Resveratrol prevents hypoxia-induced arginase II expression and proliferation of human pulmonary artery smooth muscle cells via Akt-dependent signaling

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Resveratrol prevents hypoxia-induced arginase II expression and proliferation of human pulmonary artery smooth muscle cells via Akt-dependent signaling. Am J Physiol Lung Cell Mol Physiol 307: L317–L325, 2014. First published June 20, 2014; doi:10.1152/ajplung.00285.2013—Pulmonary artery smooth muscle cell (PASMC) proliferation plays a fundamental role in the vascular remodeling seen in pulmonary hypertensive diseases associated with hypoxia. Arginase II, an enzyme regulating the first step in polyamine and proline synthesis, has been shown to play a critical role in hypoxia-induced proliferation of human PASMC (hPASMC). In addition, there is evidence that patients with pulmonary hypertension have elevated levels of arginase in the vascular wall. Resveratrol, a natural polyphenol found in red wine and grape skins, has diverse biochemical and physiological actions including antiproliferative properties. Furthermore, resveratrol has been shown to attenuate right ventricular and pulmonary artery remodeling, both pathological components of pulmonary hypertension. The present studies tested the hypothesis that resveratrol would prevent hypoxia-induced pulmonary artery smooth muscle cell proliferation by inhibiting hypoxia-induced arginase II expression. Our data indicate that hypoxia-induced hPASMC proliferation is abrogated following treatment with resveratrol. In addition, the hypoxic induction of arginase II was directly attenuated by resveratrol treatment. Furthermore, we found that the inhibitory effect of resveratrol on arginase II in hPASMC was mediated through the PI3K-Akt signaling pathway. Supporting these in vitro findings, resveratrol normalized right ventricular hypertrophy in an in vivo neonatal rat model of chronic hypoxia-induced pulmonary hypertension. These novel data support the notion that resveratrol may be a potential therapeutic agent in pulmonary hypertension by preventing PASMC arginase II induction and proliferation.

pulmonary hypertension; pulmonary vasculature; phosphatidylinositol 3-kinase; vascular smooth muscle cells; stilbene

PULMONARY HYPERTENSION (PH) is a disease characterized by increased pulmonary vascular resistance leading to the elevation of right ventricular afterload, and eventual right heart failure and death. Based on the World Health Organization Classification (28), there are five updated clinical categories of PH, each associated with high morbidity and mortality. Despite being etiologically diverse, cellular proliferation and vascular remodeling as a response to lung injury, systemic disease, or stimuli such as hypoxia, drugs, or toxins are the common hallmark pathogenic features of PH (12, 20). Currently, treatment options available for PH are limited to vasodilators that are only supportive with no curative therapy (8). Therefore, new strategies are needed for the prevention and treatment of the pathogenic vascular remodeling and vascular smooth muscle cell proliferation characteristic of PH.

We have previously shown that hypoxia-induced proliferation in human pulmonary artery smooth muscle cells (hPASMC) depends on the induction of arginase II (2). Arginase, of which there are two described isoforms, has been shown to be important in proliferation of different cell types. Both isoforms are expressed in the lung and metabolize the conversion of l-arginine to l-ornithine and urea. This is the first step in polyamine and proline synthesis, which are critical for cell proliferation, differentiation, tissue repair, and growth (14, 15).

Resveratrol (3,5,4′-trihydroxystilbene) is a natural polyphenol compound found in many plant species, including red grapes and berries, which exerts significant antioxidant, anti-inflammatory, and endothelial protective effects in the systemic circulation (24). Recent data have shown that stilbenic derivatives inhibited, in a dose-dependent manner, arginase activity and increased intracellular concentration of nitric oxide in human umbilical vein endothelial cells (33). In addition, the same stilbenic derivative resulted in decreased arginase activity and increased intracellular concentration of nitric oxide in human umbilical vein endothelial cells (33). Although there have been relatively little data on the effects of resveratrol in the pulmonary circulation, it has been shown to prevent monocrotaline-induced pulmonary hypertension in animal models and inhibit proliferation of pulmonary arterial smooth muscle cells (7). In the present study, we hypothesized that resveratrol would prevent hypoxia-induced hPASMC proliferation via inhibition of arginase II induction. Furthermore, we provide evidence that the mechanism of action of resveratrol’s inhibition of arginase II induction is at the level of transcription via PI3K-Akt-dependent cell signaling.

