Peroxisome Proliferator-Activated Receptor Gamma (PPARγ) Expression Is Decreased in Pulmonary Hypertension and Affects Endothelial Cell Growth

Shingo Ameshima, Heiko Golpon, Carlyne D. Cool, Daniel Chan, R. William Vandivier, Shyra J. Gardai, Marilee Wick, Raphael A. Nemenoff, Mark W. Geraci, Norbert F. Voelkel

Abstract—PPARγ is a member of a family of nuclear receptors/ligand–dependent transcription factors, which bind to hormone response elements on target gene promoters. An antiproliferative and proapoptotic action profile of PPARγ has been described and PPARγ may function as a tumor suppressor gene, but little is known about the role of PPARγ in vascular remodeling. One group of human diseases that shows impressive vascular remodeling exclusively in the lungs is the group of severe pulmonary hypertensive disorders, which is characterized by complex, endothelial cell–proliferative lesions of lung precapillary arterioles composed of clusters of phenotypically altered endothelial cells that occlude the vessel lumen and contribute to the elevation of the pulmonary arterial pressure and reduce local lung tissue blood flow. In the present study, we report the ubiquitous PPARγ expression in normal lungs, and in contrast, a reduced lung tissue PPARγ gene and protein expression in the lungs from patients with severe PH and loss of PPARγ expression in their complex vascular lesions. We show that fluid shear stress reduces PPARγ expression in ECV304 endothelial cells, that ECV304 cells that stably express dominant-negative PPARγ (DN-PPARγ ECV304) form sprouts when placed in matrigel and that DN-PPARγ ECV304 cells, after tail vein injection in nude mice, form lumen-obliterating lung vascular lesions. We conclude that fluid shear stress decreases the expression of PPARγ in endothelial cells and that loss of PPARγ expression characterizes an abnormal, proliferating, apoptosis-resistant endothelial cell phenotype.

Key Words: severe pulmonary hypertension ■ peroxisome proliferator-activated receptor γ ■ endothelial cell growth ■ apoptosis ■ shear stress

Peroxisome proliferator-activated receptors (PPARs) are members of a nuclear hormone receptor/transcription factor superfamily. The transcription factor PPARγ forms a heterodimeric complex with the retinoid X receptor, and the complex of PPARγ and RXR binds to specific DNA recognition sites and regulates transcriptional events. PPARγ controls expression of genes that are involved in metabolism and cellular differentiation and is highly expressed in adipose tissue. Differentiation of preadipocytes into adipocytes is regulated by PPARs, which in turn are responsive to glucocorticoids, xanthines, retinoids, and prostanoids. PPARγ expression occurs in the lung in alveolar type II cells, coincidentally with surfactant protein A expression during type II cell differentiation. Recently, antiinflammatory properties of PPARγ have been demonstrated; in particular, the attenuation of the oxidative burst in activated macrophages and decreased cytokine production by monocytes via inhibition of the AP-1 and NF-κB transcription factors. Although it has been demonstrated that oxidized alkyl phospholipids are high-affinity PPARγ ligands, which may induce PPARγ-dependent gene expression in atherosclerotic lesions, and that PPARγ can regulate vascular smooth muscle cell migration and proliferation, little information is available regarding the potential role of PPARs in pulmonary vascular remodeling. Because PPARγ can act as a tumor suppressor protein, and because prostacyclin is decreased in severe pulmonary hypertension, we wondered whether PPARγ expression is reduced in the lung vascular lesions found in severe pulmonary hypertension (PH).

Severe pulmonary hypertension is characterized by complex precapillary arteriolar plexiform lesions, which contain phenotypically altered smooth muscle and endothelial cells, which express 5-lipoxygenase but not p27 or...
caveolin-1 and 2 (R. Achcar, N.F. Voelkel, L. Taraseviciene-Stewart, M. Kasper, C.D. Cool, unpublished data, 2003). By microarray gene expression screening, we found a decrease in PPARγ transcripts in random lung tissue samples from patients with primary PH.27

