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Peroxisome proliferator-activated receptor γ inhibits pulmonary hypertension targeting store-operated calcium entry

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Abstract

In this study, we investigated the role of peroxisome proliferator-activated receptor γ (PPAR γ) on store-operated calcium entry (SOCE) and expression of the main store-operated calcium channels (SOCCs) components, canonical transient receptor potential (TRPC) in chronic hypoxia (CH) and monocrotaline (MCT)-induced PH rat models. siRNA knockdown and adenoviral overexpression strategies were constructed for both loss-of-function and gain-of-function experiments. PPAR γ agonist rosiglitazone attenuates the pathogenesis of both CHPH and MCT-PH, suppresses Hif-1 α , TRPC1, TRPC6 expression in the distal pulmonary artery (PA) and SOCE in freshly isolated rat distal pulmonary arterial smooth muscle cells (PASMCs). By comprehensive use of knockdown and overexpression studies, bioinformatic analysis of the TRPC gene promoter and luciferase reporter assay, we demonstrated that PPAR γ exerts roles of anti-proliferation, anti-migration, and pro-apoptosis in PASMCs, likely by inhibiting the elevated SOCE and TRPC expression. These effects were inhibited under the conditions of hypoxia or Hif-1 α accumulation. We also found that under hypoxia, accumulated Hif-1 α protein acts as upstream of suppressed PPAR γ level, however, targeted PPAR γ rescue acts negative feedback on suppressing Hif-1 α level and Hif-1 α mediated signaling pathway. PPAR γ inhibits PH by targeting SOCE and TRPC via inhibiting Hif-1 α expression and signaling transduction.

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DISCLOSURES

None.

AUTHOR CONTRIBUTION

JW initiated and designed the project, analyzed data and wrote the paper; WL designed the project and edited the paper; YW performed the animal, functional and molecular experiments; YW, JZ, XY and JJ performed the molecular experiments; KY and LT edited the paper; YC, QJ, BZ and XC performed the animal experiments.

Keywords

pulmonary hypertension; PPAR γ ; TRPC; Hif-1 α

INTRODUCTION

Pulmonary hypertension (PH) is a severe and progressive disease accompanied with poor prognosis and high mortality. It is pathologically characterized by excessive pulmonary vascular remodeling, including smooth muscle cell proliferation and migration. In pulmonary arterial smooth muscle cells (PASMCs), global increase in intracellular Ca²⁺ concentration ([Ca²⁺]_i) is thought to be a key mediator of both the proliferation and migration [1–3]. Moreover, the hypoxia-enhanced store-operated calcium entry (SOCE) through store-operated calcium channels (SOCs) largely accounts for the elevated [Ca²⁺]_i in PASMCs. In mammalian cells, SOCs are believed to be composed of transient receptor potential (TRP) proteins [3–5]. Previous studies demonstrated that chronic hypoxia selectively upregulates the expression of TRPC1 and TRPC6 in PASMCs, which are responsible for the triggered SOCE in PASMCs, and ultimately contributes to the pathogenesis of chronic hypoxia-induced pulmonary hypertension (CHPH) [2, 6].

Hypoxia rapidly induced protein accumulation of hypoxia-inducible factor 1 α (Hif-1 α) and leads to increased expression of genes regulated by the hypoxia-responsive element (HRE) [7]. Functional HIF-1 exists as an α , β heterodimer, the activity of which is dependent on hypoxic induction of the α subunit, as HIF-1 β is ubiquitously expressed under normoxic condition, whereas Hif-1 α is selectively stabilized under hypoxia, translocates into the nucleus and binds to the HREs (5'-RCGTG-3'), results in the transcriptional induction of multiple gene promoters and further lead to vascular remodeling, glucose metabolism, and cell proliferation/apoptosis [8–10]. Accumulating evidence indicates that Hif-1 α is involved in the pathophysiology of CHPH. Our previous studies also demonstrated that overexpression of Hif-1 α is associated with up-regulation of TRPC1 and TRPC6, and the regulation of intracellular calcium homeostasis in PASMCs [11].

