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PPARγ depletion stimulates Nox4 expression and human pulmonary artery smooth muscle cell proliferation

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Abstract

Hypoxia stimulates pulmonary hypertension (PH) in part by increasing the proliferation of pulmonary vascular wall cells. Recent evidence suggests that signaling events involved in hypoxia-induced cell proliferation include sustained nuclear factor-kappaB (NF-κB) activation, increased NADPH oxidase 4 (Nox4) expression, and downregulation of peroxisome proliferator-activated receptor gamma (PPARγ) levels. To further understand the role of reduced PPARγ levels associated with PH pathobiology, siRNA was employed to reduce PPARγ levels in human pulmonary artery smooth muscle cells (HPASMC) *in vitro* under normoxic conditions. PPARγ protein levels were reduced to levels comparable to those observed under hypoxic conditions. Depletion of PPARγ for 24 - 72 hours activated mitogen-activated protein kinase, ERK 1/2, and NF-κB. Inhibition of ERK 1/2 prevented NF-κB activation caused by PPARγ depletion indicating that ERK 1/2 lies upstream of NF-κB activation. Depletion of PPARγ for 72 hours increased NF-κB-dependent Nox4 expression and H_2O_2 production. Inhibition of NF-κB or Nox4 attenuated PPARγ depletion-induced HPASMC proliferation. Degradation of PPARγ depletion-induced H_2O_2 by PEG-catalase prevented HPASMC proliferation and also ERK 1/2 and NF-κB activation and Nox4 expression indicating that H_2O_2 participates in feed-forward activation of above signaling events. Contrary to the effects of PPARγ depletion, HPASMC PPARγ overexpression reduced ERK 1/2 and NF-κB activation, Nox4 expression and cell proliferation. Taken together these findings provide novel evidence that PPARγ plays a central role in the regulation of the ERK1/2-NF-κB-Nox4-H_2O_2 signaling axis in HPASMC. These results indicate that reductions in PPARγ caused by pathophysiological stimuli such as prolonged hypoxia exposure are sufficient to promote the proliferation of pulmonary vascular smooth muscle cells observed in PH pathobiology.

Keywords

PPARγ; NF-κB; ERK 1/2; Nox4; pulmonary hypertension; pulmonary artery smooth muscle cell

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Introduction

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors that play important roles in cell metabolism, growth, differentiation, and inflammation via regulation of a large number of gene networks [1, 2]. Three PPAR isoforms, α, β, and γ are expressed in tissue-specific patterns. Upon activation by endogenous or exogenous ligands, PPARs form heterodimers with the 9-cis retinoic acid receptor (RXR-α) and bind to peroxisome proliferator response elements (PPRE) in the promoter regions of target genes to stimulate their expression [3, 4]. Activation of PPARγ can also cause transrepression of other pro-inflammatory transcription factors [5]. Because PPARγ is expressed in numerous cells including pulmonary vascular endothelial and smooth muscle cells, the goal of the current study was to further explore the role of PPARγ in pulmonary vascular smooth muscle cell function [6, 7].

Pulmonary hypertension (PH) is characterized by increases in pulmonary artery pressure and pulmonary vascular resistance that cause significant morbidity and mortality [8]. Growing evidence supports the role of PPARγ in pulmonary vascular regulation. PPARγ activation with exogenous synthetic thiazolidinedione ligands attenuates PH and pulmonary vascular remodeling in several experimental models of PH [9-13], whereas loss of PPARγ expression is associated with PH. Expression of PPARγ is reduced in the lungs of rodents with PH caused by chronic hypoxia [11, 13]. Reduced PPARγ expression has also been observed in the vascular lesions of patients with idiopathic pulmonary arterial hypertension, and in a rat model of severe PH caused by treatment with hypoxia and a VEGF receptor antagonist [14]. Levels of PPARγ are attenuated in pulmonary artery endothelial cells isolated from patients with idiopathic pulmonary arterial hypertension [15]. Furthermore, targeted and constitutive genetic ablation of PPARγ from endothelial [16] or vascular smooth muscle cells [17] is associated with the development of spontaneous PH in mice. Taken together, these reports suggest that activation of PPARγ attenuates pulmonary vascular dysfunction and PH whereas reductions in PPARγ contribute to PH pathogenesis.

Hypoxia reduces PPARγ expression and activity via activation of oxidative stress signals [13]. Hypoxia increases Nox4 expression in the pulmonary vasculature [18], and Nox4-derived H_2O_2 reduces PPARγ expression and activity in PASMC [19], and H_2O_2 similarly reduces PPARγ in endothelial cells *in vitro* [20]. Hypoxia activates both mitogen-activated protein kinases that regulate PPARγ transcriptional activity and the pro-inflammatory transcription factor, NF-κB [21, 22]. For example, hypoxia increases Nox4 expression in HPASMC by stimulating NF-κB p65 binding to the Nox4 promoter [23]. Recent findings from our laboratory demonstrate that hypoxia induces ERK-mediated-NF-κB activation, Nox4 expression, H_2O_2 generation and PPARγ downregulation in HPASMCs and that Nox4-derived H_2O_2 is in turn required for ERK 1/2 activation suggesting the existence of cyclic signaling cascades underlying chronic hypoxia-induced derangements in pulmonary vascular wall cells [19]. Although these studies clarify mechanisms involved in hypoxia-induced reductions in PPARγ expression, the downstream signaling events attributable to PPARγ downregulation are not well defined. Therefore, the current study explores the

ability of reductions in PPARγ to stimulate proliferative signaling mechanisms associated with hypoxia-induced PH pathobiology.

Our findings demonstrate that loss of PPARγ is sufficient to promote HPASMC proliferation through ERK1/2-NF-κB-Nox4 dependent H_2O_2 generation. Taken together with previous reports, these findings further emphasize the importance of PPARγ in pulmonary vascular cell biology and elucidate mechanistic pathways by which stimuli that reduce PPARγ stimulate derangements in PASMC function. We postulate that sustained activation of these pathways caused by PPARγ downregulation contributes to PH pathobiology. Strategies targeting suppression or reversal of these pathways may preserve PPARγ function in the pulmonary vascular wall and provide a novel therapeutic strategy in PH.

Materials and Methods

Reagents

The ERK 1/2 inhibitor (PD98059) and PEG-catalase were purchased from Calbiochem (La Jolla, CA) and Sigma-Aldrich (St. Louis, MO), respectively. Antibodies against phospho-(Thr202/Tyr204)-ERK 1/2, total ERK 1/2, and phospho-(Ser536)-NF-κB were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against PPARγ, total NF-κB, IκBα, Nox4, and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against PGC-1α was purchased from Millipore (Billerica, MA). Antibody against GAPDH was purchased from Sigma-Aldrich (St. Louis, MO). All other materials were purchased from VWR Scientific Corp. (Gaithersburg, MD) and Fisher Scientific (Pittsburg, PA). The Nox4 inhibitor, GKT137831 was obtained through a material transfer agreement from GenKyoTex (Geneva, Switzerland).

Cell Culture and siRNA transfections

Human pulmonary artery smooth muscle cells (HPASMC) were purchased from Lonza (Basel, Switzerland). HPASMC monolayers (passages 3-4) were grown at 37°C in a 5% CO_2 atmosphere in culture media (SmGM-2, Lonza) containing 2% fetal calf serum, growth factors, and antibiotics as previously reported [19]. Upon reaching 50-60% confluency, the cells were transfected with 50-100 nM non-targeting siRNA (control siRNA) or siRNA targeting human PPARγ using Dharmafect transfection reagent (Dharmacon, Waltham, MA) for 12 hours. Cells were then washed with serum-free media and recovered for 24 - 72 hours in complete growth media under normoxic conditions (21% O_2, 5% CO_2) at 37°C in a cell culture incubator.

Overexpression of PPARγ in HPASMC

HPASMC monolayers were grown at 37°C in a 5% CO_2 atmosphere in culture media (SmGM-2, Lonza) containing 5% fetal calf serum, growth factors, and antibiotics. Human PPARγ in adenovirus (Ad-hPPARγ) or Ad-GFP (Vector Biolabs, Philadelphia, PA) were applied to cells at 3-28 MOI (Ad-GFP was applied at 3 MOI and Ad-PPARγ was applied at 28 MOI) for 4 hours in 2% FBS media. Media were replaced with fresh SmGM-2 media, and HPASMC were cultured for 72 hrs.