In the present study, we assessed the PPARγ gene and protein expression in normal human lungs and in lungs from patients with severe primary and secondary pulmonary hypertension, and we used immunohistochemistry to localize PPARγ in normal and pulmonary hypertensive lungs. Because in severe PH the plexiform lesions form at sites of bifurcations21 where shear stress is likely high,28 we examined whether fluid shear stress affects PPARγ expression. Indeed, increased fluid shear stress reduced the expression of PPARγ in a human cell (ECV304 cell) line which has endothelial cell features.29–33 We found that in normal human lung tissue, PPARγ is abundantly expressed especially in endothelial cells, but that the PPARγ expression is reduced or lacking in all of the angiogenic plexiform lesions of the pulmonary hypertensive lungs and in the vascular lesions of a rat model of severe PH.34 We also found that ECV304 cells that stably express dominant-negative PPARγ are hyperproliferative, relatively apoptosis-resistant, and rapidly form tubes when embedded in matrigel, whereas ECV304 cells that overexpress PPARγ do not. We conclude that lack of endothelial cell PPARγ expression is a marker of an abnormal endothelial cell phenotype and lack of PPARγ expression inhibits apoptosis and facilitates endothelial cell growth and angiogenesis.

Materials and Methods

Patients With Severe Pulmonary Hypertension and COPD, Tissue Samples, Immunohistochemistry

Lung tissue was obtained from 6 patients with primary pulmonary hypertension and from 3 patients with collagen vascular disease associated pulmonary hypertension and 1 patient with PH and Eisenmenger physiology. All of these patients had severely elevated pulmonary artery pressures as documented by right heart catheterization (online Table, in the online data supplement available at http://www.circresaha.org). We also obtained lungs from 6 patients with severe emphysema who were undergoing lung transplantation, lung volume reduction surgery, or lobectomy. All 6 patients were chronic cigarette smokers with pulmonary obstructive changes documented by lung function studies. Histologically normal lung tissue was obtained from 6 patients (3 males, 3 females; 62.5±6.2 [SEM] years;) undergoing lung biopsy for diagnostic purposes (localized inflammation [n=1] or primary or metastatic malignancies [n=5]).

Maternal Rat Model of Severe Pulmonary Hypertension

Adult Sprague-Dawley rats (Harlan, Indianapolis, Ind; UCHSC-approved animal protocol was used.) (n=5) received a single sc injection of the VEGF receptor inhibitor SU5416 (Sugen) and then the animals were housed in a hypobaric pressure chamber at a simulated altitude of 15 000 feet for 3 weeks. The combination of SU5416 treatment and chronic hypoxia causes severe pulmonary hypertension and lumen-obliterating pulmonary vascular lesions as described previously.20

Quantitative PCR

Quantitative RT-PCR was performed using the SYBR Green PCR Core reagents (Perkin-Elmer). Primers were designed to meet specific criteria by using the Primer Express software (Perkin-Elmer). The primers used were for human-specific PPARγ (forward: 5’-GGGATGTTCTCTACCCGTA-3’; reverse: 5’-GGGATGTTCTCTACCCGTA-3’; and for PPARγ2 (forward: 5’-CCCAGAAAGGCATTCCTTC-3’; reverse: 5’-AATGCGTCTGTGGTCAACCA-3’). We isolated total RNA from human lung tissue and ECV304 cells using the RNeasy Mini Kit (Qiagen). Five microliters (200 ng total RNA) was used as a template for the one-step RT-PCR. To obtain the copy numbers for the PPARγ gene, we subtracted the copy number of PPARγ from that of total PPARγ.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections (5 μm) of normal, emphysemaous, and pulmonary hypertensive lungs were deparaffinized and microwave-treated for 15 minutes. Endogenous peroxidase was blocked with 3% H2O2 for 30 minutes. Immunohistochemical staining was performed using the Vectastain Universal Quick kit (Vector Laboratories). To block nonspecific binding of Biotin/Avidin system reagents, the Avidin/Biotin blocking kit from Vector laboratories was used. The sections were incubated with mouse monoclonal anti-PPARγ (Santa Cruz) at 1:200 dilution in a humid chamber at room temperature for 30 minutes. The universal secondary antibody was incubated for 10 minutes, developed with DAB (Vector laboratories) and counterstained with Gill’s Hematoxylin (solution No. 1; Electron Microscopy Sciences). Negative controls were performed using a polyclonal rabbit IgG control primary antibody (Vector Laboratories) at 1:200. To stain the lung tissues for cleaved caspase 3 (the large fragment of activated caspase 3), we used a polyclonal antibody (Cell Signaling Technology).