MATERIALS AND METHODS

Cell culture. Human PASMC (Lanza, Walkersville, MD) were grown in 21% O2, 5% CO2, balance N2 at 37°C in smooth muscle growth media (SmGM, Lonza), which includes smooth muscle basal medium (Lonza), 5% fetal bovine serum, 0.5 ng/ml human recombinant epidermal growth factor, 2 ng/ml human recombinant fibroblast growth factor, 5 μg/ml insulin, and 50 μg/ml of gentamicin. Human PASMC from at least two donors were used between the fifth and eighth passages. The cells were grown to ~80% confluence and washed, and fresh medium was placed on the cells. Varying concentrations of resveratrol (Acros Organics, part of Thermo Fisher Scientific, Geel, Belgium; 40, 80, or 100 μM), LY294002 (Calbiochem, Billerica, MA; 10 μM), or vehicle was added to the cells and were incubated in 21% O2, 5% CO2, balance N2 (normoxia) or 1% O2, 5%
CO₂, balance N₂ (hypoxia) for 30 min, 24, 48, or 120 h. In addition, hPASMC were exposed to hypoxia for 5, 10, 15, 20, or 30 min to confirm Akt activation. For the 30-min time points, resveratrol or LY294002 were added to the cells for 30 min prior to hypoxia exposure. After completion of exposure, cells were isolated for RNA or protein as described below.

**RNA isolation, reverse transcription, and quantitative real-time PCR.** Human PASMC were denatured with 4 M guanidine thiocyanate, mixed sequentially with 2 M sodium acetate (pH 4), extracted with phenol and phenol/chloroform/isoamyl alcohol, and then precipitated with isopropanol. RNA pellet was washed with 75% ethanol and then dissolved in diethylpyrocarbonate water. The RNA was reverse transcribed and quantitative real-time PCR was performed as previously described (2, 3). Relative arginase I and II mRNA amounts were normalized to 18S mRNA expression by the ΔΔCt method (18). Samples were analyzed in triplicate.

**Protein isolation.** Protein was isolated from hPASMC as previously described (22). Briefly, hPASMC were washed with phosphate-buffered saline (PBS), and 50–150 μl of lysis buffer (0.2 M NaOH, 0.2% SDS) was added to each plate or each well of a six-well plate. Before use, the following protease inhibitors were added to each milliliter of lysis buffer: 1 μM aprotonin (10 mg/ml double distilled (dd) H₂O), 1 μM leupeptin (10 mg/ml ddH₂O), and 1 μL of phenylmethylsulfonyl fluoride (34.8 mg/ml methanol). The lysis buffer solution was added to each plate or each well of a six-well plate and placed on ice for 30 min. The hPASMC were scraped, pipetted into sterile centrifuge tubes. The cell lysates were centrifuged at 12,000 g for 10 min. The supernatant was stored in 1.5-ml tubes at −80°C. Total protein concentration was determined by the Bradford method (1) using a commercially available assay (Bio-Rad, Hercules, CA).