Cell proliferation assays

HPASMC proliferation was determined with a quantitative colorimetric assay employing dimethylthiazol (MTT assay; ATCC) as described earlier [19]. Briefly, cells transfected with non-targeting control siRNA (si-Con) or with siRNA against PPARγ (si-PPARγ) were treated with or without PEG-catalase (1000 u/ml) during the last 24 hours of the 72 hour recovery period. The cells were then incubated with the MTT reagent for 4 hours. The mitochondrial reductase present in living cells reduces MTT to purple formazan, which is detected by spectrophotometry. Samples were then analyzed using an ELISA plate reader (λ = 570 nm), and values from treated cells were normalized to values from corresponding control cells. To assess proliferation by cell counting, HPASMCs were counted using a hemocytometer and cell viability was determined by Trypan blue exclusion assay as described previously [24]. We previously reported that MTT assays and cell counting methods produced similar results in hypoxia-exposed pulmonary vascular wall cells [24].

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA), and RNA was quantified by Nanodrop spectrophotometry (Thermo Scientific, Wilmington, DE). cDNA was prepared using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative PCR was performed to assess the expression level of PPARγ1 RNA using primers based on human PPARγ1 mRNA sequence: F, 5′-gtggc cgcag atttg aaaga ag-3′ and R, 5′-tgtca accat ggtca tttcg-3′. Real-time PCR was performed using iQ SYBR Green Supermix and the iCycler real-time PCR detection system (Bio-Rad). Amplicon expression in each sample was normalized to 9s RNA levels. The relative abundance of target mRNA in each sample was calculated using ΔΔCT methods (Applied Biosystems, Carlsbad, CA).

Western blot analysis

HPASMC protein lysates were resolved by SDS-PAGE and subjected to Western blot analysis for PPARγ, phospho-ERK 1/2, phospho-NF-κB/p65, Nox4 or PGC-1α and normalized to their respective total forms (non-phosphorylated) or β-actin or GAPDH as appropriate. Relative levels of immunoreactive proteins were quantified using the Licor or the ChemiDoc XRS imaging systems and Quantity One software (Bio-Rad Laboratories).

Amplex Red H_2O_2 assay

H_2O_2 production was measured with Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Molecular Probes, Eugene, OR). The assay is based on the detection of H_2O_2 which reacts with 1:1 stoichiometry with Amplex® Red reagent in combination with horseradish peroxidase to produce highly fluorescent resorufin red. H_2O_2 released from HPASMC was quantified by fluorometric detection on a plate reader (λ_{ex} = 590 nm and λ_{em} = 560 nm), followed by plotting sample values against a standard curve containing known concentrations of H_2O_2. The concentration of H_2O_2 was normalized to the total protein concentration of each sample.

Statistical analysis

When comparing more than 2 groups, data were analyzed using analysis of variance (ANOVA). Post hoc analysis using the Student Neuman Keuls test was employed to detect differences between individual groups. In studies comparing only two experimental groups, data were analyzed with Student's t-test to determine significance of treatment effects. The level of statistical significance was taken as $p<0.05$.

Results

Depletion of PPARγ activates ERK 1/2 in HPASMC

Hypoxia promotes proliferation of HPASMC through reduction in PPARγ levels via upstream activation of ERK 1/2 followed by activation of NF-κB, Nox4 expression and H_2O_2 generation [19, 23]. In the current study, we further hypothesized that reduced PPARγ levels in HPASMCs promote sustained NF-κB activation and cell proliferation associated with chronic hypoxia. To address this possibility, siRNA was used to deplete PPARγ levels in HPASMCs under normoxic conditions (21% O_2, 5% CO_2). To mimic alterations in HPASMC caused by exposure to hypoxia for 72 hours, HPASMC were transfected with control or PPARγ siRNA for 24 or 72 hours. As shown in Figure 1A, PPARγ mRNA levels were significantly reduced 72 hours following transfection with PPARγ siRNA. Furthermore, compared to control siRNA, treatment with PPARγ siRNA significantly reduced PPARγ protein levels by approximately 25 to 50% at both 24 and 72 hours transfection duration (Figs. 1B and C). These levels of PPARγ depletion are comparable in magnitude to those observed in hypoxia-exposed HPASMC [19]. The impact of PPARγ protein depletion with siRNA was confirmed by immunoblotting for its target gene, PGC-1α (peroxisome proliferator-activated receptor-gamma coactivator) [Fig 1C].

Because hypoxia reduced PPARγ levels and activated HPASMC ERK 1/2 [19], the effect of PPARγ depletion on ERK 1/2 activation was examined. PPARγ depletion enhanced ERK 1/2 activation detected as Thr202/Tyr204 phosphorylation at both 24 and 72 hours of siRNA transfection indicating that PPARγ downregulation promotes early and sustained MAPK activation (Figs. 2A and B). Compared to untransfected cells, neither transfection reagent alone nor si-Con significantly altered phospho-ERK 1/2 levels (data not shown).

PPARγ depletion promotes ERK 1/2-mediated activation of NF-κB in HPASMC

PPARγ depletion for 72 hours stimulated degradation of IκBα, an NF-κB pathway inhibitory protein (Fig. 3A). In addition to IκBα degradation, a critical determinant of NF-κB activation is its phosphorylation at serine residue(s) (Ser536, 276, 529, 311) required for its complete transactivation [25-28]. We previously reported that hypoxia reduces PPARγ and stimulates phosphorylation of NF-κB p65 at Ser536 in HPASMC [19]. Therefore the effect of normoxic PPARγ depletion on the phosphorylation status of NF-κB p65 at Ser536 was examined. Compared to control cells, PPARγ depletion promoted phosphorylation of NF-κB p65 at both 24 and 72 hours of siRNA transfection (Figs. 3B and C). Thus, consistent with ERK 1/2 activation, PPARγ depletion promotes both early and sustained activation of NF-κB. Since hypoxia promotes NF-κB activation in HPASMC through the activation of ERK 1/2 [19], the possibility that ERK 1/2 could also mediate PPARγ

depletion-induced NF-κB activation was examined. As observed in Figure 3C, pharmacological inhibition of ERK 1/2 using PD98059 during the last 24 hours of the 72 hours transfection period significantly attenuated phosphorylation of NF-κB p65 in PPARγ depleted cells.

PPARγ depletion promotes NF-κB-dependent Nox4 expression and Nox4-dependent H_2O_2 generation in HPASMC

We previously reported that exposure to hypoxia for 72 hours reduced PPARγ expression and stimulated Nox4 expression through an NF-κB- dependent pathway in HPASMC [19, 23]. To further examine the role of PPARγ depletion alone in these signaling events, the current study examined HPASMC after 72 hours of treatment with si-PPARγ. Consistent with NF-κB activation (Figs. 3A-C), a significant increase in Nox4 protein expression was observed in PPARγ depleted cells compared to untreated or si-Con cells (Fig. 4A). Furthermore, pharmacological inhibition of NF-κB using CAPE (caffeic acid phenethyl ester) compound attenuated Nox4 protein expression caused by PPARγ depletion (Fig. 4A) suggesting that NF-κB regulates Nox4 expression in this response. Because Nox4 is a major source of hypoxia-induced H_2O_2 generation in HPASMC [15, 19, 23], the effect of PPARγ depletion on H_2O_2 generation was examined. Consistent with increases in Nox4 expression levels, PPARγ depletion for 72 hours caused a significant increase in HPASMC H_2O_2 generation. Evidence that Nox4 upregulation increased HPASMC H_2O_2 generation was provided by studies using a novel Nox4 inhibitor, GKT137831. Treatment with GKT137831 significantly attenuated H_2O_2 generation caused by PPARγ depletion (Fig. 4B).

