression levels are increased in response to exogenous H2O2 administration (100 μM). Although the physiological concentrations of H2O2 are unknown, the concentrations used [30–32] are consistent with previous studies. Additionally, our results coincide with previous work indicating that ROS release stimulates ALOX5 [17].

This study also reveals that prolonged hypoxia exposure stimulates endothelial cell proliferation. Although previous studies indicate that hypoxia failed to stimulate endothelial cell proliferation [33], several studies demonstrate that hypoxia exposure produces significant elevations in endothelial cell proliferation [18,34]. Though it is widely-accepted that hypoxia promotes pulmonary vascular cell proliferation, the underlying mechanism remains unclear. Endothelial cells contribute to hypoxic pulmonary remodeling by increasing the release of pro-proliferative mediators such as endothelin-1 and angiotensin II and reducing the release of anti-proliferative agents such as nitric oxide. Similarly, hypoxia stimulates cell proliferation by increasing the production of pro-proliferative stimuli from smooth muscle cells, platelets, fibroblasts, and endothelial cells [35–38]. Our data indicate that hypoxia increases endothelial cell proliferation by stimulating the ALOX5 pathway. Endothelial proliferation was reduced following the inhibition of the ALOX5 pathway by siRNA knockdown of ALOX5 as well as zileuton, MK-886, and montelukast administration. Zileuton inhibits ALOX5 by binding to the iron atom needed for catalytic function [39]. MK-886, however, inhibits leukotriene synthesis [40] by binding to the membrane-bound FLAP and preventing translocation of ALOX5 from the cytoplasm to the plasma membrane for activation. Furthermore, targeted reductions in ALOX5 using siRNA similarly inhibited hypoxia-induced proliferation. These data implicate a contributing role of leukotrienes in hypoxia-induced endothelial proliferation.

The results of the present study also indicate that ROS such as H2O2 contribute to the hypoxia-induced endothelial cell proliferation, as PEG-Catalase treatment prevents hypoxia-induced endothelial proliferation. Interestingly, our data demonstrate a prolonged exposure to hypoxia (72 hours) is needed to induce pulmonary endothelial cell proliferation, whereas 24- and 48-hours of hypoxia exposure do not significantly impact endothelial cell proliferation. It is plausible that accumulation of H2O2 is needed for the induction of cellular proliferation and our H2O2 measurements demonstrate a delay in hypoxia-induced increases in H2O2 (Figure 6C). Interestingly, bolus H2O2 administration promotes ALOX5 expression and HPAEC proliferation following only 24 hours. This discrepancy most likely results from the exogenous administration of H2O2, which surpasses the normal

Figure 5. Hypoxia exposure stimulates endothelial ROS release. Human pulmonary artery endothelial cells were exposed to normoxic or hypoxic (1% O2) conditions for 24-, 48- or 72-hours. Following exposure, HPAEC ROS release was assessed by DCF staining (A, n = 3) and Amplex Red assay (C, n = 4). Results demonstrate that prolonged hypoxia exposure significantly increases endothelial ROS production whereas administration with the antioxidants, PEG- catalase or superoxide dismutase reduces these effects (B). Amplex Red Assay indicates that chronic hypoxia exposure promotes H2O2 release (C). * p<0.0001 when compared to normoxic controls.

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cellular processes necessary for H$_2$O$_2$ production during hypoxia exposure. Moreover, bolus H$_2$O$_2$ administration may also stimulate ALOX5 expression and HPAEC proliferation through the activation of downstream signaling pathways. Recent reports indicate that H$_2$O$_2$ activates endothelial p38 MAPK [41,42] and NF-kappaB activity [43,44], two mediators associated with increases in ALOX5 expression and activity. Furthermore, the addition of PEG-catalase is shown to double endogenous endothelial catalase activity within 4 hours [45]. Taken together, these findings are consistent with previous reports demonstrating that the highly reactive oxidant, peroxynitrite stimulates pulmonary artery endothelial cell proliferation [46] and catalase inhibits cell proliferation [47]. The mechanism responsible for an ALOX5-mediated increase in proliferation is unclear however, it is possible that ALOX5 promotes cell proliferation via its nuclear localization or through interaction with cytoskeletal proteins [48,49]. Nonetheless, the excessive proliferation of pulmonary endothelial cells is thought to contribute to the obliteration of the pulmonary artery vessel lumen. Therefore, it is likely that hypoxia-induced pulmonary artery endothelial proliferation contributes to pulmonary hypertension (PH) development and progression.