**Western blot.** The lysed hPASMC were assayed for phosphorylated Akt (p-Akt), total Akt, arginase I, arginase II, and cleaved caspase 3 protein by Western blot analysis as previously described (21, 22). Aliquots of cell lysate were diluted 4:1 with NuPAGE LDS sample buffer (Life Technologies, Grand Island, NY), heated to 70°C for 10 min, and then quickly spun down at room temperature. Aliquots of the supernatant were used for SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membranes and blocked for 2 h in PBS with 0.1% Tween (PBS-T) containing 5% nonfat dried milk. The membranes were then incubated with primary antibody (1:1,500: Cell Signaling Technology, Danvers, MA), total Akt (1:1,000; Cell Signaling), arginase I (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), arginase II (1:500; Santa Cruz), or cleaved caspase 3 (1:1,000; Cell Signaling) overnight and then washed three times with PBS-T. The membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:15,000, Bio-Rad) or sheep anti-mouse secondary antibody (1:15,000, GE Healthcare, Piscataway Township, NJ) for 1 h and then washed three times with PBS-T. The protein bands were visualized with enhanced chemiluminescence (Luminata Classic/Forte Western HRP Substrate; EMD Millipore, Billerica, MA) and quantified by densitometry (SigmaPlot 11.0, Jandel Scientific, Carlsbad, CA).

**Western immunoblot.** Western immunoblot was performed as described (21, 22). For each sample, 20 μg of protein were loaded on sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Membranes were blocked with 5% nonfat dried milk in TBS-T for 1 h, incubated with primary antibody (1:1,500) overnight and then washed three times with TBS-T. The membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:15,000, Bio-Rad) or sheep anti-mouse secondary antibody (1:15,000, GE Healthcare, Piscataway Township, NJ) for 1 h and then washed three times with PBS-T. The protein bands were visualized with enhanced chemiluminescence (Luminata Classic/Forte Western HRP Substrate; EMD Millipore, Billerica, MA) and quantified by densitometry (SigmaPlot 11.0, Jandel Scientific, Carlsbad, CA). Differences were considered significant when P < 0.05.

**Results**

**Resveratrol prevents hypoxia-induced arginase II protein expression.** To determine the effects of resveratrol on arginase protein expression, hPASMC treated with resveratrol or vehicle were incubated in either normoxia or hypoxia for 48 h and
Resveratrol prevents hypoxia-induced arginase II mRNA expression. To determine whether resveratrol exerted its effects on arginase II at the level of transcription, hPASMC treated with resveratrol or vehicle were incubated in normoxia or hypoxia for 24 h and analyzed by quantitative real-time PCR. Hypoxia increased arginase II mRNA expression after 24 h ($P < 0.001$). Treatment with resveratrol prevented the hypoxia-induced arginase II mRNA expression ($P < 0.001$), such that there was no difference in arginase II expression in the resveratrol-treated, hypoxia group compared with the vehicle-treated, normoxia controls. Hypoxia did not have any significant effects on arginase I mRNA expression (Fig. 2A). Similarly, resveratrol did not affect arginase I mRNA expression in normoxia or hypoxia (Fig. 2A).

Fig. 1. Resveratrol prevents hypoxia-induced arginase II expression. Human pulmonary artery smooth muscle cell (PASMC) were incubated with vehicle or resveratrol, then exposed to 21% O$_2$ or 1% O$_2$ for 24 or 48 h. A: arginase II mRNA levels were analyzed by quantitative real-time PCR and normalized to 18S expression. B: representative Western blots are shown for arginase II and β-actin. C: Western blot data summarized and shown as fold change ± SE relative to vehicle-treated, normoxia controls. *Different from normoxia, vehicle treated, $P < 0.0001$; †different from hypoxia, vehicle treated, $P < 0.001$ ($n = 3–16$).