PPARγ depletion promotes HPASMC proliferation

Given the requirement for H_2O_2 in hypoxia-induced proliferation of pulmonary vascular wall cells [19], we next examined whether PPARγ depletion promotes proliferation of HPASMC via an H_2O_2-dependent mechanism. Determination of HPASMC proliferation by MTT assay revealed that PPARγ depletion increases HPASMC proliferation and that degradation of H_2O_2 by PEG-catalase treatment during the final 24 hours of the 72 hour transfection period attenuated proliferation of PPARγ-depleted cells (Fig. 5A). Cell proliferation caused by PPARγ depletion was also verified by cell counting which produced results similar to those observed with the MTT assay (Fig. 5B). Consistent with these observations, pharmacological inhibition of NF-κB or Nox4 significantly attenuated HPASMC proliferation caused by PPARγ depletion (Fig. 5C).

PPARγ depletion-induced H_2O_2 production mediates feed-forward ERK 1/2 and NF-κB activation and Nox4 expression

To further understand the role of H_2O_2 generated by PPARγ depletion in HPASMC signaling, HPASMC transfected with si-Con or siPPARγ were treated with PEG-catalase (Pcat) to degrade H_2O_2 or with DMSO as a control vehicle during the final 24 hours of the 72 hours transfection period. Immunoblots of cell lysates revealed that Pcat attenuated ERK 1/2 and NF-κB activation and Nox4 protein expression caused by PPARγ depletion suggesting that H_2O_2 mediates sustained activation of these signaling events in HPASMC proliferation (Fig. 6A-C).

Overexpression of PPARγ inhibits ERK 1/2 and p65 activation, Nox4 protein expression and HPASMC proliferation

To provide proof-of-concept for the regulatory role of PPARγ in these signaling pathways, reciprocal experiments were performed defining the impact of PPARγ overexpression on ERK 1/2 and p65 activation and Nox4 protein expression in HPASMC. As expected, adenoviral-mediated PPARγ overexpression in HPASMC reduced basal ERK and p65 activation, Nox4 expression and cell proliferation (by manual cell counting) compared to control cells (Figs. 7A-D). Cell proliferation was also verified by MTT assay which yielded similar results (data not shown).

Discussion

Excessive proliferation of pulmonary vascular wall cells with increases in pulmonary vascular remodeling and resistance are crucial hallmarks of pulmonary hypertension (PH) pathogenesis. The nuclear hormone receptor, PPARγ plays an important role in normal vascular function [7, 12, 15]. PPARγ levels are reduced in lungs of various rodent models of hypoxia-induced PH and in vascular cells and lesions of patients with idiopathic pulmonary hypertension [11, 13-15]. However, the contribution of reduced PPARγ levels to the pathobiology of PH continues to be defined. We recently demonstrated that hypoxia reduced PPARγ protein and stimulated HPASMC proliferation via sustained activation of ERK 1/2 and NF-κB, which increased Nox4 protein expression and H_2O_2 generation [19]. To better understand the contributions of altered PPARγ expression to vascular cell signaling and proliferation, the current study employed siRNA to reduce PPARγ in normoxic HPASMC and investigate downstream pathways that cause HPASMC proliferation.

Exposure to hypoxia (1% O_2) for 72 hours reduced HPASMC PPARγ protein levels by approximately 50% [19]. To mimic this observation, the current study employed siRNA to induce comparable reductions in PPARγ protein levels under normoxic conditions (Figs. 1B and C). The increase in HPASMC proliferation caused by PPARγ depletion (1.34 fold) was similar to that observed under hypoxic conditions (1.5 fold) [19]. The MAP kinase, ERK1/2, is required for cell proliferation and growth [29], and increased ERK1/2 activity mediates PPARγ phosphorylation and proteosomal degradation leading to decreased PPARγ expression and transcriptional activity [30, 31]. Using HPASMC, we previously demonstrated that hypoxic ERK 1/2 activation was required for downregulation of PPARγ expression and transcriptional activity [19]. Further, neither inhibition of p38 nor JNK altered hypoxia-induced reductions in PPARγ expression and activity [19]. Therefore, in the current study, we focused on ERK 1/2 activation during PPARγ depletion. Our findings demonstrate that 24 or 72 hrs of PPARγ depletion is sufficient to induce ERK 1/2 activation (Fig. 2). Taken together, these findings suggest that potential feed forward cycles of PPARγ depletion and ERK1/2 activation contribute to altered smooth muscle cell phenotypes.

Hypoxia also activated NF-κB p65 which binds to the Nox4 promoter to stimulate H_2O_2 generation and HPASMC proliferation [19, 23]. In the current study, siRNA-mediated loss of PPARγ at both 24 and 72 hrs caused a significant increase in p65 phosphorylation (Fig. 3), which indicates that PPARγ depletion engages upstream kinases to mediate p65 phosphorylation and NF-κB activation. Based on the observed ERK 1/2 activation (Fig. 2),

we addressed the effect of ERK 1/2 inhibition on p65 phosphorylation caused by PPARγ depletion. Indeed pharmacological inhibition of ERK 1/2 with PD98059 significantly attenuated p65 phosphorylation caused by PPARγ depletion (Fig. 3C) suggesting that ERK 1/2 lies upstream of p65 to mediate this effect.

Previous evidence demonstrated that Nox4 constitutes an important downstream target of hypoxic signaling. Hypoxia stimulated ERK 1/2 and NF-κB activation to increase Nox4 expression in HPASMC, and Nox4-derived H_2O_2 reduced PPARγ expression and promoted HPASMC proliferation [19, 23]. In addition, pharmacological inhibition of Nox4 attenuated experimental PH and reductions in PPARγ *in vitro* and *in vivo* [15]. The current study therefore sought to determine if reductions in PPARγ were not only sufficient to activate signaling and transcriptional pathways such as ERK1/2 and NF-κB, respectively but also capable of modulating the expression of Nox4. Our results demonstrate that PPARγ depletion not only stimulated ERK 1/2 and p65 activation but also increased Nox4 protein expression, H_2O_2 generation, and HPASMC proliferation (Figs. 4 and 5). Our observations with GKT137831 are consistent with our previous findings in which Nox4 inhibition attenuated hypoxia-induced HPASMC proliferation [15]. GKT137831 is a novel Nox1/Nox4 inhibitor that is potent and orally bioavailable [32]. This compound was employed in the current study due to its ability to inhibit Nox4 activity, which is selectively up-regulated in the pulmonary vasculature under hypoxic conditions [18] and associated with hypoxia-induced HPASMC proliferation [23, 33], vascular remodeling, and pulmonary hypertension [13, 18].

Previously we reported that hypoxia-induced H_2O_2 production promoted cyclic ERK 1/2 activation and Nox4 expression in HPASMC [19]. Similar observations with PPARγ depletion in the current study (Fig. 6A-C) suggest that, under hypoxic conditions, H_2O_2 production remains sustained not only due to hypoxia-induced signaling events but also likely due to hypoxic-reductions in PPARγ levels. Such a sustained level of H_2O_2 is crucial for maintaining sustained ERK and NF-κB activation and Nox4 expression thereby contributing to PH pathology associated with chronic hypoxia. Taken together, the downregulation of ERK 1/2, p65 and Nox4 and reductions in cell proliferation caused by overexpression of PPARγ (Fig. 7) provides novel proof-of-concept on the regulatory role of PPARγ and is consistent with previous reports that the PPARγ ligand, rosiglitazone, attenuated hypoxia-induced HPASMC Nox4 expression [23]. The findings in the current study are consistent with the postulate that reductions in SMC PPARγ generate proliferative mediators in the pulmonary vasculature.

