PPAR γ Regulates Mitochondrial Structure and Function and Human Pulmonary Artery Smooth Muscle Cell Proliferation

Samantha M. Yeligar^{1,2*}, Bum-Yong Kang^{1,2*}, Kaiser M. Bijli^{1,2*}, Jennifer M. Kleinhenz^{1,2}, Tamara C. Murphy^{1,2}, Gloria Torres³, Alejandra San Martin³, Roy L. Sutliff^{1,2}, and C. Michael Hart^{1,2}

¹Division of Pulmonary, Allergy, Critical Care and Sleep Medicine, Department of Medicine, Atlanta Veterans Affairs Medical Center, Decatur, Georgia; ²Emory University, Atlanta, Georgia; and ³Division of Cardiology, Department of Medicine, Emory University, Atlanta, Georgia

ORCID IDs: 0000-0001-9309-0233 (S.M.Y.); 0000-0001-8251-1637 (B.-Y.K.).

Abstract

Pulmonary hypertension (PH) is a progressive disorder that causes significant morbidity and mortality despite existing therapies. PH pathogenesis is characterized by metabolic derangements that increase pulmonary artery smooth muscle cell (PASMC) proliferation and vascular remodeling. PH-associated decreases in peroxisome proliferator-activated receptor γ (PPAR γ) stimulate PASMC proliferation, and PPAR γ in coordination with PPAR γ coactivator 1 α (PGC1 α) regulates mitochondrial gene expression and biogenesis. To further examine the impact of decreases in PPAR γ expression on human PASMC (HPASMC) mitochondrial function, we hypothesized that depletion of either PPAR γ or PGC1 α perturbs mitochondrial structure and function to stimulate PASMC proliferation. To test this hypothesis, HPASMCs were exposed to hypoxia and treated pharmacologically with the PPAR γ antagonist GW9662 or with siRNA against PPAR γ or PGC1 α for 72 hours.

HPASMC proliferation (cell counting), target mRNA levels (qRT-PCR), target protein levels (Western blotting), mitochondria-derived H₂O₂ (confocal immunofluorescence), mitochondrial mass and fragmentation, and mitochondrial bioenergetic profiling were determined. Hypoxia or knockdown of either PPAR γ or PGC1 α increased HPASMC proliferation, enhanced mitochondria-derived H₂O₂, decreased mitochondrial mass, stimulated mitochondrial fragmentation, and impaired mitochondrial bioenergetics. Taken together, these findings provide novel evidence that loss of PPAR γ diminishes PGC1 α and stimulates derangements in mitochondrial structure and function that cause PASMC proliferation. Overexpression of PGC1 α reversed hypoxia-induced HPASMC derangements. This study identifies additional mechanistic underpinnings of PH, and provides support for the notion of activating PPAR γ as a novel therapeutic strategy in PH.

Keywords: HPASMC; hypoxia; mitochondria; PGC1 α ; PPAR γ

Pulmonary hypertension (PH) causes significant morbidity and mortality (1). Complex mechanisms, including alterations in cellular metabolism, derangements in the

production of vasoactive mediators, and enhanced proliferation of cells in the pulmonary vascular wall, contribute to PH pathogenesis (2). These alterations in

vascular structure and function stimulate increases in pulmonary vascular remodeling and pulmonary vascular resistance, increasing right ventricular

(Received in original form September 9, 2016; accepted in final form August 10, 2017)

*These authors are co-first authors and contributed equally to this work.

This work was supported in part by grants from the National Institute on Alcohol Abuse and Alcoholism (1K99AA021803 and 4R00AA021803 to S.M.Y.), the American Heart Association (13SDG14150004 to B.-Y.K. and 11SDG5440016 to K.M.B.), the National Heart, Lung, and Blood Institute (2P01HL095070-06A1 to A.S.M. and HL133053-01A1 to B.-Y.K., and HL102167 to R.L.S. and C.M.H.), and the Veterans Affairs Basic Laboratory Research and Development Merit Review Award Program (1101BX001910 to C.M.H.). The contents of this report do not represent the views of the Department of Veterans Affairs or the United States government.

Author Contributions: S.M.Y., B.-Y.K., and K.M.B. designed and analyzed experiments, and prepared the manuscript; J.M.K. and T.C.M. designed and analyzed experiments; G.T. and A.S.M. designed and analyzed mitochondria structure experiments; R.L.S. and C.M.H. designed experiments and prepared the manuscript.

Correspondence and requests for reprints should be addressed to C. Michael Hart, M.D., Atlanta VA Medical Center (151-P), 1670 Clairmont Road, Decatur, GA 30033. E-mail: michael.hart3@va.gov.

This article has a data supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 58, Iss 5, pp 648–657, May 2018

Copyright © 2018 by the American Thoracic Society

Originally Published in Press as DOI: 10.1165/rcmb.2016-0293OC on November 28, 2017

Internet address: www.atsjournals.org

Clinical Relevance

Pulmonary hypertension pathogenesis is characterized by metabolic derangements, increased pulmonary artery smooth muscle cell proliferation, and decreases in peroxisome proliferator-activated receptor γ (PPAR γ). The current study provides novel evidence that either hypoxia-induced or siRNA-mediated knockdown of PPAR γ causes decreases in PGC1 α , a major regulator of mitochondrial biogenesis. Depletion of PPAR γ is sufficient to increase human pulmonary artery smooth muscle cell proliferation, enhance mitochondria-derived H₂O₂ production, decrease mitochondrial mass, increase mitochondrial fragmentation, and impair mitochondrial bioenergetics. These findings reveal additional mechanisms linking diminished PPAR γ expression to altered pulmonary vascular cell phenotypes with metabolic dysregulation, and provide support for strategies targeting PPAR γ as a novel therapeutic approach in pulmonary hypertension.

pressure (2, 3). Despite recent pharmacological advances in PH therapy that ameliorate imbalances in the production of vasodilating and vasoconstricting mediators, current therapeutic approaches largely fail to reverse the underlying derangements in metabolism and proliferation in cells of the pulmonary vasculature. These observations suggest that interventions that target and reverse metabolic and proliferative abnormalities in pulmonary vascular wall cells might be more effective for PH treatment.