Previous research demonstrates that chronic hypoxia significantly increases endothelial cell proliferation. In vivo studies indicate that endothelial cells in the main pulmonary artery and in the small muscular arteries are increased in chronically hypoxic rats [50,51]. Endothelial proliferation is also increased in neonatal calves following exposure to 8% oxygen for 14 days [52]. Additionally, excessive endothelial proliferation leads to plexiform lesion formation in idiopathic PAH patients [53]. Our research, performed in vitro, similarly demonstrates that hypoxia promotes endothelial proliferation. We also show that hypoxia-induced endothelial proliferation is ROS-dependent as the administration of PEG-catalase attenuates these events. These hypoxia-induced ROS are likely produced by NADPH oxidases (Noxes) as previous studies indicate that Nox4 expression is elevated in low oxygen environments [25]. Interestingly, previous studies also suggest that...

Figure 6. ROS mediate hypoxia-induced increases in endothelial ALOX5 expression and cell proliferation. Human pulmonary artery endothelial cells (HPAEC) were exposed to 0, 10, 100, and 200 mM hydrogen peroxide (H$_2$O$_2$) for 24 hours. Following exposure, supernatants were collected to assess cell toxicity by adenylate kinase release. Results demonstrate no significant changes in cell death as indicated by adenylate kinase release (n=4–6; data not shown). HPAEC were collected and total RNA was isolated for quantitative real-time PCR gene expression analysis. ALOX5 was normalized to the housekeeping gene b-globin. Relative expression was calculated using the Delta-Delta CT method and values were expressed as percent of control (A, n=4–5). * p<0.05 when compared to untreated controls. H$_2$O$_2$ exposure stimulates HPAEC ALOX5 protein levels as analyzed by western blot (B, n=4). PEG-Catalase (10U - 1000 U/ml) administration during the final 24 hours of the 72 hour hypoxia exposure prevents hypoxia-induced elevations in endothelial ALOX5 expression (C, n=5) and cell proliferation (D, n=6). * p<0.01 when compared to normoxic groups. ** p<0.05 when compared to untreated hypoxia controls.

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NADPH oxidase activity is required for endothelial cell proliferation [34]. Additionally, studies by our group and others have implicated Nox4 in the hypoxia-induced proliferation of human pulmonary artery smooth muscle cells [19,55]. Hypoxia-induced increases in ROS may also result from reductions in antioxidant availability, as hypoxia exposure significantly decreases glutathione levels in porcine pulmonary artery endothelial cells [56].

Although our evidence indicates that hypoxia increases ALOX5 in a redox-sensitive manner, the mechanisms underlying these events remain unclear. Two major pathways are implicated in regulating ALOX5 expression: promoter methylation and transcription factor activation [57]. Research demonstrates that leukocyte cell lines that have methylated promoters, U-937 and HL-60TB, do not express ALOX5, while the HL-60 cell line, which is unmethylated expresses ALOX5 [58,59]. Moreover, the treatment of the U-937 and HL-60TB cell lines with the demethylating agent, 5-aza-2’deoxyctidine resulted in the restoration of ALOX5 expression [58]. There are also 5 GC boxes that bind Sp1 and have been shown to be important in regulating expression of ALOX5 [60] however, ablation of all GC-boxes only reduces ALOX5 promoter activity 47%, implicating other transcription factors such as hypoxia-inducible factor (HIF) in ALOX5 regulation. The oxygen-sensing molecule, HIF-1α [61] regulates the adaptive response by activating genes associated with energy metabolism, erythropoiesis, vasomotor tone, and angiogenesis [62], and recent work demonstrates that HIF-1α mediates hypoxia-induced FLAP expression in human pulmonary microvascular endothelial cells [63]. HIF-2α also contributes to the vascular response to chronic hypoxia including the expression of genes involved in pulmonary vascular cell proliferation [64]. Hypoxia may also modulate ALOX5 via the early growth response protein-1, or Egr-1. This hypoxia-inducible transcription factor [65] has a binding site within the ALOX5 promoter region [66] and is expressed in a variety of pulmonary vascular cells, including endothelial cells [67]. Research also demonstrates that Egr-1 acts in a redox-sensitive manner, as Egr-1 is upregulated by ROS release and attenuated following antioxidant overexpression [68,69]. In addition to HIF and Egr-1, the ALOX5 promoter region also contains binding sites for TGF-β [70] and NF-κB [71,72], which are also implicated in the endothelial dysfunction [73,74] and vascular remodeling [75] in PH pathogenesis [76,77].

In summary, the current study demonstrates that hypoxia-induced ROS plays a critical role regulating ALOX5 and endothelial proliferation. Our data suggests that ALOX5 mediates hypoxia-induced endothelial cell proliferation and implicates increased ALOX5 expression and activity in vascular remodeling in experimental and clinical PH. Overall, these results suggest that ALOX5 inhibition merits additional investigation as a therapeutic target for the prevention or treatment of PH.

Author Contributions
Conceived and designed the experiments: KMP RLS. Performed the experiments: KMP BYK SEA TCM. Analyzed the data: KMP BYK SEA TCM CMH RLS. Contributed reagents/materials/analysis tools: RLS. Wrote the paper: KMP CMH RLS.

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