Previous work has suggested that targeted smooth muscle cell PPARγ depletion promotes PH *in vivo*. Mice with constitutive smooth muscle-targeted PPARγ depletion had elevated right ventricular systolic pressure (RVSP), right ventricular hypertrophy (RVH), and muscularization of small pulmonary arteries under normoxic conditions [17]. The current results provide novel insights into mechanisms by which loss of PPARγ function may contribute to PASMC proliferation and PH pathobiology. These results are also consistent with previous reports that endothelial-targeted loss of vascular PPARγ function enhanced aortic NF-κB activity [34]. PPARγ depletion also increased basal and stimulated systemic vascular SMC proliferation [35]. The current findings demonstrate that constitutive PPARγ

function limits the activity of pulmonary vascular wall signaling cascades that lead to the upregulation of proliferative pathways including Nox4. The current findings also support the previously reported mutually repressive relationship between PPARγ and NF-κB in vascular wall cells [19, 23]. Hypoxia downregulates PPARγ by post-transcriptional mechanisms via upregulation of miRNA27a [36] and via post-translational mechanisms by activating NF-κB which induces PPARγ suppression [19]. Although hypoxia-induced ERK activation is partly dependent on Nox4-derived H_2O_2 production [19], the exact signaling mechanisms upstream of ERK 1/2 are not well defined. However, based on evidence that chronic hypoxia increased calcium influx [37-39] and on our unpublished observations that the calcium-dependent kinase, Pyk2 is involved in hypoxic ERK 1/2 activation in HPASMC, we speculate that hypoxic increases in calcium play a proximal role in stimulating these proximal signaling events. As a master regulatory switch in metabolic function, alterations in PPARγ may also contribute to metabolic derangements in pulmonary vascular wall cells to mediate the glycolytic, apoptosis-resistant, hyperproliferative pulmonary vascular cell phenotype associated with the pathobiology of PH. As illustrated in Figure 8, we postulate that derangements in PPARγ play a critical role in pulmonary vascular smooth muscle cell proliferation. Targeted interruption of these feed-forward signaling cascades could provide novel therapeutic strategies to attenuate sustained signaling derangements and alterations in gene expression and redox signaling that promote pulmonary vascular cell phenotypic alterations and PH pathogenesis.

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Highlights

- Reduced PPARγ expression is sufficient to activate pathophysiological signaling mechanisms in pulmonary artery smooth muscle cells (PASMC).
- PPARγ depletion promotes ERK 1/2-dependent NF-κB activation and NF-κB-dependent Nox4 expression.
- PPARγ-depletion stimulates PASMC proliferation by increasing Nox4-derived H_2O_2.
- These observations demonstrate that reductions in PPARγ caused by hypoxia or other pathophysiological stimuli are sufficient to promote alterations in PASMC signaling and proliferation that can contribute to the pathobiology of pulmonary hypertension.

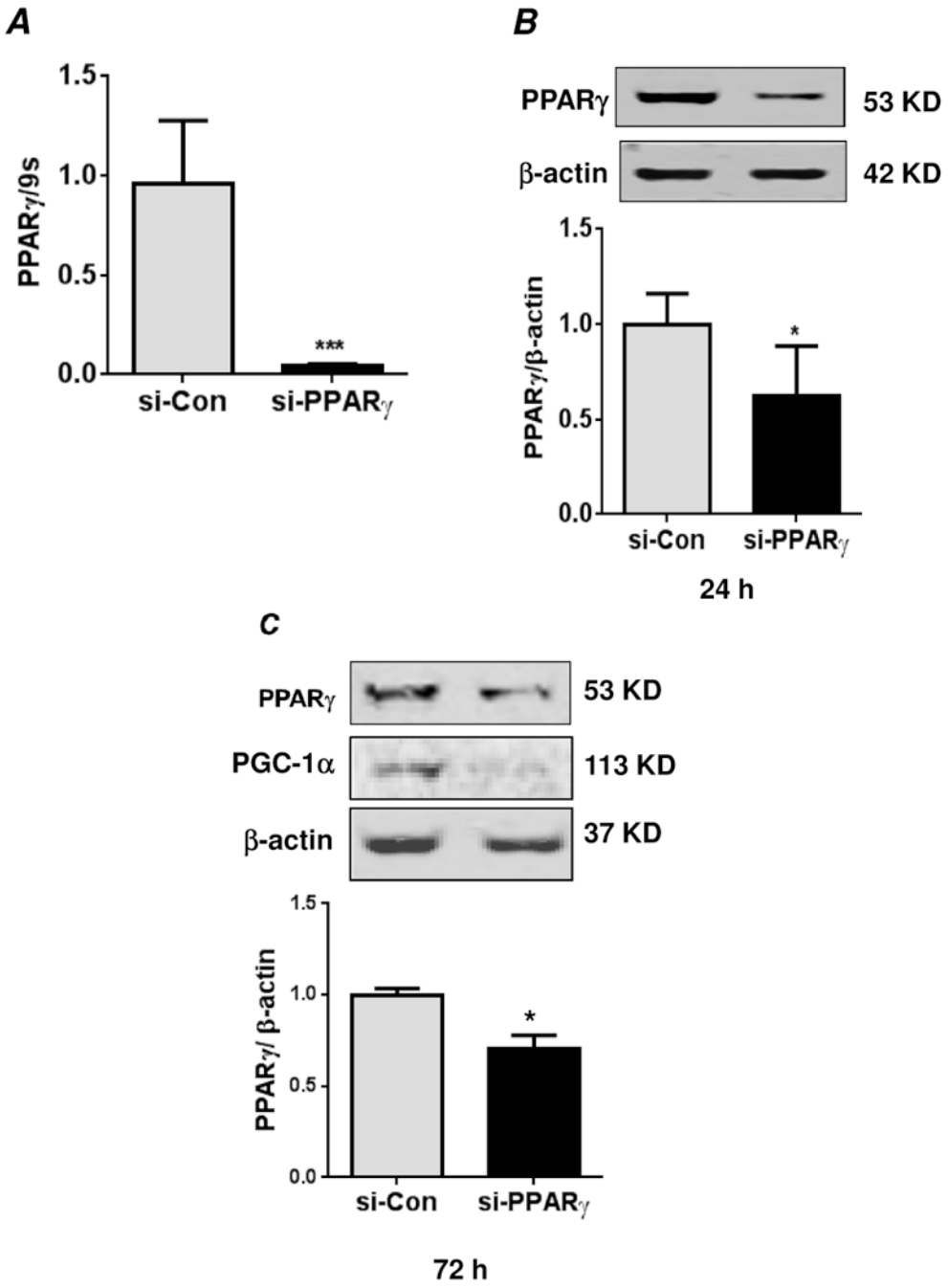

Figure 1. siRNA-mediated knockdown of PPARγ

(A) Human pulmonary artery smooth muscle cells (HPASMC) were transfected with control siRNA (si-Con) or PPARγ siRNA (si-PPARγ) under normoxic conditions (21% O_2 and 5% CO_2 at 37°C) for 72 hours. RNA was isolated, and PPARγ mRNA levels were determined by qRT-PCR. The mRNA levels were normalized to 9S ribosomal RNA. Each bar represents the mean ± SEM of levels of PPARγ mRNA relative to 9S ribosomal RNA in the same sample expressed as fold-change versus control. n=6, ***p<0.001. In related experiments, HPASMC were transfected with control siRNA or PPARγ siRNA under

normoxic conditions for 24 hours ***(B)*** or 72 hours ***(C)***. Cells were lysed and analyzed for PPARγ protein levels or its target gene, PGC-1α by western blot. The PPARγ protein levels were normalized with β-actin levels. Each bar represents the mean ± SEM of levels of PPARγ protein relative to β-actin in the same sample expressed as fold-change versus control. n=3, *p<0.05.