Peroxisome proliferator-activated receptor γ (PPAR γ), a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors, plays an important role in regulating a variety of fundamental cellular processes, including metabolism, proliferation, and inflammation (4). For example, PPAR γ is expressed in pulmonary vascular endothelial cells and smooth muscle cells (SMCs), where it participates in the regulation of normal pulmonary vascular

function (5). PPAR γ expression is decreased in the vascular lesions of patients with idiopathic pulmonary arterial hypertension (PAH) (6), and in cellular studies, depletion of PPAR γ was sufficient to promote development of the PH phenotype by upregulating cell cycle-related genes and angiogenesis-related genes in pulmonary artery endothelial cells (7), and to promote the proliferation of human pulmonary artery SMCs (HPASMCs) (8). In contrast, pharmacological PPAR γ activation decreased PH in experimental models (9, 10) and favorably altered transcriptional and post-transcriptional pathways and the levels of vasoactive mediators involved in PH pathogenesis (9–11). PPAR γ activation using the thiazolidinedione ligand rosiglitazone decreased pulmonary artery remodeling, muscularization of distal pulmonary arterioles, and SMC proliferation in rats subjected to chronic hypoxia (HYP) (12), and reversed right ventricular hypertrophy and PH in insulin-resistant, apolipoprotein E-deficient mice fed a high-fat diet (13). Combined with evidence that mice with vascular-targeted PPAR γ deletion develop PH (8), these studies indicate that decreases in PPAR γ contribute to PH pathogenesis. The current study seeks to further explore the role of PPAR γ in the underlying metabolic derangements that contribute to SMC proliferation.

PPAR γ activates the promoter of PPAR γ coactivator 1 α (PGC1 α), a 795-amino-acid protein that coordinates nuclear and mitochondrial gene expression and also is a transcriptional coactivator of PPAR (14). In PH, metabolic derangements related to reductions in the number of mitochondria and fragmentation of existing mitochondria (15–17) underlie phenotypic changes in pulmonary vascular wall cells (18), such as SMC proliferation. In human and experimental PH, decreased expression of PGC1 α contributes to mitochondrial fragmentation and proliferation, which are in part related to changes in mitochondrial number, structure, and function (19). PGC1 α also contributes to the adaptive response of PASMCs to HYP (20). Collectively, these observations suggest that losses of PPAR γ and PGC1 α may mutually contribute to PASMC proliferation. The current study identifies

novel roles for PPAR γ in the regulation of SMC mitochondria that contribute to metabolic dysfunction and proliferation in pulmonary vascular wall cells.

This study also further defines the molecular mechanisms whereby decreases in PPAR γ stimulate metabolic derangements that are associated with increased pulmonary vascular cell proliferation. We previously reported that HYP decreased PPAR γ *in vitro* and *in vivo* (21). Our current findings demonstrate that HYP exposure also decreases PGC1 α expression, attenuates expression of mitochondrial proteins, and increases SMC proliferation. In addition, they demonstrate that short interfering RNA (siRNA)-mediated depletion of either PPAR γ or PGC1 α is sufficient to stimulate mitochondria-derived H₂O₂ and alter mitochondrial metabolism and function, which induce SMC proliferation. PGC1 α activation using adenovirus overexpression reversed these HYP-induced HPASMC derangements. Taken together, these findings identify PPAR γ as a critical regulator of PASMC mitochondrial metabolism and proliferation, and illustrate mechanisms whereby decreases in this receptor could potentially contribute to PH pathogenesis.

Methods

In Vitro HPASMC Studies

HPASMCs were purchased from Lonza and Sci-Cell. HPASMC monolayers were grown at 37°C in a 5% CO₂ atmosphere and cultured in complete SmGM-2 medium (Lonza) containing 5% fetal calf serum, growth factors, and antibiotics as previously described (22). Cells were cultured in a HYP chamber (1% O₂; Biospherix) or cell culture incubator (normoxia [NOR], 21% O₂) for 72 hours. HPASMC passages 2–9 were used for all experimental studies.

Knockdown or Overexpression of PPAR γ and PGC1 α

To knock down PPAR γ in HPASMC, the PPAR γ antagonist GW9662 (5–10 μ M; Sigma-Aldrich) or PPAR γ siRNA (100 nM; Dharmacon) was used as previously described (8). To attenuate PGC1 α , PGC1 α siRNA (10 nM; Integrated DNA Technologies Inc.) was used. An adenovirus overexpressing PGC1 α ,

AdPGC1 α (a gift from Dr. Russ Price, Emory University), was used. Briefly, cells (40–50% confluent) were incubated for 3 hours with Opti-MEM (Life Technologies), transfected with siRNA using Lipofectamine RNAi reagent (Life Technologies) or treated with AdPGC1 α for 4 hours, and grown in complete growth media for 72 hours. These HPASMCs were compared with cells transfected with scrambled siRNA (SCR) or treated with an adenovirus expressing green fluorescent protein (AdGFP) as an experimental control.

HPASMC Proliferation

HPASMCs were cultured in 12-well plates in complete medium, followed by 72 hours of HYP or GW9662 treatment or siRNA transfection. HPASMCs (75–85% confluent) were trypsinized in 500 μ l trypsin, and a 1:1 cell suspension dilution with trypan blue was performed. Cells were counted in triplicate using a hemocytometer and expressed as mean cell counts $\pm$ SEM relative to NOR, control DMSO vehicle, or SCR.

Analysis of mRNA, Protein, and Mitochondrial Alterations

Target mRNA levels were measured and quantified by qRT-PCR with specific human, mouse, and rat primer sequences as outlined in Table E1 in the data supplement. Target protein levels were assessed by Western blotting. HPASMC

mitochondria number, volume, and H₂O₂ generation were determined by confocal microscopy. The mitochondrial bioenergetics were by measuring the oxygen consumption rate (OCR) (23) and expressed as mean $\pm$ SEM. See the data supplement for additional details.