Immunoblots

Lung tissue was homogenized with 20 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl2, 0.15 mmol/L spermine, 0.5 mmol/L spermidine, 0.25 mol/L sucrose, 100 mmol/L NaCl, 0.2 mmol/L EDTA, 200 μmol/L PMSF, 0.5 mmol/L DTT, 1 μg/μL leupeptin, and 1 μg/μL aprotinin, using a Tissuemizer (Tekener, Cincinnati, OH) at 4°C, centrifuged at 10 000g for 10 minutes at 4°C to remove tissue fragments, and the supernatant collected. Protein (25 μg, measured with Dc protein assay kit; Bio-Rad) was loaded in each lane of a 4% to 20% Tris–HCl Ready Gel (Bio-Rad). After 1 hour-incubation in a primary antibody (either 1:1000 rabbit polyclonal IgG against the full-length (32kDa) and cleaved fragments of human caspase 3 or β-actin; both Santa Cruz Biotechnology), the membranes were incubated with horseradish-peroxidase–conjugated secondary antibody for 2 hours and visualized by chemiluminescence (ECL kit; Amersham Pharmacia Biotech). The densitometry results for PPARγ were normalized to β-actin using the Gel Doc 2000 Gel Documentation System (Bio-Rad).

Shear Stress of ECV304 Cells

ECV304 cells were grown to 90% confluence, trypsinized, pooled, resuspended in media with 1% fetal bovine serum (FBS), and inoculated into polystyrene capillaries contained within a sterile cartridge (CELLMAX Cell Culture Systems, Spectrum Laboratories), which contain artificial capillaries lined with fibronectin. The ECV304 cells were exposed to no flow, low flow (2 dyne/cm2), or high flow (14.3 dyne/cm²) for 24 hours. The cartridge containing the capillary tubes was dismantled, and the cells within the capillary lumens were stripped with trypsin and washed in preparation for quantitative RT-PCR. Alternatively, the capillary tubes were fixed in formalin and embedded in paraffin for routine histology and immunohistochemistry.

Stable Transfection of ECV304 Cells

For stable transfections,35–37 WT or DN PPARγ cDNA (gift of Carl Clay, Bowman Gray School of Medicine, Winston-Salem, NC) was inserted into the pLNCS, retroviral expression vector (Clontech). The cDNAs for the WT and DN PPARγ were as reported by Gurnell et al.35 Recombinant virus was prepared in 293T cells by transfecting cells with Gag/Pol/Env vectors using Lipofectin (Gibco BRL). Polybrene (8 μg/mL) was added to the retroviral-containing medium from the packaging cells and filtered before two 24-hour
incubations with subconfluent layers of ECV304 cells. The infected cells were replated, selected for G418 resistance, and expanded. Clones were selected for expression of PPARγ by immunoblotting with specific antibodies.

**ECV304 Cell Tube Formation in Matrigel**
Matrigel (50 μL; Collaborative Biomedical Products) at 4°C was coated on each well of a 96-well culture plate and allowed to gel at 37°C for 1 hour. ECV304 (2×10⁴, wild-type or ECV-PPARγ dominant-negative or PPARγ-overexpressing ECV304 cells) were plated in each well in 200 μL RPMI 1640 supplemented with 10% FCS. The cells were then treated with saline solution (control), ciglitazone, or 15-deoxy-Delta12,14-prostaglandin J2 at various concentrations indicated for 24 hours or more. Pictures were taken under an Olympus microscope (10×10 or 10×40 magnifications) equipped with a Nikon Coolpix 995 camera.

**ECV304 Cell Apoptosis**
Wild-type, PPARγ overexpressing, and PPARγ dominant-negative endothelial cells were incubated in the presence of medium alone, human TNFα (1000 U/mL; R&D Systems), and cycloheximide (10 μg/mL; Calbiochem), or H2O2 (1 mmol/L; Sigma-Aldrich) for 6 hours at 37°C, 5% CO2. Endothelial cells were centrifuged onto slides (Cytospin; Shandon), stained with modified Wright’s Giemsa stain, and analyzed blindly for apoptosis using nuclear condensation. In some experiments, endothelial cells were processed for Western blotting as described in the immunoblot section above.