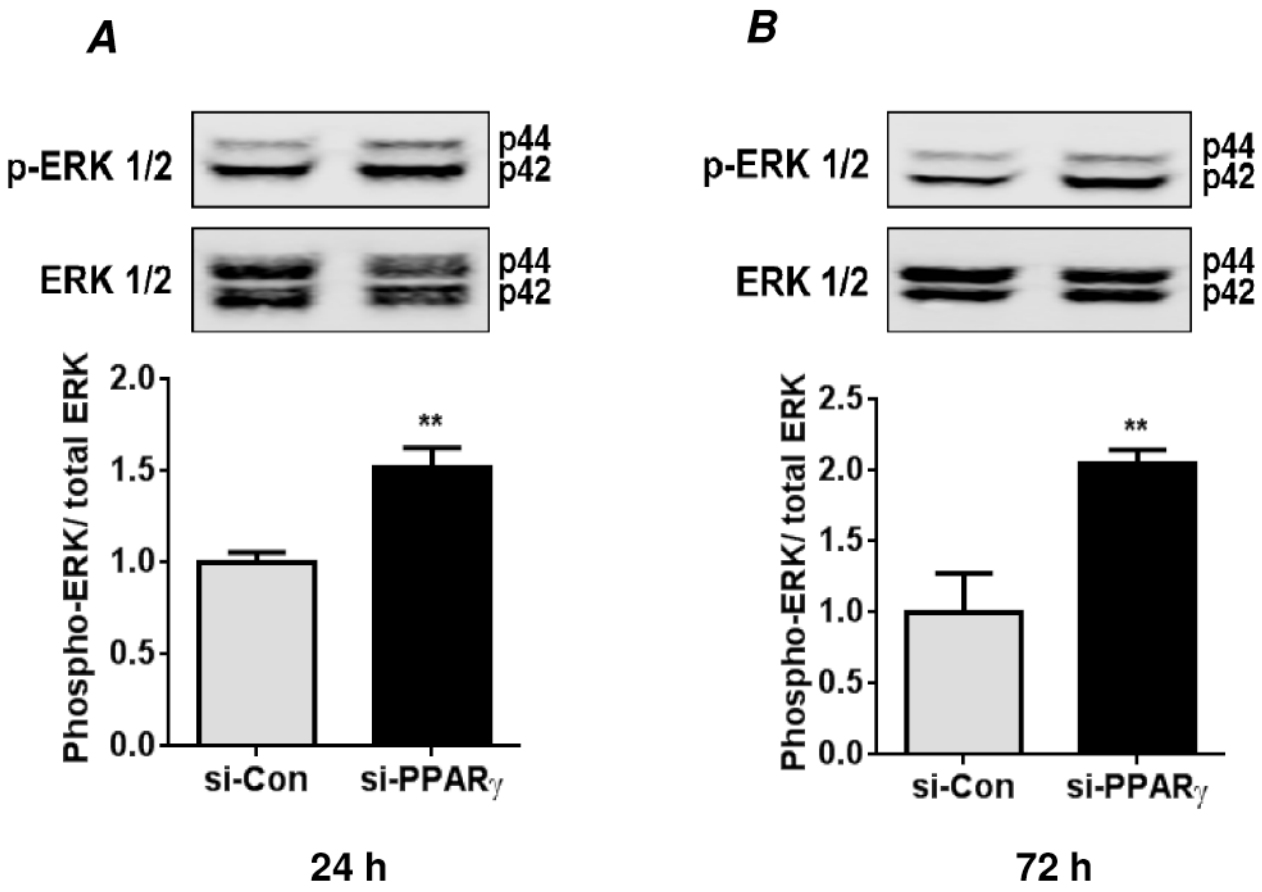

Figure 2. PPARγ knockdown promotes HPASMC ERK 1/2 activation

HPASMC were transfected with control siRNA (si-Con) or PPARγ siRNA (si-PPARγ) under normoxic conditions for 24 hours ***(A)*** or 72 hours ***(B)***. Cells were lysed and analyzed by western blot for activation of extracellular regulated kinase (ERK)-1/2 using an anti-phospho-(Thr202/Tyr204)-ERK1/2 antibody. The phospho-ERK 1/2 levels were normalized with total ERK 1/2 levels. Each bar represents levels of phospho-ERK 1/2 relative to total ERK 1/2 in the same sample expressed as fold-change versus control. n=3, **p<0.01.

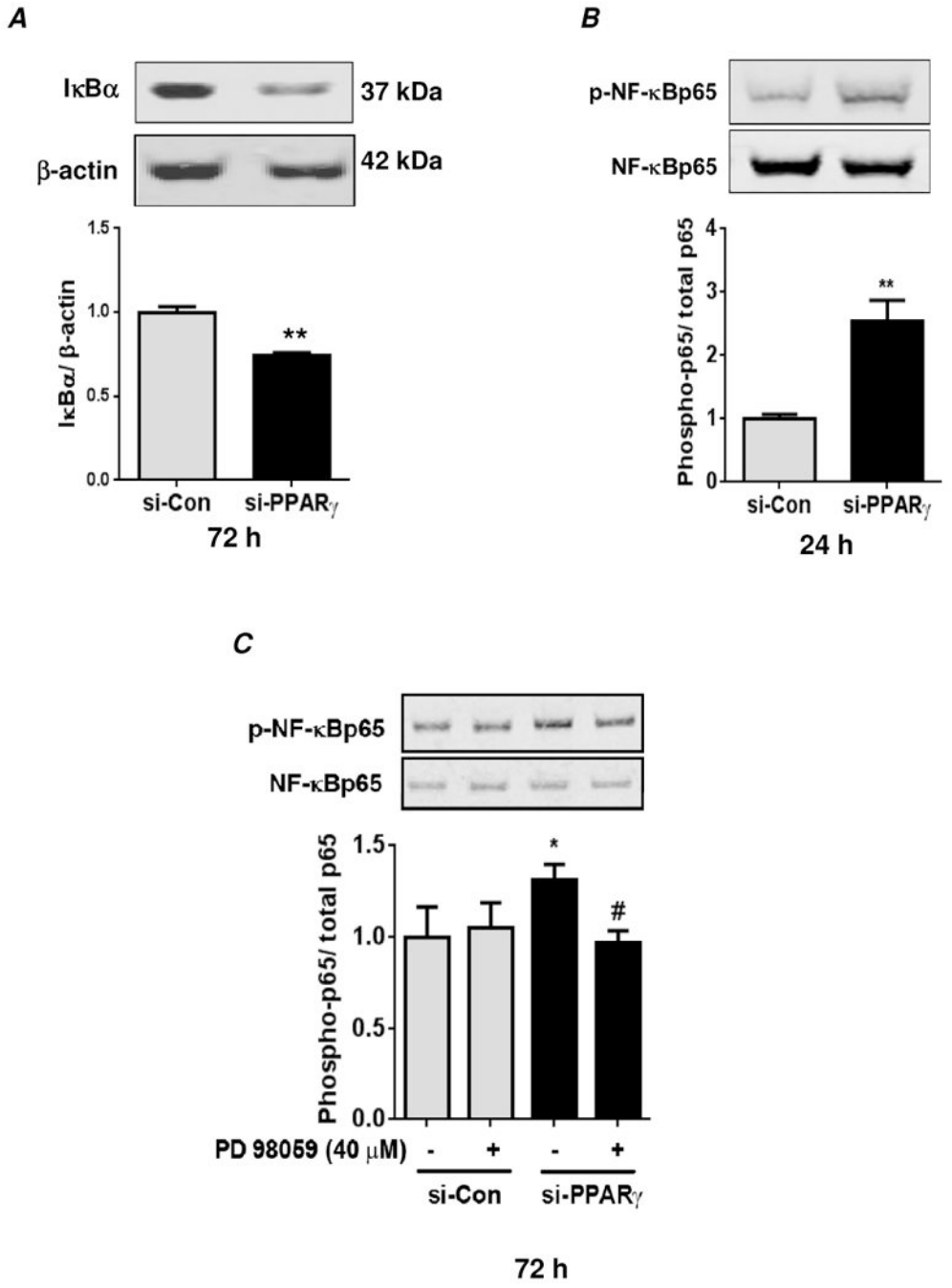

Figure 3. PPARγ knockdown in HPASMC promotes ERK 1/2-mediated activation of NF-κB

HPASMC were transfected with control siRNA (si-Con) or PPARγ siRNA (si-PPARγ) under normoxic conditions for 72 hours ***(A)*** or 24 hours ***(B)***. Cells were lysed and analyzed by western blot for IκBα ***(A)*** or phospho-p65 using an anti-phospho-(Ser536)-NF-κBp65 antibody ***(B)***. In ***(A)***, the levels of IκBα were normalized with β-actin. Each bar represents mean ± SEM IKBα levels relative to β-actin in the same sample expressed as fold-change versus control. n=3, **p<0.01. In ***(B)***, phospho-NF-κBp65 levels were normalized with total NF-κBp65 levels. Each bar represents mean ± SEM of phospho-NF-κBp65 levels relative to

total NF-κBp65 in the same sample expressed as fold-change versus control. n=3-6, **p<0.01. **(C)** Cells were transfected with siRNA as described above, and treated with the ERK 1/2 inhibitor, PD98059 (40 μM) or vehicle (DMSO) during the final 24 hours of the 72 hour transfection period. Cells were lysed and analyzed for phospho-NF-κBp65 by western blot as described above. Each bar represents mean ± SEM phospho-NF-κBp65 levels relative to total NF-κBp65 in the same sample expressed as fold-change versus control. n=3, *p<0.05 vs si-Con; $^{\#}$p<0.05 vs vehicle treated si-PPARγ.