In Vivo Rodent Models of PH

Male C57BL/6J mice (The Jackson Laboratory), 8–12 weeks old, were exposed to HYP (10% O₂) or NOR (room air, 21% O₂) for 3 weeks (24). Male Sprague-Dawley rats, 8 weeks old, were injected subcutaneously with SU5416, a vascular endothelial growth factor receptor 2 inhibitor (25). See the data supplement for further details. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Atlanta Veterans Affairs Medical Center, and all animal studies were performed in accordance with guidelines outlined in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Statistical Analysis

Data were analyzed using Student's *t* test or two-way ANOVA followed by Tukey's post hoc test in GraphPad Prism version 6 (GraphPad) to determine the significance of the treatment effects. Statistical significance was set at an α -value of $P \leq 0.05$.

Results

HYP, PPAR γ Knockdown, or PGC1 α Knockdown Increases HPASMC Proliferation

In previous studies, exposure to HYP regimens sufficient to induce PH in experimental animals *in vivo* (24) or to stimulate proliferation in HPASMC *in vitro* (9, 21, 22) was shown to significantly decrease lung-tissue or cellular levels of PPAR γ , respectively. Here, to further explore the relationships among PPAR γ , PGC1 α , and PASC proliferation, we treated cells with HYP, GW9662, siPPAR γ , or siPGC1 α for 72 hours. HPASMC proliferation was measured by cell counting. We previously reported that HYP (21) or siPPAR γ (8) treatment regimens depleted PPAR γ levels by 50%. Figure 1 establishes that treatment with HYP (Figure 1A), GW9662 (Figure 1B), or siPPAR γ (Figure 1C) comparably stimulated HPASMC proliferation by roughly 40%. This confirms our previous reports that HYP and loss of PPAR γ increase HPASMC proliferation (8, 9). Treatment with siPGC1 α decreased HPASMC PGC1 α mRNA and protein levels by $42 \pm 8.4\%$ (Figure E1A) and $27 \pm 3.3\%$ (Figure E1B), respectively, and stimulated proliferation (Figure 1C). Although treatment with combined siPPAR γ and siPGC1 α also increased HPASMC proliferation, the incremental

Figure 1. Hypoxia (HYP) and knockdown of PPAR γ or PGC1 α increases human pulmonary artery smooth muscle cell (HPASMC) proliferation. Cell counting was used to measure HPASMC proliferation. (A) HPASMCs were treated with HYP (1% O₂) or NOR (21% O₂) for 72 hours. (B) HPASMCs were treated with vehicle (DMSO) or the PPAR γ antagonist GW9662 (10 μ M) for 72 hours. (C) HPASMCs were transfected with scrambled siRNA control (SCR), 100 nM siPPAR γ , 100 nM siPGC1 α , or combined 100 nM siPPAR γ and 100 nM siPGC1 α for 72 hours. $n = 4$, * $P < 0.05$ versus NOR, DMSO, or SCR. NOR = normoxia; PGC1 α = PPAR γ coactivator 1 α ; PPAR γ = peroxisome proliferator-activated receptor γ .

Figure 2. PPAR γ knockdown induces mitochondrial H₂O₂ generation and attenuates mitochondrial mass in HPASMCs. HPASMCs were transfected with SCR control or 100 nM siPPAR γ for 72 hours. (A) Representative confocal fluorescence images of treated HPASMC monolayers stained with DAPI, MitoPY1, and Mitotracker Red. Merged images confirm colocalization of H₂O₂ production with mitochondria labeling. Magnification $\times 90$. (B) The fluorescence intensity of MitoPY1 is quantified in the bar graph. $n = 6$, $*P < 0.05$ versus SCR. (C) TFAM mRNA levels were measured by qRT-PCR ($n = 5-6$). (D and E) The protein levels of mitochondrial-specific GRP75 (D) and VDAC (E) were measured by Western blot analysis ($n = 8$). (F) The mRNA levels of the mitochondrial fusion gene MFN2 were measured by qRT-PCR. The mRNA and protein levels of the targets were normalized to housekeeping genes and proteins and expressed as mean $\pm$ SEM fold change relative to SCR. (G) Representative confocal fluorescence images of treated HPASMC monolayers stained with Mitotracker Orange. Magnification $\times 63$. The fluorescence intensity of Mitotracker Orange and the number of mitochondria are quantified in the bar graphs. $n = 6$, $*P < 0.05$ versus SCR. GRP75 = heat shock protein family A member 9; MFN2 = mitofusin-2; RFU = relative fluorescence units; TFAM = mitochondrial transcription factor A; VDAC = voltage-dependent anion-selective channel.

increase in proliferation caused by the combination of siRNAs was not significantly greater than that observed with each siRNA alone (Figure 1C). Collectively, these findings illustrate that loss of PPAR γ activity alone (GW9662), loss of PPAR γ protein and activity (by treatment with HYP or siPPAR γ), or loss of PGC1 α is sufficient to stimulate HPASMC proliferation.

PPAR γ or PGC1 α Knockdown Induces Mitochondrial Derangements in HPASMCs

Because metabolic derangements play a significant role in the pathogenesis of pulmonary vascular cell proliferation in PH (18), and because PPAR γ regulates both metabolism and PGC1 α , a master regulator of mitochondrial biogenesis (26), we sought to further define the relationships between alterations in PPAR γ and mitochondrial structure and function. As illustrated in Figure 2, treatment with siPPAR γ for 72 hours enhanced mitochondria-derived H₂O₂ generation, as measured with MitoPY1 (Figures 2A and 2B). Further, siPPAR γ decreased the mRNA levels of mitochondrial transcription factor A (TFAM; Figure 2C); the expression of mitochondrial-specific proteins, including heat shock protein family A member 9 (GRP75; Figure 2D) and voltage-dependent anion-selective channel (VDAC; Figure 2E); and mRNA levels of mitofusin-2 (MFN2; Figure 2F). These data, demonstrating decreases in mitochondrial mass, along with evidence of increased mitochondrial reactive oxygen species (ROS) generation (Figures 2A and 2B), suggested an increase in mitochondrial fission (27). Consistent with this, confocal microscopy of HPASMCs labeled with MitoTracker Orange (Thermo Fisher Scientific) demonstrated that siPPAR γ decreased the overall volume of mitochondria but increased the number of labeled mitochondria per cell (Figure 2G). To further examine the functional correlates of these siPPAR γ -induced alterations in mitochondrial structure, we determined the HPASMC mitochondrial bioenergetics. Treatment with siPPAR γ decreased HPASMC OCR (Figure 3A) and diminished basal respiration (Figure 3B), ATP production (Figure 3C), proton leak (Figure 3D), and maximal respiration (Figure 3E).