Fig. 2. Resveratrol has no effect on arginase I expression. Human PASMC were incubated with vehicle or resveratrol, then exposed to 21% O$_2$ or 1% O$_2$ for 24 or 48 h. A: arginase I mRNA levels were analyzed by quantitative real-time PCR and normalized to 18S expression. B: representative Western blots are shown for arginase I and β-actin. C: Western blot data summarized and shown as fold change ± SE relative to vehicle-treated, normoxia controls ($n = 3–9$).
Resveratrol inhibits arginase activity with no effect on nitrite levels. To determine the effects of resveratrol on arginase activity, hPASMC were treated with resveratrol or vehicle and incubated in normoxia or hypoxia for 48 h. The cell lysates were incubated with increasing concentrations of L-arginine and urea production was determined. Hypoxia resulted in a significantly greater \( V_{\text{max}} \) for arginase than in vehicle-treated, normoxia control cells (Fig. 3; \( V_{\text{max}} \) 0.32 ± 0.01 \( \mu \text{mol-mg protein}^{-1}\cdot\text{min}^{-1} \), hypoxia vs. 0.15 ± 0.01 \( \mu \text{mol-mg protein}^{-1}\cdot\text{min}^{-1} \), normoxia; \( P < 0.0001 \)). The arginine \( K_{m} \) for arginase was not different between the two groups (1.11 ± 0.48 mM, hypoxia vs. 1.56 ± 0.62 mM, normoxia). The addition of resveratrol to hPASMC during the 48-h incubation period had no significant effect on the \( V_{\text{max}} \) for arginase in normoxic hPASMC (Fig. 3). However, resveratrol completely inhibited the hypoxia-induced increase in \( V_{\text{max}} \) (Fig. 3; \( V_{\text{max}} \) 0.10 ± 0.01 \( \mu \text{mol-mg protein}^{-1}\cdot\text{min}^{-1} \), resveratrol; \( P < 0.0001 \)) to levels not significantly different from the baseline \( V_{\text{max}} \) in normoxic, vehicle-treated cells. The arginine \( K_{m} \) for arginase was not different between the hypoxic, vehicle-treated and hypoxic, resveratrol-treated hPASMC (1.56 ± 0.62 mM, vehicle vs. 4.88 ± 3.12 mM, resveratrol).

Nitrite levels were measured from the media of normoxic and hypoxic hPASMC treated with resveratrol. Hypoxia significantly decreased nitrite levels, relative to normoxia (\( P < 0.1 \); Fig. 3C). Interestingly, resveratrol had no measurable effect on nitrite levels (Fig. 3C).

Resveratrol prevents hypoxia-induced hPASMC proliferation with no evidence of apoptosis. To determine the role of resveratrol on hypoxia-induced cell proliferation, viable cell numbers were determined by Trypan blue exclusion after resveratrol treatment of hPASMC (seeded at 10^4 cells/well) in normoxia and hypoxia for 120 h. Data are shown as viable cell numbers as a percentage change from number of seeded cells. Overall, hypoxia resulted in significantly greater viable cell numbers (Fig. 4A). In both normoxia and hypoxia, resveratrol decreased the viable number of hPASMC at all of the tested concentrations (Fig. 4A). Indeed, the viable cell numbers in the highest resveratrol concentration group in both normoxia and hypoxia were less than the number of cells seeded.

hPASMC were evaluated for apoptosis by measuring cleaved caspase 3 by Western blot analyses. There was no difference in cleaved caspase 3 protein expression in normoxia or hypoxia with or without resveratrol treatment (Fig. 4, B and C).

Hypoxia activates Akt. The PI3K-Akt pathway has been implicated in hypoxia-induced PASMC proliferation (13) and our group has previously shown Akt activation by hypoxia in hPASMC (4). We used Western blot analysis to assess activation of Akt by hypoxia in hPASMC. Phosphorylated Akt levels were barely detectable at 0 min, were dramatically elevated, and were highest at 30 min following hypoxic exposure (figure not shown). The PI3K inhibitor LY294002 was utilized to confirm inhibition of hypoxia-induced Akt activation at 30 min (Fig. 5, A and B).

Hypoxia induces arginase II expression via Akt-dependent signaling. To determine the effects of PI3K-Akt inhibition on arginase II protein expression, hPASMC were treated with LY294002 or vehicle, incubated in either normoxia or hypoxia for 48 h, and analyzed by Western blot. The PI3K-Akt inhibitor LY294002 had no appreciable effect on arginase II protein.
DISCUSSION