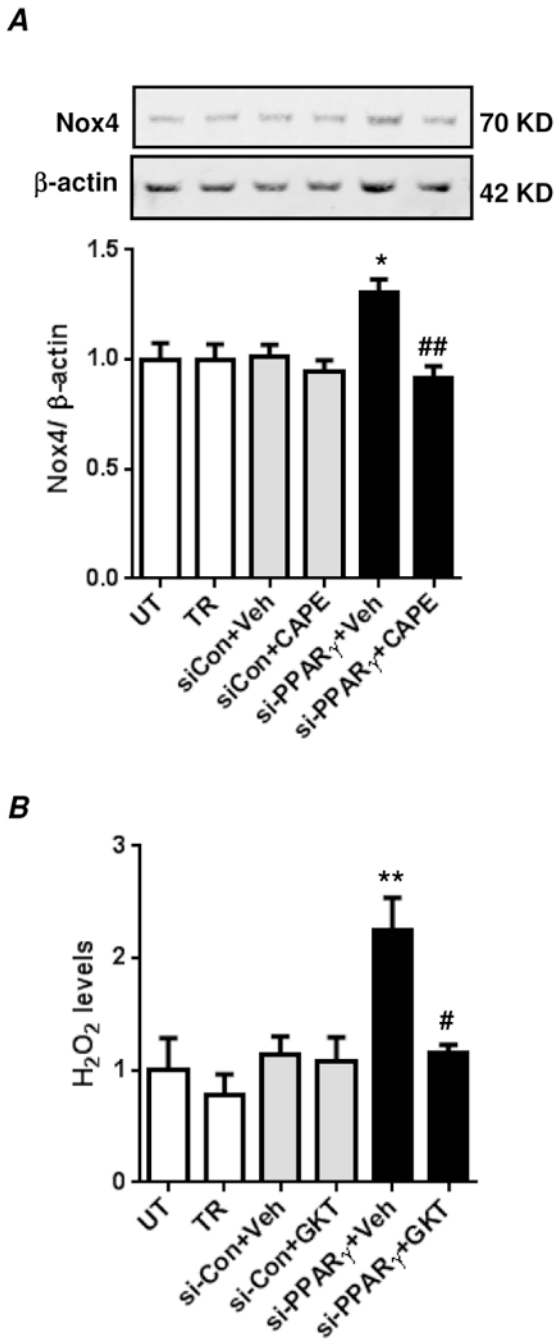

Figure 4. PPARγ knockdown promotes NF-κB-dependent Nox4 expression and Nox4-derived H_2O_2 generation in HPASMC

(A) HPASMC were untransfected (UT) or transfected with transfection reagent alone (TR), control siRNA (si-Con) or PPARγ siRNA (si-PPARγ) under normoxic conditions for 72 hours. Cells were treated with CAPE compound (10 μM) or equivalent volume of DMSO (vehicle) during the final 24 hours of the 72 hours transfection period. Cells were lysed and analyzed by western blot for Nox4 protein expression. Nox4 levels were normalized with β-actin levels. Each bar represents mean ± SEM Nox4 relative to β-actin in the same sample

expressed as fold-change versus UT. n=6-7, *p<0.05 compared to UT, ##p<0.01 compared to PPARγ siRNA transfected cells that were treated with DMSO. **(B)** HPASMC were transfected as above under normoxic conditions for 72 hours. Cells were treated with GKT137831 (20 μM) or equivalent volume of DMSO (vehicle) during the final 24 hours of the 72 hour transfection period, and H_2O_2 concentration was measured with the Amplex Red assay. Each bar represents mean ± SEM H_2O_2 concentration as fold-change versus UT. n=6-7, **p<0.01 compared to UT; #p<0.05 compared to si-PPARγ+Veh.

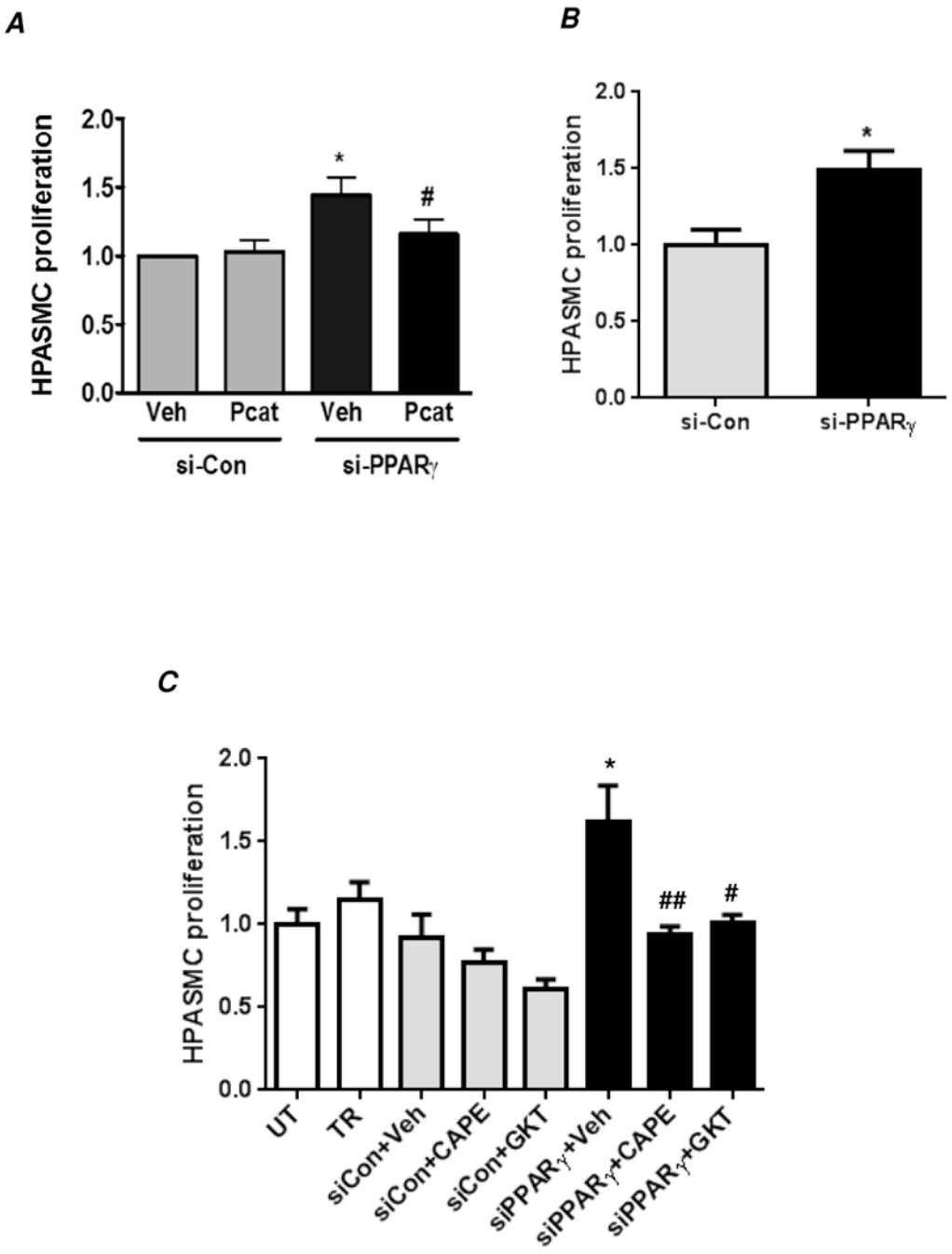

Figure 5. PPARγ knockdown promotes HPASMC proliferation via an NF-κB-Nox4-H_2O_2-dependent mechanism