Figure 3. PPAR γ knockdown attenuates mitochondrial bioenergetics in HPASMCs. HPASMCs were transfected with SCR control or 100 nM siPPAR γ for 72 hours. (A) Mitochondrial bioenergetic profiling was performed with a Seahorse XF96 Extracellular Flux Analyzer, and the oxygen consumption rate (OCR) was recorded and normalized to protein levels. (B–E) OCR profiles were used to determine basal respiration (B), ATP production (C), proton leak (D), and maximal respiration (E). $n = 6$, * $P < 0.05$ versus SCR. FCCP = carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone; Oligo = oligomycin; R/A = rotenone/antimycin A.

Loss of PPAR γ Decreases PGC1 α Expression *In Vitro* and *In Vivo*

PPAR γ and PGC1 α are mutually activating central metabolic regulators (14, 28). Because the PGC1 α promoter is regulated by PPAR γ , we sought to determine whether alterations in PPAR γ expression modulate PGC1 α levels. HPASMCs were treated with the PPAR γ antagonist GW9662 or with siPPAR γ for 72 hours. Compared with vehicle, GW9662 decreased the levels of PGC1 α mRNA (Figure 4A) and protein (Figure 4B). Similarly, compared with SCR, siPPAR γ decreased both PGC1 α mRNA (Figure 4C) and protein (Figure 4D) levels. These data demonstrate that loss of PPAR γ decreases PGC1 α expression in HPASMCs.

Because HYP was previously shown to downregulate PPAR γ expression in HPASMCs *in vitro* (21) and in mouse

lungs *in vivo* (9), stimulating PASC proliferation and PH, respectively, we examined lung PGC1 α in C57BL/6J mice exposed to HYP (10% O₂) or NOR (21% O₂) for 3 weeks. HYP decreased mouse lung PGC1 α mRNA (Figure 5A) and protein levels (Figure 5B). Similarly, PGC1 α mRNA (Figure 5C) and protein (Figure 5D) levels were depleted in HPASMC treated with HYP (1% O₂) for 72 hours. Collectively, these data indicate that HYP decreases expression of both PPAR γ and PGC1 α in mouse lungs and HPASMCs.

Because depletion of PPAR γ perturbed mitochondrial structure and function, and because PGC1 α is a critical PPAR γ cofactor in regulating mitochondrial mass and bioenergetics, we further examined the relationship between decreases in HPASMC PPAR γ and PGC1 α . HPASMCs

Figure 4. Loss of PPAR γ attenuates PGC1 α expression in HPASMCs. (A and B) HPASMCs were treated with vehicle (DMSO) or the PPAR γ antagonist GW9662 (10 μ M) for 72 hours. PGC1 α mRNA (A) and protein (B) levels were measured by qRT-PCR and Western blot (Calbiochem) analysis, respectively. (C and D) HPASMCs were transfected with SCR control or 100 nM siPPAR γ for 72 hours. PGC1 α mRNA (C) and protein (D) levels were measured. The mRNA ($n = 5-6$) and protein ($n = 8$) levels of the targets were normalized to housekeeping genes and proteins and expressed as mean $\pm$ SEM fold change relative to control. * $P < 0.05$ versus DMSO or SCR. Readers may view the uncut gels for Figures 4B and 4D in the data supplement.

were transfected with SCR or siPPC1 α for 72 hours, which knocked down PGC1 α mRNA (Figure E1A) and protein (Figure E1B) levels. Treatment with siPPC1 α did not affect PPAR γ mRNA levels (Figure E1C), suggesting that the effects of PPAR γ are upstream of PGC1 α in HPASMCs. Similar to the effects of induced knockdown of PPAR γ , siPPC1 α diminished TFAM mRNA levels (Figure E2A)

and perturbed the HPASMC mitochondrial bioenergetic profile (Figures E2B–E2F). Taken together, these data demonstrate that decreases in either PPAR γ or PGC1 α are sufficient to alter HPASMC mitochondrial structure and function. Because decreases in PPAR γ or PGC1 α are sufficient to stimulate HPASMC proliferation, these data indicate that targeting the PPAR γ -PGC1 α axis is a promising

therapeutic strategy for reversing metabolic and proliferative derangements in pulmonary vascular wall cells.

HYP Decreases Expression of Mitochondrial Proteins in HPASMCs

Because HYP downregulated PPAR γ expression in HPASMCs *in vitro* (21) and siPPAR γ induced HPASMC mitochondrial fragmentation (Figure 2), we sought to determine the effect of HYP on the expression of HPASMC mitochondrial proteins. Treatment with HYP caused decreases in HPASMC TFAM (Figure 6A), GRP75 (Figure 6B), and VDAC (Figure 6C) protein expression, comparable to those caused by siRNA-mediated PPAR γ knockdown. To confirm these results in an *in vivo* experimental model of PH, we used a hypoxic SU5416 rat model of PH. Compared with the lungs isolated from normoxic SU5416 rats, the lungs from hypoxic SU5416 rats showed decreased mRNA levels of PGC1 α (Figure E3A), TFAM (Figure E3B), GRP75 (Figure E3C), VDAC (Figure E3D), and MFN2 (Figure E3E). These data further support the postulate that HYP-induced loss of PPAR γ mediates derangements in mitochondrial structure and function in HPASMC, and that these mitochondrial derangements are not specific to a single rodent model of PH.