**Tail Vein Injection of ECV304 Cells in Nude Mice**
Atlasmic nude mice (nu/nu) were obtained from NIH-NCI (Rockville, Md). Experiments were performed with an approved IACUC protocol. One million ECV304 cells each were injected intravenously via the tail vein of each mouse under anesthesia. Animals were monitored closely for any changes in health or activity. Animals were euthanized 3 months later. Lungs were isolated, fixed in buffered formalin, and examined histologically. An expanded Materials and Methods section is available in the online data supplement at http://www.circresaha.org.

**Results**

**Decreased PPARγ-1 Gene Expression in the Lung Tissue From Patients With Severe Pulmonary Hypertension**
Quantitative PCR analysis of lung tissue samples showed that total PPARγ mRNA was decreased in patients with severe pulmonary hypertension when compared with normal lung tissue or tissue from patients with emphysema. Using primer sets designed to specifically identify PPARγ₁, we found that the majority of the PPARγ expressed in the lung tissue was accounted for by PPARγ₁, and not PPARγ₂ (Figure 1A). PPARγ protein expression was decreased in whole lung tissue extracts from patients with both primary and secondary PH (Figure 1B).

Immunohistochemistry revealed in normal lungs the ubiquitous expression of PPARγ in alveolar septal structures and in small vessel endothelium; bronchial epithelial cells did not express PPARγ (Figures 2A and 2B). In PH, remodeled, muscularized precapillary arterioles showed frequently reduced endothelial cell PPARγ expression. Most remarkable was the reduction or lack of PPARγ staining of the lumen-obliterating cells of the plexiform lesions (Figures 2C and 2D) in the lungs from patients with primary or secondary PH (Figure 2E). In fact, all of the plexiform lesions (n=38) in the lungs from the 9 patients with severe PH were characterized by pale centers, ie, relative or total absence of the nuclear PPARγ staining. Figure 2 also shows serial sections of plexiform lesions. The lack of smooth muscle cell actin staining of the cells in the lesion (Figure 2D) indicates that the PPARγ-negative cells of the lesion are not smooth muscle cells. Lack of cells undergoing apoptosis in this lesion (Figure 2F) and in the lesion from a patient with Eisenmenger physiology (VSD) is also apparent (Figure 2G). Two different lesions shown in Figure 2, with diminished PPARγ expression also demonstrate a lack of expression of active caspase 3 (Figures 2F and 2H). An example of a complex vascular lesion in a rat lung with severe pulmonary hypertension, documenting that most of the lesion cells do not express PPARγ is shown in Figure 2I.
Decreased Expression of PPARγ Protein in the Lung Vascular Lesions of Rats With Severe PH

A single sc injection of 25 mg/kg of the VEGF receptor blocker SU5416 causes severe pulmonary hypertension and intense vascular remodeling in the lungs of rats exposed to chronic hypobaric hypoxia. Immunohistochemistry was performed on such lungs using the antibody directed against PPARγ and lung sections from 5 different rats. Figure 2I shows that the lung vascular lesion, which is composed of proliferating endothelial cells lacks PPARγ protein expression. Thus, absence of PPARγ staining serves as a marker for easy recognition of abnormal, proliferating pulmonary vascular endothelial cells also in this animal model of severe PH.

Decreased PPARγ Expression of ECV304 Cells by Fluid Shear Stress

PPARγ gene expression in cultured confluent ECV304 cells, which had been placed on a rocking platform that was tilted 15 times a minute for 8 hours in order to move the cell culture medium rhythmically back and forth over the monolayer and thus apply fluid shear stress, was decreased in the tilted cells when compared with resting cells. Similar results were also obtained with human umbilical vein endothelial cells (HUVECs) (data not shown). Subsequently, ECV304 cells were seeded into the CellMax system and exposed to no shear, low fluid shear, or high fluid shear stress for 24 hours. High shear stress for 24 hours dramatically reduced PPARγ gene expression (Figure 3A) and so did chronic shear stress applied to the CellMax system for 3 weeks (Figure 3B).