HPASMC were untransfected (UT) or transfected with transfection reagent alone (TR), control siRNA (si-Con) or PPARγ siRNA (si-PPARγ) under normoxic conditions for 72 hours. Selected HPASMC were treated with PEG-catalase (1000 U/ml) ***(A)***, CAPE (10 μM) or GKT137831 (20 μM) ***(C)*** or with vehicle (Veh, DMSO) during the last 24 hours of the 72 hour transfection period. Cell proliferation was determined by MTT assay. **(B)** Cell proliferation upon PPARγ depletion was also verified by manual cell counting method. In

(A), each bar represents mean ± SEM HPASMC proliferation as fold-change versus control. n=3, *p<0.05 compared to Veh/si-Con; #p<0.05 compared to Veh/si-PPARγ. In ***(B)***, each bar represents mean ± SEM HPASMC proliferation (cells/ml) as fold-change versus control. n=3, *p<0.05 compared to si-Con. In ***(C)***, each bar represents mean ± SEM HPASMC proliferation as fold-change versus UT. n=5-7, **p<0.01 compared to UT; # ##p<0.05 and 0.01, respectively, compared to si-PPARγ+Veh.

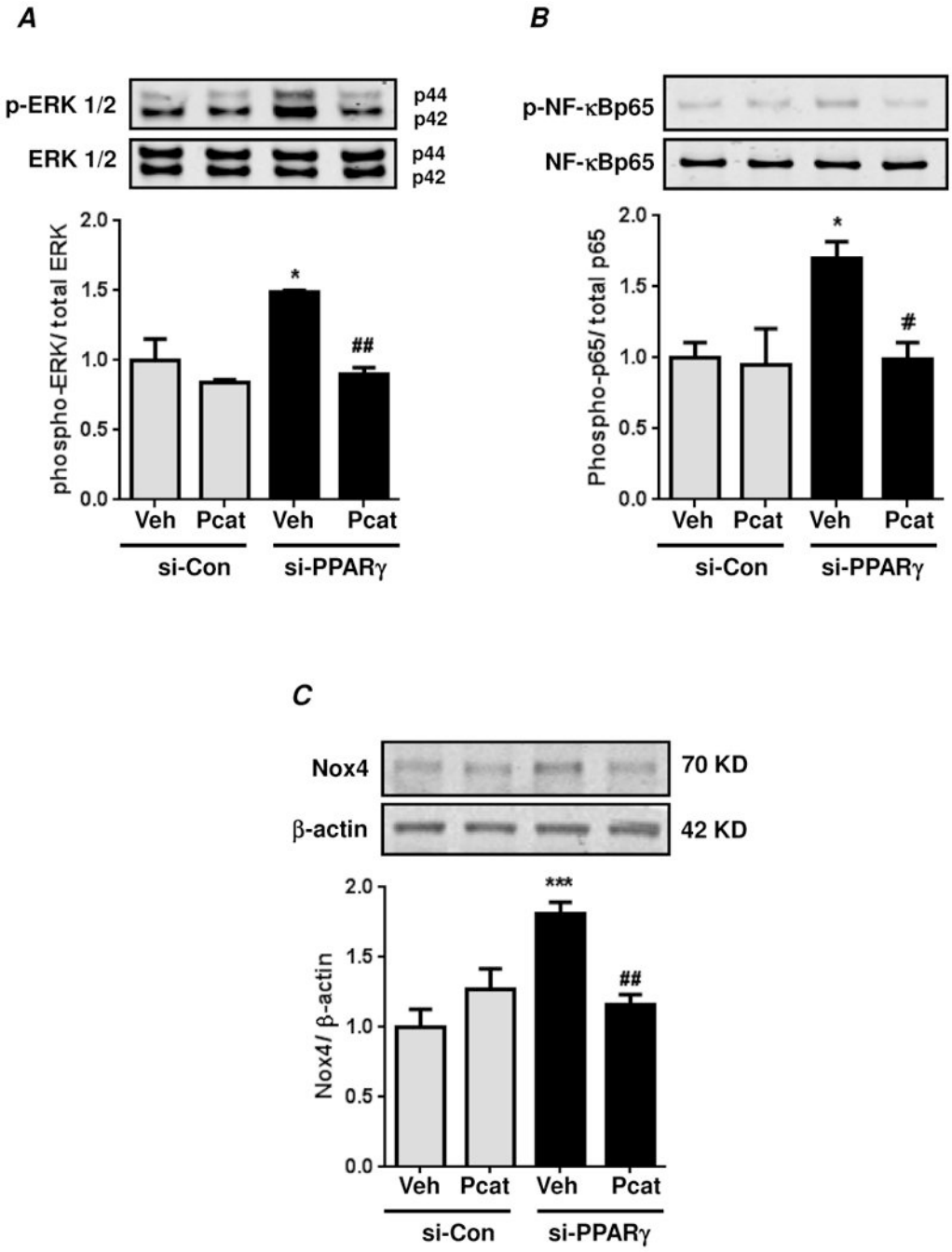

Figure 6. Production of H_2O_2 caused by PPARγ knockdown is required for sustained ERK 1/2 and NF-κB activation as well as Nox4 expression

HPASMC were transfected with control siRNA (si-Con) or PPARγ siRNA (si-PPARγ) under normoxic conditions for 72 hours. During final 24 hours of transfection, cells were treated with PEG-catalase (Pcat, 1000 U/ml) or with DMSO as control vehicle (Veh). Cell lysates were immunoblotted for phospho-ERK 1/2 ***(A)*** phospho-p65 ***(B)*** or Nox4 ***(C)***. In ***(A)***, each bar represents mean ± SEM phospho-ERK 1/2 relative to total ERK 1/2 in the same sample expressed as fold-change versus control. n=3, *p<0.05 vs si-Con; ##p<0.01 vs

vehicle treated si-PPARγ. In ***(B)***, each bar represents mean ± SEM phospho-NF-κBp65 relative to total NF-κBp65 in the same sample expressed as fold-change versus control. n=3, *p<0.05 vs si-Con; $^{\#}$p<0.05 vs vehicle treated si-PPARγ. In ***(C)***, each bar represents mean ± SEM Nox4 relative to β-actin in the same sample expressed as fold-change versus control. n=6, ***p<0.001 vs si-Con; $^{\#\#}$p<0.01 vs vehicle treated si-PPARγ.