Overexpression of PGC1 α Reverses HYP-Induced HPASMC Proliferation

Because HYP decreased PGC1 α expression in mouse lungs and HPASMCs (Figure 5), and knockdown of PGC1 α increased HPASMC proliferation (Figure 1C), we sought to determine whether an adenovirus overexpressing PGC1 α could reverse HYP-induced HPASMC derangements. The PGC1 α overexpression adenovirus increased PGC1 α mRNA and protein levels (Figures E4A and E4B) in a dose-dependent manner, but did not affect PPAR γ mRNA and protein levels (Figures E4C and E4D). PGC1 α adenoviral overexpression reversed HYP-induced HPASMC proliferation (Figure E5). Collectively, these data indicate the critical importance of PPAR γ and PGC1 α for HYP-induced HPASMC derangements.

Discussion

The pathogenesis of PH is complex and characterized by alterations in metabolism and proliferation in cells of the pulmonary

Figure 5. HYP decreases PGC1 α expression in mouse lungs and HPASMCs. (A and B) Male C57BL/6J mice were exposed to HYP (10% O₂) or NOR (room air, 21% O₂) for 3 weeks. PGC1 α mRNA (A) and protein (B) levels were measured by qRT-PCR and Western blot (Calbiochem) analysis, respectively. (C and D) HPASMCs were treated with HYP (1% O₂) or NOR (21% O₂) for 72 hours. PGC1 α mRNA (C) and protein (D) levels were measured. The mRNA ($n = 5-6$) and protein ($n = 8$) levels of the targets were normalized to housekeeping genes and proteins and expressed as mean $\pm$ SEM fold change relative to NOR. * $P < 0.05$ versus NOR. Readers may view the uncut gels for Figure 5B in the data supplement.

vascular wall (2). Chronic HYP constitutes a common stimulus for experimental PH in rodent models (29). HYP promotes a variety of molecular and cellular changes in the lung, including decreases in PPAR γ expression and activity (21). PPAR γ expression is downregulated in the lungs of rodents with HYP-induced PH *in vivo* (24, 30) and in HPASMCs exposed to HYP

in vitro (21). PPAR γ downregulation in these models is comparable to that reported in the vascular lesions (6) of patients with PAH (31). Further, SMC-targeted PPAR γ deletion is sufficient to cause PH in mice (8). Taken together, these observations suggest that decreases in PPAR γ represent a common pathogenic factor in PH. The precise mechanisms by which reductions in

PPAR γ promote PASMC proliferation and pulmonary vascular remodeling continue to be defined. The current study further clarifies molecular pathways linking decreases in PPAR γ to alterations in cell metabolism and proliferation by showing that HYP or decreases in PPAR γ in HPASMCs deplete PGC1 α and cause derangements in mitochondrial structure and function that stimulate HPASMC proliferation (Figure 7). These novel results identify PPAR γ and PGC1 α as potential targets in PH pathogenesis and therapy.

Current evidence indicates that metabolic derangements (18), including decreased mitochondrial numbers, mitochondrial fragmentation (15–17), and a shift toward glycolysis-derived ATP production, contribute to pulmonary vascular wall cell phenotypic changes that cause pulmonary vascular remodeling. PPAR γ is a central metabolic regulator that activates PGC1 α , which coordinates mitochondrial gene expression and in turn activates PPAR. Our data demonstrate that HYP decreases PPAR γ and PGC1 α , and that decreases in PPAR γ are sufficient to diminish PGC1 α expression (Figures 4 and 5). Our findings further demonstrate that loss of PPAR γ or PGC1 α is sufficient to stimulate HPASMC proliferation (Figure 1). PGC1 α stimulates mitochondrial biogenesis (32) via activation of nuclear respiratory factor 1, which increases expression of TFAM (33) and nuclear respiratory factor 2 (32). TFAM is a mitochondrial-specific transcription factor that is required for maintenance of mitochondrial DNA copy number and the integrity of respiration (33). Depletion of PPAR γ (Figure 2) or PGC1 α (Figure E2) by siRNA or HYP (Figure 6) decreased expression of the mitochondrial-specific proteins TFAM, GRP75, and VDAC in HPASMCs, and these same targets were decreased in lung tissue from HYP-exposed, SU5416-treated rats (Figure E3). Further, activation of PPAR γ (34) or PGC1 α overexpression (Figure E5) attenuated HYP-induced HPASMC proliferation, supporting the importance of PPAR γ and PGC1 α in HYP-induced HPASMC derangements. VDAC is located on the outer membrane of the mitochondria and forms the permeability transition pore complex, which is responsible for the release of mitochondrial products that trigger apoptosis (35). GRP75 interacts with VDAC and modulates its channel properties (36). Therefore, PPAR γ or PGC1 α depletion-mediated decreases in GRP75 and

Figure 6. HYP decreases expression of mitochondrial proteins in HPASMCs. HPASMCs were treated with HYP (1% O₂) or NOR (21% O₂) for 72 hours. The protein levels of TFAM (A), GRP75 (B), and VDAC (C) were measured by Western blot analysis. The protein levels of the targets were normalized to housekeeping proteins and expressed as mean $\pm$ SEM fold change relative to NOR. $n = 8$, * $P < 0.05$ versus NOR. Readers may view the uncut gels for Figures 6A, 6B, and 6C in the data supplement.

VDAC expression may promote HPASMC proliferation (Figure 1) by inhibiting apoptosis. Consistent with this postulate, siRNA-mediated PPAR γ knockdown decreased HPASMC apoptosis (caspase-3 activity) (34). siPPAR γ -mediated decreases in MFN2 mRNA levels, coupled with decreases in mitochondrial volume and increased numbers of mitochondria per HPASMC (Figure 2), also suggest fragmentation of existing mitochondria, which may contribute to the impaired mitochondrial bioenergetics shown in Figure 3.