Increased Angiogenic Potential of Cells Expressing DN-PPAR

We stably transfected ECV304 cells with either full-length WT-PPARγ or a construct encoding DN-PPARγ. Cells overexpressing WT-PPARγ had a marked increase in activation of a PPARγ responsive promoter, whereas promoter
activity was inhibited in cells expressing DN-PPARγ (data not shown). In 3-dimensional collagen gels, wild-type ECV304 cells formed clumps, whereas cells DN-PPARγ consistently formed reticular tube-like structures (Figure 4), suggesting that lack of PPARγ results in an angiogenic potential. PPARγ overexpressing ECV304 cells did not form tubes at all. Treatment of DN-PPARγ ECV304 cells with the PPARγ agonists 15-deoxy Δ12,14 prostaglandin J2 and ciglitazone suppressed the formation of tube-like structures at concentrations of 6 μmol/L, but not of concentrations of 3 μmol/L (Figure 4).

Tail vein injection into nude mice of ECV304 cells expressing DN-PPARγ caused the growth of intravascular tumors exclusively in the lungs but not in other organs (Figure 5), whereas wild-type or PPARγ overexpressing cells did not grow after similar injection.

**Apoptosis of ECV304 Cells**

ECV cells exposed to the combination of TNF-α plus cycloheximide or to H₂O₂ underwent apoptosis as assessed by nuclear morphology (Figure 6A). Overexpression of PPARγ significantly increased the number of apoptotic cells, i.e., facilitated apoptosis. The combination of TNF-α plus cycloheximide did not facilitate apoptosis in the DN-PPARγ cells when compared with the unchallenged cells, indicating that DN-PPARγ cells were relatively apoptosis resistant. Although H₂O₂ exposure increased the number of apoptotic cells when compared with the untreated groups, DN-PPARγ ECV cells showed a trend toward apoptosis protection when compared with the PPARγ-overexpressing ECV cells (Figure 6A). The effect of DN-PPARγ as an inhibitor of apoptosis was confirmed in separate experiments (Figure 6B), where DN-PPARγ prevented the loss of procaspase 3 compared with either wild-type or overexpressing PPARγ ECV cells.

**Discussion**

The remodeling of precapillary arterioles in severe pulmonary hypertension is in part caused by the uncontrolled growth of endothelial cells contributing to the lumen obliteration of these arterioles.22–25 The phenotype of these proliferating endothelial cells is abnormal as these cells express VEGF and 5-lipoxygenase, have absent or decreased expression of the gene encoding prostacyclin synthase,22 decreased expression of the p27 protein, and loss of expression of the tumor suppressor TGF-β RI protein in the plexiform lesions in the lungs from patients with primary pulmonary hypertension.38 Our present study characterizes the phenotype of the proliferating endothelial cells further. All of the plexiform lesions examined had reduced staining or lacked staining when the lung sections from the patients with severe PH were probed with the antibody directed against PPARγ; a similar loss of staining (expression) was observed in the vascular lesions, which characterize our rat model of severe PH.34 In addition, PPARγ gene expression was reduced by fluid shear stress in cultures of human endothelial cell–like (ECV304) cells.

Activation of PPARγ can induce cell growth inhibition, even in cancer cells11–13; therefore, our data that show a global tissue decrease in both PPARγ gene and protein expression in the lungs from patients with severe primary or secondary PH—but not in the lungs from patients with chronic obstructive lung disease (Figure 1), which are characterized by apoptosis of endothelial cells—suggest to us the loss of a cell growth inhibitor in the vascular lesions in severely pulmonary hypertensive lungs.