The main objective of the present study was to investigate the effects of resveratrol on hypoxia-induced arginase II expression, arginase activity, and hypoxia-induced proliferation in hPASMC. The major findings of this study were that 1) consistent with our previous findings (2, 3), hypoxia increased arginase activity, arginase II mRNA and protein expression, and proliferation in hPASMC; 2) resveratrol prevented the hypoxia-induced increase in arginase II mRNA and protein expression and arginase activity; 3) resveratrol prevented hypoxia-induced hPASMC proliferation; 4) Akt was activated by hypoxia and resveratrol prevented hypoxia-induced Akt activation; 5) inhibition of Akt prevented hypoxia-induced arginase II protein expression; and 6) resveratrol attenuated chronic hypoxia-induced RVH in neonatal rats as evidenced by normalization of RV/(LV+S) ratios. Taken together, these findings support our hypothesis that resveratrol prevents hypoxia-induced hPASMC proliferation via Akt-dependent inhibition of arginase II induction.

Vascular smooth muscle cell proliferation and resultant vascular remodeling are important pathogenic features of pulmonary hypertensive diseases; therefore, understanding mechanistic pathways involved in smooth muscle cell proliferation may isolate novel targets for drug therapies that may be able to reverse vascular remodeling. Arginase II has been demonstrated to be elevated in the pulmonary vasculature of patients with pulmonary hypertension (34). In addition to greater arginase expression, the authors also found a decrease in NO synthesis in the pulmonary artery endothelial cells in the same patients. This is not surprising because L-arginine is the substrate for both NO synthase (NOS) and arginase and an increase in arginase activity may decrease the bioavailability of L-arginine for NOS (5). Furthermore, NO tends to be antiproliferative; thus in conditions where arginase activity is increased and NO production is decreased there will be a net proliferative phenotype as well as decreased production of an important pulmonary vasodilator. Consistent with these findings, in our cell culture model in both hPASMC and human pulmonary microvascular endothelial cells, hypoxia-induced cellular proliferation is dependent on arginase II (2, 30), with a concomitant decrease in nitrite levels (Fig. 3C).
The natural dietary polyphenolic compound resveratrol exerts significant antioxidant, anti-inflammatory, and endothelial protective effects in the systemic circulation (9, 16). It has also been shown to have an antiproliferative effect on both rat and cultured human aortic smooth muscle cells (32, 36). More recently in the pulmonary circulation, resveratrol and other stilbene derivatives have been shown to inhibit rat PASMC proliferation (10). The treatment of isolated carotid arteries and aortic rings, as well as chronic treatment of rats with resveratrol, stimulates endothelium-dependent relaxation in both normotensive and hypertensive rat models (26, 29, 31). In addition, resveratrol prevented monocrotaline-induced pulmonary hypertension in rats (7). In the present studies, we found a striking inhibition of the hypoxia-induced arginase II protein expression and activity in hPASMC. Upon further analysis, we determined that the inhibitory effect of resveratrol occurs at the level of transcription. Despite an inhibitory effect of resveratrol on arginase expression, nitrite production did not change. We speculate that this may be due to low levels of NO synthase in the PASMC. Furthermore, resveratrol prevented the hypoxia-induced increase in viable cell numbers and even induced cell death at the highest tested concentration. Thus the potential for resveratrol as a therapeutic option for pulmonary hypertension and other cardiovascular diseases exists.

Fig. 5. Inhibition of Akt prevents hypoxia-induced arginase II protein expression. Human PASMC were treated with LY29002 or siRNA targeting Akt, then exposed to 21% O2 or 1% O2 for 0.5 or 48 h. A: representative Western blots are shown for phosphorylated Akt (p-Akt) and total Akt after hypoxia exposure. B: data are shown as fold change of p-Akt/total Akt ± SE relative to hypoxia, vehicle treated. C: representative Western blots are shown for arginase II and β-actin. D: data are shown as fold change of arginase II/β-actin ± SE relative to normoxia, vehicle treated. E: validation of Akt knockdown after siRNA treatment. Representative Western blots are shown for totalAkt and β-actin. F: data are shown as fold change of total Akt/β-actin ± SE relative to hypoxia, scramble siRNA. G: representative Western blots are shown for arginase II and β-actin after siRNA knockdown of Akt. H: data are shown as fold change of arginase II/β-actin ± SE relative to normoxia, vehicle treated. *Different from normoxia, vehicle treated, \( P < 0.01 \); †different from hypoxia, vehicle treated, \( P < 0.05 \); ‡different from hypoxia, siRNA Akt treated, \( P < 0.05 \) (\( n = 5–6 \)).
There is a balance between cell proliferation and cell death, and in hypoxia this balance normally favors cell proliferation, i.e., a substantial increase in viable cell numbers through 120 h. Our findings suggest that by preventing proliferation, the highest doses of resveratrol tip the balance toward cell death such that there are fewer viable cells at 120 h than there were at 0 h. This is consistent with previous data in which Gao et al. (10) showed increased apoptosis in rat PASMC following what they considered to be intermediate concentrations of resveratrol (100 µM), compared with low concentrations of resveratrol (50 µM). Although we did not find evidence for apoptosis by caspase 3 activation, other cell cycle regulatory mechanisms were not evaluated in the present study that may be responsible for the cell death seen at the higher resveratrol doses.