Loss of PPAR γ (Figure 3A) or PGC1 α (Figure E2B) decreased the overall mitochondrial bioenergetic profile in HPASMCs and suppressed basal OCR, proton leak, ATP production, and maximal respiration, consistent with broad derangements in mitochondrial function. Our results indicate that these alterations in mitochondrial function, secondary to depletion of PPAR γ or PGC1 α , are related in part to (1) decreased mitochondrial biogenesis, as evidenced by decreases in PGC1 α (Figures 4C and 4D), TFAM, GRP75, and VDAC (Figures 2C–2E), and (2) increased mitochondrial fission, as evidenced by decreases in MFN2 and mitochondrion volume and increases in the number of mitochondria per cell (Figures 2F and 2G). In a previous study, SMCs isolated from the pulmonary vessels of rats with monocrotaline-induced PH showed decreases in mitochondrial function

due to loss of complex I assembly, decreased activities of complexes II and III, and increased levels of mitochondrial-derived ROS, membrane potential, and glycolysis (37). Our findings extend those observations by demonstrating that decreases in PPAR γ are sufficient to increase mitochondrial-derived ROS generation (Figure 2) and decrease mitochondrial function (Figure 3). Ongoing studies will further characterize the mitochondrial derangements caused by loss of PPAR γ by defining the uncoupling protein activity, mitochondrial membrane potential, and integrity of the electron transport chain.

Several limitations in our study merit additional consideration. First, it is well recognized that the mouse model of HYP-induced PH used in our studies fails to recapitulate many characteristics of PH in humans (29). Determining the relevance of these pathways to human PH will require additional studies employing cells or tissues isolated from PH patients. However, because PPAR γ is also decreased in the lungs and pulmonary vascular cells of patients with PAH (6), and because decreases in PPAR γ are sufficient to induce PH in experimental animals (38), our findings suggest that decreases in PPAR γ may represent a common pathogenic stimulus in PH regardless of etiology or species. Second, although our *in vivo* HYP studies in mice suggest that pathways defined in hypoxic HPASMCs *in vitro* are also seen *in vivo*, our

data do not define which specific pulmonary vascular cells sustain HYP-induced depletion of PPAR γ and PGC1 α *in vivo*. Therefore, the effects of alterations in PPAR γ or PGC1 α in other cell types in the vascular wall or in circulating immune cells on PH pathogenesis must also be considered (39–41). For example, previous work demonstrated depleted PPAR γ levels in pulmonary artery endothelial cells (10) in PH. In addition, loss of bone morphogenetic protein receptor type 2 led to reductions in PGC1 α , mitochondrial dysfunction, and a propensity to undergo apoptosis after HYP reoxygenation (42), suggesting that these derangements are not confined to a single cell compartment. Third, although the present study does not specifically address the mechanisms by which HYP decreases PPAR γ *in vitro* or *in vivo*, our published studies have defined both transcriptional (21, 22) and post-translational (10) mechanisms that are activated by HYP to decrease PPAR γ levels. Finally, the length of time in which pulmonary vascular SMCs display proliferative responses to HYP *in vivo* is not well defined and may be limited (43–45). Therefore, translating our *in vitro* findings to the pathobiology of PH *in vivo* will require more rigorous studies linking temporal and cell compartment-specific alterations in PPAR γ and PGC1 α to mitochondrial, metabolic, and proliferative derangements that cause pulmonary vascular remodeling.

Figure 7. Summary schematic. HYP decreases PPAR γ expression, leading to diminished PGC1 α expression. Loss of PPAR γ and the subsequent loss of PGC1 α alter mitochondrial structure through decreased expression of TFAM, GRP75, VDAC, and MFN2, and impair mitochondrial function and bioenergetics. These mitochondrial derangements may contribute to the proliferation of HPASMCs observed under chronic hypoxic conditions.

The current study extends previous reports to demonstrate a novel role for PPAR γ , through modulation of PGC1 α , in regulating mitochondrial structure, function, and cellular metabolism in pulmonary vascular wall cells. Because PPAR γ or PGC1 α knockdown stimulated HPASMC proliferation (Figure 1) and comparable derangements in mitochondrial protein expression and bioenergetics (Figures 2, 3, and E2), and because manipulation of

PPAR γ regulated PGC1 α , whereas manipulation of PGC1 α had no effect on PPAR γ (Figures E1 and E4), our findings collectively support a model in which PPAR γ regulates mitochondrial bioenergetic function through PGC1 α . Although a metabolomic analysis of late-stage human PAH lung samples suggested that derangements in metabolism may differ according to the stage and severity of disease (46), our studies along with previous reports provide evidence that

depletion of PPAR γ causes metabolic derangements in pulmonary vascular wall cells and proliferative cellular phenotypes. Because loss of PPAR γ function has been implicated in diverse disorders (5), discovering common pathways that link PPAR γ downregulation to mitochondrial dysfunction may shed light on other disorders that share similar pathobiological mechanisms. Our findings identify additional mechanistic pathways whereby PPAR γ activation could potentially decrease metabolic derangements. Pharmacological activation of PPAR γ using rosiglitazone was previously shown to decrease chronic HYP-induced PH, right ventricular hypertrophy, and vessel thickness (24). Taken together, these considerations suggest that therapeutic strategies that target PPAR γ have the potential to mitigate the fundamental metabolic derangements that cause vascular cell hyperproliferation and vascular remodeling during PH pathogenesis. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Sherry Adesina, Ph.D., for her contributions to Figure 2, and Dr. Russ Price (Emory University) for providing the adenovirus overexpressing PGC1 α and GFP. They also thank Brian C. Brockway, M.S. (medical illustrator at Medical Media Services, Atlanta VA Medical Center), and Brandy E. Wade, Ph.D., for their contributions to the illustration depicted in Figure 7.

Note Added in Proof: While the current paper was under review, Calvier and colleagues (47) reported that PPAR γ also regulates PASMC proliferation through modulation of TGF β -induced alterations in signaling and cellular metabolism.