We offer two pathobiological explanations: either loss of a tumor suppressor gene and protein facilitates endothelial cell proliferation in PH, or the loss of PPARγ expression is another marker of angiogenic endothelial cell growth22 and impaired apoptosis. We could not find any information regarding control mechanisms of PPARγ expression in the lung or in endothelial cells; for example, it is not known whether hypoxia or shear stress regulate PPARγ expression. What is known, however, is that PPARγ ligands are potent inhibitors of angiogenesis.19,20 Although pulmonary blood flow is low in many patients with severe pulmonary hypertension, we postulate that regional shear stress is high, in particular at sites of precapillary arteriolar bifurcations, where plexiform lesions form.21 We thus examined whether fluid shear stress applied for 24 hours or 3 weeks affects endoto-
lial PPARγ gene expression. Indeed in the CellMax endothelial cell model, shear stress reduced PPARγ expression (Figure 3). The mechanism whereby shear stress decreases PPARγ expression is unclear because the promoter region of the PPARγ gene does not contain the known shear stress response motif (GAGACC).41 Because shear stress inhibits endothelial cell apoptosis,42-44 it is an intriguing hypothesis that endothelial cell apoptosis resistance might be related to decreased or lost PPARγ expression.

Because it had been shown previously that 15-deoxy-\(\Delta^{12,14}\) prostaglandin \(J_2\) induces endothelial cell,15 synoviocyte,16 and T-lymphocyte18 apoptosis, we conducted experiments to assess the effect of PPARγ activation on the growth of stably expressing DN-PPARγ, overexpressing PPARγ, and wild-type ECV304 cells. In comparison to the wild-type cells, the ECV304 cells expressing DN-PPARγ sprouted tubes; ECV304 cells overexpressing PPARγ did not grow any sprouts. Both ciglitazone (data not shown) and 15-deoxy-\(\Delta^{12,14}\) prostaglandin \(J_2\) (Figure 4) decreased sprout formation in ECV304 cells expressing DN-PPARγ at higher concentrations (6 \(\mu\)mol/L), but had little effect at the 3 \(\mu\)mol/L concentration; this is consistent with the concept that diminished or impaired expression of the nuclear receptor PPARγ permits angiogenesis and that ligand activation of PPARγ induces endothelial cell apoptosis.15 In fact, when ECV304 cells overexpressing PPARγ were subjected to TNF-\(\alpha\) plus cycloheximide, apoptosis was facilitated when compared with the DN-PPARγ cells (Figure 6).

Finally, when we injected ECV304 cells into the tail-veins of nude mice, only the ECV304 cells expressing DN-PPARγ formed tumors in the lungs (Figure 5). No tumor formation was observed in liver, spleen, or kidney, perhaps because the lung vessels provided a filter for the injected cells. As we observed tumor formation after injection of ECV304 cells carrying dominant-negative mutations of PPARγ, anti-tumor growth effects of PPARγ ligands have been observed in mice injected with breast cancer cells.13

In conclusion, we propose that lack of PPARγ expression is an important aspect of an abnormal, apoptosis-resistant, angiogenesis-promoting endothelial cell phenotype, and that in severe forms of pulmonary hypertension, lumen-obliterating endothelial cell growth may be facilitated by the loss of the tumor suppressor function of PPARγ. Although our data indicate that PPARγ plays a role in endothelial cell sprout formation, in rapid growth of endothelial cells in an artificial tube system and also in endothelial cell apoptosis resistance, it remains unclear what the initiating events leading to severe pulmonary hypertensive remodeling are. In the SU5416/chronic hypoxia model of severe PH,34 it could be shown that initial endothelial cell apoptosis is critical for the subsequent endothelial cell growth, which results eventually in lumen obliteration. Whether serotonin excess,45 potassium channel activity,46 protease/anti-protease imbalances,47,48 or germline mutations of the bone morphogenic protein receptor II49 relate to loss of pulmonary vascular endothelial growth control, the evolution of apoptosis-resistant endothelial cell phenotypes, which also have lost PPARγ expression, requires further investigation. Although we can show that the cells of the plexiform lesions in severe PH lack both apoptotic events and PPARγ expression, inhibition of apoptosis and lack of PPARγ expression may be associated or causally linked as suggested by our experiments that used PPARγ-overexpressing and DN-PPARγ ECV304 cells.