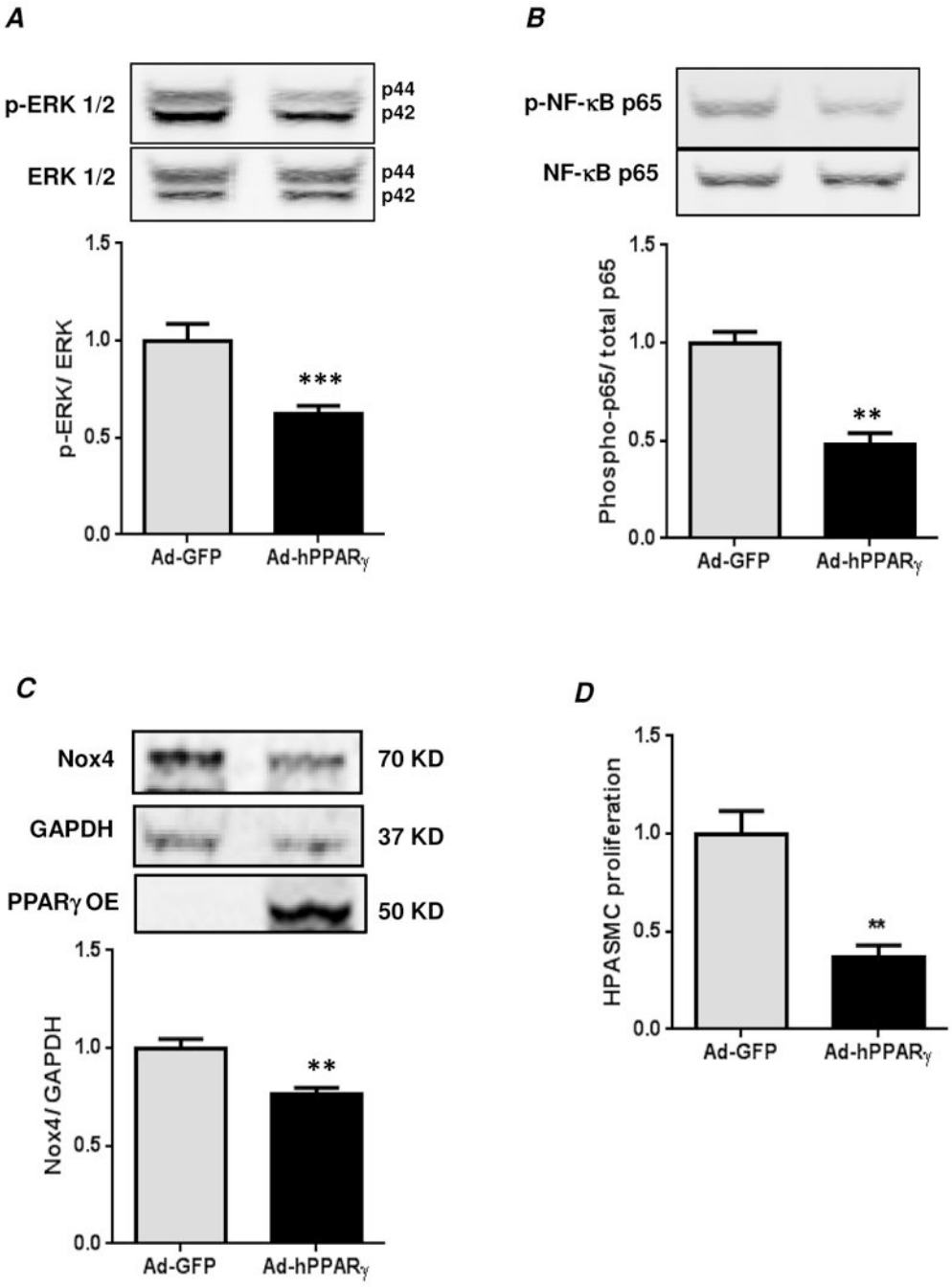

Figure 7. PPARγ overexpression reduces basal ERK 1/2 and NF-κB activation and Nox4 protein expression and HPASMC proliferation

Confluent HPASMC monolayers were transfected with human PPARγ in adenovirus (Ad-hPPARγ) or Ad-GFP (Vector Biolabs, Philadelphia, PA) as described in Materials and Methods. Cells were lysed and immunoblotted for phospho-ERK 1/2 ***(A)***, phospho-p65 ***(B)***, or Nox4 ***(C)***. In ***(D)***, HPASMC proliferation was measured by manual cell counting method. In ***(A)***, each bar represents mean ± SEM phospho-ERK 1/2 relative to total ERK 1/2 in the same sample expressed as fold-change versus Ad-GFP. n=4, ***p<0.001. In ***(B)***, each bar

represents mean ± SEM phospho-p65 relative to total p65 in the same sample expressed as fold-change versus Ad-GFP. n=4, **p<0.01. In ***(C)***, each bar represents mean ± SEM Nox4 relative to GAPDH in the same sample expressed as fold-change versus Ad-GFP. n=4, **p<0.01. Overexpression of PPARγ (PPARγ-OE) was confirmed by immunoblotting. The image was captured at a lower intensity to highlight the expression of exogenously introduced PPARγ. In ***(D)***, each bar represents mean ± SEM HPASMC proliferation (cells/ml) expressed as fold-change versus Ad-GFP. n=4, **p<0.01. Similar results were obtained by MTT assay (data not shown).

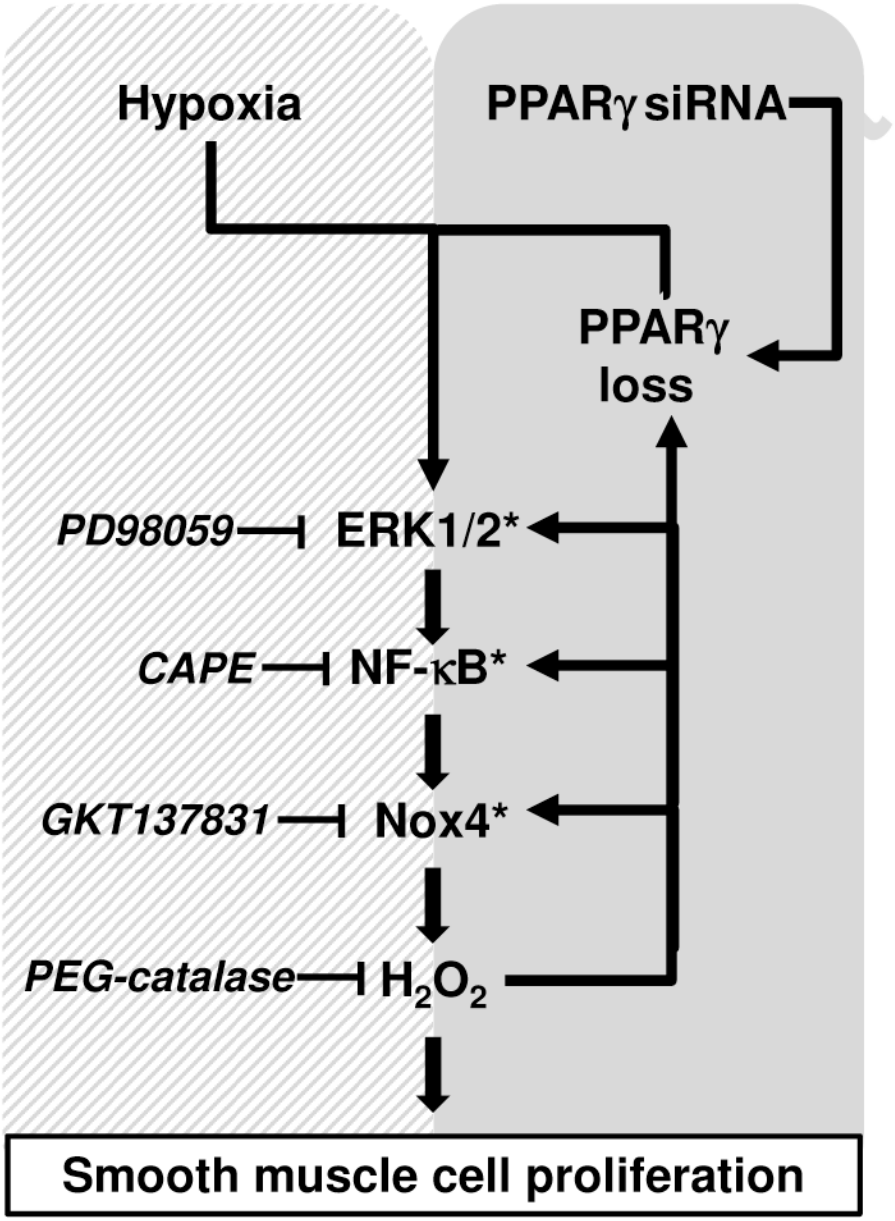

Figure 8. Schematic representation of ERK1/2-p65-Nox4-mediated pathways of cell proliferation in HPASMC caused by PPARγ depletion

We previously demonstrated that hypoxia promotes HPASMC proliferation and PPARγ downregulation via an ERK1/2-NF-κB p65-Nox4-dependent mechanism [19] *(hatched box)*. The findings in the current study *(gray box)* demonstrate that loss of PPARγ is sufficient to activate ERK1/2 and increase NF-κB p65 activity, Nox4 expression, H_2O_2 production, and HPASMC proliferation whereas PPARγ overexpression attenuated ERK1/2, NF-κB, and Nox4 *(denoted by asterisks)*. These findings indicate that PPARγ provides constitutive inhibition of ERK, NF-κB, and Nox4 signaling pathways critical in PASMC proliferation. These findings further emphasize that targeting PPARγ may provide a useful therapeutic strategy to attenuate PASMC proliferation and pulmonary vascular remodeling.