References

- Humbert M, Sitbon O, Chaouat A, Bertocchi M, Habib G, Gressin V, *et al.* Pulmonary arterial hypertension in France: results from a national registry. *Am J Respir Crit Care Med* 2006;173:1023–1030.
- Morrell NW, Adnot S, Archer SL, Dupuis J, Jones PL, MacLean MR, *et al.* Cellular and molecular basis of pulmonary arterial hypertension. *J Am Coll Cardiol* 2009;54(1, Suppl):S20–S31.
- Archer SL, Weir EK, Wilkins MR. Basic science of pulmonary arterial hypertension for clinicians: new concepts and experimental therapies. *Circulation* 2010;121:2045–2066.
- Ahmadian M, Suh JM, Hah N, Little C, Atkins AR, Downes M, *et al.* PPAR γ signaling and metabolism: the good, the bad and the future. *Nat Med* 2013;19:557–566.
- Green DE, Sutliff RL, Hart CM. Is peroxisome proliferator-activated receptor gamma (PPAR γ) a therapeutic target for the treatment of pulmonary hypertension? *Pulm Circ* 2011; 1:33–47.
- Ameshima S, Golpon H, Cool CD, Chan D, Vandivier RW, Gardai SJ, *et al.* Peroxisome proliferator-activated receptor gamma (PPAR γ) expression is decreased in pulmonary hypertension and affects endothelial cell growth. *Circ Res* 2003;92:1162–1169.
- Tian J, Smith A, Nechtman J, Podolsky R, Aggarwal S, Snead C, *et al.* Effect of PPAR γ inhibition on pulmonary endothelial cell gene expression: gene profiling in pulmonary hypertension. *Physiol Genomics* 2009;40:48–60.
- Bijli KM, Kleinhenz JM, Murphy TC, Kang BY, Adesina SE, Sutliff RL, *et al.* Peroxisome proliferator-activated receptor gamma depletion stimulates Nox4 expression and human pulmonary artery smooth muscle cell proliferation. *Free Radic Biol Med* 2015;80:111–120.
- Green DE, Murphy TC, Kang BY, Searles CD, Hart CM. PPAR γ ligands attenuate hypoxia-induced proliferation in human pulmonary artery smooth muscle cells through modulation of microRNA-21. *PLoS One* 2015;10:e0133391.
- Kang BY, Park KK, Kleinhenz JM, Murphy TC, Green DE, Bijli KM, *et al.* Peroxisome proliferator-activated receptor γ and microRNA 98 in hypoxia-induced endothelin-1 signaling. *Am J Respir Cell Mol Biol* 2016;54:136–146.