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Figure 4. Neither wild-type (WT) ECV304 cells nor ECV304 cells overexpressing the PPARγ gene (middle) form reticular tube structures at 18 hours after being embedded in matrigel. DN-PPARγ cells form tube-like structures (A). Treatment of the ECV304 cells with the PPARγ agonist 15-deoxy-\(\Delta^{12,14}\) prostaglandin \(J_2\) had little effect on ECV304 cell tube formation at a concentration of 3 \(\mu\)mol/L (B) or 6 \(\mu\)mol/L (C).
References


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On-Line Supplement

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Pulmonary Hypertension Center¹, Division of Renal Medicine², Cancer Center³ and Department of Pathology⁴, University of Colorado Health Sciences Center, Denver, CO 80262

National Jewish Research Center, Denver, CO⁵

Pulmonary Division, Universitätsklinik Magdeburg, Germany⁶

(Short Title) PPARγ Expression in Pulmonary Hypertension

Subject Codes: 18, 95, 129

Corresponding Author:
Norbert F. Voelkel, MD
Division of Pulmonary Sciences and Critical Care Medicine
4200 East Ninth Avenue, C272
Denver, CO 80262
phone: 303/315-4211
fax: 303/315-5632
e-mail: Norbert.Voelkel@uchsc.edu
Methods:

Quantitative PCR: We confirmed that the primer pairs amplified the expected genes by cloning and sequencing the PCR products. The absence of non-specific amplification was confirmed either by running dissociation curves or examining PCR amplification products by agarose gel electrophoresis. RT and amplification were performed by using one-step RT-PCR. Fifty-microliter reactions were used as suggested by the manufacturer, using 200 nM each forward and reverse primer and 100 nM of the probe. All reagents used in the RT-PCR were purchased from Perkin-Elmer. The RT reaction was performed for 30 min at 48° C using MultiScribe Reverse Transcriptase (final concentration 0.25 U/μl). AmpliTaq Gold polymerase (final concentration: 0.025 U/μl) was activated by incubation of the reactions at 95° C for 10 min followed by 40 cycles of amplification (95° C for 15 s and 60° C for 1 min) with the Gene Amp 5700 Sequence Detection System (ABI Prism). Samples were run in duplicate, and a control reaction without reverse transcriptase was also run for each sample. In addition, reactions without RNA were used to establish baseline levels for fluorescence. As an internal standard, a plasmid (pGEM-T Easy Vector Systems, JM109 cells, Promega, Madison, WI) containing the PPAR-γ PCR-product was used at dilutions of 0.1 ng to 0.0001ng/μl.

Immunoblots: In additional experiments, carbonic anhydrase protein (Sigma chemicals, St. Louis, MO) was loaded at a concentration of 250 ng and 2.5 μg each lane. Transfer of proteins to PVDF (Polyvinylidene difluoride) membrane (Hybond-P, Amersham Pharmacia Biotech, Piscataway, NJ) was performed by electrophoresis at 100 V for 1 h in
a Bio-Rad Western blot apparatus. Membranes were then blocked for 1 h in PBS-Tween 20 (0.1%) with 5% non-fat dried milk. Mouse polyclonal anti-PPARγ (Santa Cruz, CA), or mouse monoclonal anti-β-actin antibodies (Sigma-Aldrich, St. Louis, MO) were used at a 1:1000 dilution.

**ECV304 Cells Shear Stress:** Each cartridge contains 50 capillary tubes. After inoculation, the cartridges were placed in a 37°C/5% CO2 incubator for approximately two hours to allow attachment of the cells to the capillary tubes. After a second inoculation and reattachment period (two to four hours), the endothelial cell cartridge was connected to the flow path of the CELLMAX Quad instrument (Spectrum Laboratories, CA) and a reservoir of 10% FBS supplemented media. A pump circulates the sterile media from the reservoir through a sealed cartridge at a regulated rate. Each cartridge has a separate reservoir and tubing system, and can be subjected to variable flow rates. Flow rates in the system can range from none to over 30 dynes/cm2 to simulate high shear stress conditions.
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<td>M</td>
<td>COPD</td>
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<tr>
<td>16</td>
<td>53</td>
<td>F</td>
<td>COPD</td>
<td></td>
<td>Transplant</td>
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PPH = Primary Pulmonary Hypertension  
CREST = Severe pulmonary hypertension associated with CREST variant of Scleroderma  
SLE = Scleroderma  
VSD = Ventricular Septal Defect  
COPD = Chronic Obstructive Pulmonary Disease