Supporting our findings in vitro, we provide evidence that right ventricular hypertrophy is improved following treatment with resveratrol in a chronic hypoxia-induced neonatal rat model of pulmonary hypertension. Although changes in vessel remodeling are not specifically shown in the present study, Liu et al. (17) reveal that in a similar model of chronic hypoxia-induced rat model of pulmonary hypertension the ratio of vessel wall thickness to total external diameter, ratio of wall area to total vessel area, right ventricular systolic pressures, and right ventricular hypertrophy were all attenuated following resveratrol treatment.

There are several limitations to the present study that should be noted. Our studies use commercially available hPASMC that are isolated from main pulmonary artery segments. The PASMC proliferation seen in more proximal arteries may potentially contribute to arterial stiffening, given that increased arginine leads to an increase in proline, which is responsible for collagen synthesis; however, this was not tested in the present study. Although we do not use the resistance-level pulmonary arteries that are classically affected in pulmonary hypertensive diseases, we chose these cells, in part, because they are human cells. We acknowledge that these findings may need to be validated in resistance-level PASMC. Secondly, we use perhaps more severe levels of hypoxia of 1% O2 to induce PASMC proliferation. Based on numerous trials in our laboratory utilizing various conditions, which include different

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**Fig. 6.** Resveratrol prevents Akt phosphorylation. Human PASMC were incubated with vehicle or resveratrol, then exposed to 1% O2 for 0.5 h. A: representative Western blots are shown for p-Akt and total Akt. B: data are shown as fold change of p-Akt/total Akt ± SE relative to hypoxia, vehicle treated. †Different from hypoxia, vehicle treated, $P < 0.02$ ($n = 3$).

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**Fig. 7.** Resveratrol attenuates chronic hypoxia-induced right ventricular hypertrophy in neonatal rats. Neonatal rats were treated with resveratrol and exposed to normoxia or hypobaric hypoxia for 14 days. Right ventricle (RV) and left ventricle + septum (LV+S) weights were measured. Data are shown as RV/(LV+S) ratios. *Different from normoxia, vehicle treated, $P < 0.0001$; †different from hypoxia, vehicle treated, $P < 0.001$ ($n = 8–12$).
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oxygen levels, our cell culture model has proven consistently to be a reliable and reproducible model (2–4). Additionally, our findings are consistent with those in the literature that show increased Akt phosphorylation after exposure to even less severe levels of hypoxia (11, 35).

In conclusion, our data indicate that hypoxia induces arginase II mRNA and protein expression and arginase activity resulting in increased viable cell numbers demonstrating proliferation in hPASMC and that this hypoxia-induced effect on arginase II is via the PI3K-Akt pathway. We show that resveratrol attenuates chronic hypoxia-induced RV/(LV+S) changes in neonatal rats and may play a role in preventing hypoxia-induced hPASMC proliferation by inhibition of arginase II induction. Furthermore, we provide evidence that targeting the PI3K-Akt signaling pathway may provide novel therapies in the prevention or treatment of the pathological PASMC proliferation associated with hypoxia.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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