11. Zhang D, Wang G, Han D, Zhang Y, Xu J, Lu J, *et al.* Activation of PPAR- γ ameliorates pulmonary arterial hypertension via inducing heme oxygenase-1 and p21(WAF1): an in vivo study in rats. *Life Sci* 2014;98:39–43.
12. Crossno JT Jr, Garat CV, Reusch JE, Morris KG, Dempsey EC, McMurtry IF, *et al.* Rosiglitazone attenuates hypoxia-induced pulmonary arterial remodeling. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L885–L897.
13. Hansmann G, Wagner RA, Schellong S, Perez VA, Urashima T, Wang L, *et al.* Pulmonary arterial hypertension is linked to insulin resistance and reversed by peroxisome proliferator-activated receptor- γ activation. *Circulation* 2007;115:1275–1284.
14. Hondares E, Mora O, Yubero P, Rodriguez de la Concepción M, Iglesias R, Giral M, *et al.* Thiazolidinediones and rexinoids induce peroxisome proliferator-activated receptor-coactivator (PGC)-1 α gene transcription: an autoregulatory loop controls PGC-1 α expression in adipocytes via peroxisome proliferator-activated receptor- γ coactivation. *Endocrinology* 2006;147:2829–2838.
15. Bonnet S, Michelakis ED, Porter CJ, Andrade-Navarro MA, Thébaud B, Bonnet S, *et al.* An abnormal mitochondrial-hypoxia inducible factor-1 α -Kv channel pathway disrupts oxygen sensing and triggers pulmonary arterial hypertension in fawn hooded rats: similarities to human pulmonary arterial hypertension. *Circulation* 2006;113:2630–2641.
16. Fijalkowska I, Xu W, Comhair SA, Janocha AJ, Mavrikis LA, Krishnamachary B, *et al.* Hypoxia inducible-factor1 α regulates the metabolic shift of pulmonary hypertensive endothelial cells. *Am J Pathol* 2010;176:1130–1138.
17. Marsboom G, Toth PT, Ryan JJ, Hong Z, Wu X, Fang YH, *et al.* Dynamin-related protein 1-mediated mitochondrial mitotic fission permits hyperproliferation of vascular smooth muscle cells and offers a novel therapeutic target in pulmonary hypertension. *Circ Res* 2012;110:1484–1497.
18. Paulin R, Michelakis ED. The metabolic theory of pulmonary arterial hypertension. *Circ Res* 2014;115:148–164.
19. Ryan JJ, Marsboom G, Fang YH, Toth PT, Morrow E, Luo N, *et al.* PGC1 α -mediated mitofusin-2 deficiency in female rats and humans with pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2013;187:865–878.
20. Rao J, Li J, Liu Y, Lu P, Sun X, Sugumaran PK, *et al.* The key role of PGC-1 α in mitochondrial biogenesis and the proliferation of pulmonary artery vascular smooth muscle cells at an early stage of hypoxic exposure. *Mol Cell Biochem* 2012;367:9–18.
21. Lu X, Bijli KM, Ramirez A, Murphy TC, Kleinhenz J, Hart CM. Hypoxia downregulates PPAR γ via an ERK1/2-NF- κ B-Nox4-dependent mechanism in human pulmonary artery smooth muscle cells. *Free Radic Biol Med* 2013;63:151–160.
22. Lu X, Murphy TC, Nanes MS, Hart CM. PPAR γ regulates hypoxia-induced Nox4 expression in human pulmonary artery smooth muscle cells through NF- κ B. *Am J Physiol Lung Cell Mol Physiol* 2010;299:L559–L566.
23. Xu W, Janocha AJ, Leahy RA, Klatte R, Dudzinski D, Mavrikis LA, *et al.* A novel method for pulmonary research: assessment of bioenergetic function at the air-liquid interface. *Redox Biol* 2014;2:513–519.
24. Nisbet RE, Bland JM, Kleinhenz DJ, Mitchell PO, Walp ER, Sutliff RL, *et al.* Rosiglitazone attenuates chronic hypoxia-induced pulmonary hypertension in a mouse model. *Am J Respir Cell Mol Biol* 2010;42:482–490.
25. Taraseviciene-Stewart L, Kasahara Y, Alger L, Hirth P, Mc Mahon G, Waltenberger J, *et al.* Inhibition of the VEGF receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension. *FASEB J* 2001;15:427–438.
26. Bhalla K, Hwang BJ, Dewi RE, Ou L, Twaddell W, Fang HB, *et al.* PGC1 α promotes tumor growth by inducing gene expression programs supporting lipogenesis. *Cancer Res* 2011;71:6888–6898.
27. Casanova E, Baselga-Escudero L, Ribas-Latre A, Arola-Arnal A, Blade C, Arola L, *et al.* Epigallocatechin gallate counteracts oxidative stress in docosahexaenoic acid-treated myocytes. *Biochim Biophys Acta* 2014;1837:783–791.
28. Singh S, Simpson RL, Bennett RG. Relaxin activates peroxisome proliferator-activated receptor γ (PPAR γ) through a pathway involving PPAR γ coactivator 1 α (PGC1 α). *J Biol Chem* 2015;290:950–959.
29. Stenmark KR, Meyrick B, Galie N, Mooi WJ, McMurtry IF. Animal models of pulmonary arterial hypertension: the hope for etiological discovery and pharmacological cure. *Am J Physiol Lung Cell Mol Physiol* 2009;297:L1013–L1032.
30. Kim EK, Lee JH, Oh YM, Lee YS, Lee SD. Rosiglitazone attenuates hypoxia-induced pulmonary arterial hypertension in rats. *Respirology* 2010;15:659–668.
31. Meloche J, Courchesne A, Barrier M, Carter S, Bisserier M, Paulin R, *et al.* Critical role for the advanced glycation end-products receptor in pulmonary arterial hypertension etiology. *J Am Heart Assoc* 2013;2:e005157.
32. Shertzer HG, Krishan M, Genter MB. Dietary whey protein stimulates mitochondrial activity and decreases oxidative stress in mouse female brain. *Neurosci Lett* 2013;548:159–164.
33. Larsson NG, Wang J, Wilhelmsson H, Oldfors A, Rustin P, Lewandoski M, *et al.* Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nat Genet* 1998;18:231–236.
34. Green DE, Murphy TC, Kang BY, Bedi B, Yuan Z, Sadikot RT, *et al.* Peroxisome proliferator-activated receptor- γ enhances human pulmonary artery smooth muscle cell apoptosis through microRNA-21 and programmed cell death 4. *Am J Physiol Lung Cell Mol Physiol* 2017;313:L371–L383.
35. Sampson MJ, Lovell RS, Craigen WJ. Isolation, characterization, and mapping of two mouse mitochondrial voltage-dependent anion channel isoforms. *Genomics* 1996;33:283–288.
36. Isarangkul D, Wiyakrutta S, Kengkoom K, Reamtong O, Ampawong S. Mitochondrial and cytoskeletal alterations are involved in the pathogenesis of hydronephrosis in ICR/Mac-hydro mice. *Int J Clin Exp Med* 2015;8:9192–9204.
37. Rafikov R, Sun X, Rafikova O, Meadows ML, Desai AA, Khalpey Z, *et al.* Complex I dysfunction underlies the glycolytic switch in pulmonary hypertensive smooth muscle cells. *Redox Biol* 2015;6:278–286.
38. Rabinovitch M. PPAR γ and the pathobiology of pulmonary arterial hypertension. *Adv Exp Med Biol* 2010;661:447–458.
39. El Chami H, Hassoun PM. Immune and inflammatory mechanisms in pulmonary arterial hypertension. *Prog Cardiovasc Dis* 2012;55:218–228.
40. Huertas A, Perros F, Tu L, Cohen-Kaminsky S, Montani D, Dorfmueller P, *et al.* Immune dysregulation and endothelial dysfunction in pulmonary arterial hypertension: a complex interplay. *Circulation* 2014;129:1332–1340.
41. Rabinovitch M, Guignabert C, Humbert M, Nicolls MR. Inflammation and immunity in the pathogenesis of pulmonary arterial hypertension. *Circ Res* 2014;115:165–175.
42. Diebold I, Hennigs JK, Miyagawa K, Li CG, Nickel NP, Kaschwich M, *et al.* BMPR2 preserves mitochondrial function and DNA during reoxygenation to promote endothelial cell survival and reverse pulmonary hypertension. *Cell Metab* 2015;21:596–608.
43. Paddenberger R, Stieger P, von Lilien AL, Faulhammer P, Goldenberg A, Tillmanns HH, *et al.* Rapamycin attenuates hypoxia-induced pulmonary vascular remodeling and right ventricular hypertrophy in mice. *Respir Res* 2007;8:15.
44. Quinlan TR, Li D, Laubach VE, Shesely EG, Zhou N, Johns RA. eNOS-deficient mice show reduced pulmonary vascular proliferation and remodeling to chronic hypoxia. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L641–L650.
45. Sheikh AQ, Lighthouse JK, Greif DM. Recapitulation of developing artery muscularization in pulmonary hypertension. *Cell Reports* 2014;6:809–817.
46. Zhao Y, Peng J, Lu C, Hsin M, Mura M, Wu L, *et al.* Metabolomic heterogeneity of pulmonary arterial hypertension. *PLoS One* 2014;9:e88727.
47. Calvier L, Chouvarine P, Legchenko E, Hoffmann N, Geldner J, Borchert P, *et al.* PPAR γ links BMP2 and TGF β 1 pathways in vascular smooth muscle cells, regulating cell proliferation and glucose metabolism. *Cell Metab* 2017;25:1118–1134.