Peroxisome proliferator activated receptors (PPARs), a kind of ligand-activated nuclear hormone receptor superfamily, are ubiquitously expressed in pulmonary vascular endothelial and smooth muscle cells [12, 13] and are composed of three distinct isoforms: α , β/δ and γ [14]. Transcriptional activation of the PPAR γ receptor requires heterodimerization with the retinoid receptor, retinoid X receptor (RXR) [15], then the activated PPAR γ /RXR heterodimer binds to PPAR response elements (PPRE) in the promoter region of responsive genes to enhance or reduce the transcription of target genes in a broad variety of tissues [16]. Emerging evidence indicates that loss of PPAR γ expression and function may be associated with PH, while stimulating PPAR γ may benefit PH. Recent research even confirmed that activation of PPAR γ with thiazolidinediones (TZDs) like troglitazone and rosiglitazone (RSG), attenuated PH caused by monocrotaline (MCT) [17] or hypobaric hypoxia [18] in rats. Since both PPAR γ and Hif-1 α are strongly evidenced associated with PH pathogenesis, the interaction between PPAR γ and Hif-1 α , however, is still unknown.

In the present study, we detected the role of PPAR γ on PH pathogenesis in both CHPH and MCT induced PH (MCT-PH) animal models and especially determine the role of PPAR γ on hypoxia-induction of TRPC expression, SOCE and basal $[Ca^{2+}]_i$ in PSMCs. We also examined the molecular mechanisms underlying the transcriptional regulation of TRPC by PPAR γ and Hif-1 α under hypoxic condition.

Method

Establishment of the rat CHPH model and rosiglitazone (RSG) treatment

Adult 6 to 8-wk-old wild-type Sprague-Dawley (SD) rats were exposed to chronic hypoxia (10% O₂) or ambient room air for 21 days, with or without intragastric RSG administration (10 mg·Kg⁻¹·d⁻¹), as previously described [19]. This protocol was in accordance with National Institutes of Health guidelines for use of live animals and was approved by Animal Care and Use Committee of The first Affiliated Hospital of Guangzhou Medical University.

Primary culture of rat distal PSMCs

Rat PSMCs were isolated and cultured as previously described [2]. The cellular purity of PSMCs in all the experiments was assessed to be >95%.

Calcium imaging experiment

Calcium-imaging experiments were performed as described previously [2]. Fluorescence images of the cells were recorded by a Ratio Vision digital fluorescence microscopy system and analyzed by InCyte software.

Cell proliferation, transwell chamber migration assay

Cell proliferation was evaluated according to the operation manual by a CytoSelect™ BrdU Cell Proliferation ELISA Kit (Roche). Cell migration was assessed on 8 μ M polycarbonate membrane of Transwell Permeable Support (24 mm, Corning Inc., Corning, NY) (2 $\times$ 10⁴ cells/well). Cells were counted in 5 random fields and expressed as the average number of cells per field under a light microscope.

Measurement of apoptosis

Cell apoptosis was determined by the detection of cleavage of caspase-3. Proteins extracted from PSMCs were used to analyze the protein expression of cleaved caspase-3 by using Western blot.

Small interfering RNA silencing

All siRNA duplexes were synthesized by Shanghai GenePharma. Knockdown efficiency was determined by quantitative real-time PCR (qRT-PCR) and western immunoblotting.

Adenoviral Infection

Adenovirus (Ad) expressing constitutively active PPAR γ , Ad-negative shuttle, and Ad-green fluorescent protein. PSMCs were infected with the adenoviruses for 6 hours and then maintained in serum-free DMEM.

Real-time Quantitative PCR

Total RNA was extracted from PSMCs using RNAi Plus Reagent (Takara, Japan). Reverse transcription was performed using Script cDNA synthesis kit (Takara, Japan). Real-time quantitative PCR (qPCR) was performed using QuantiTect SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA) in Real-time PCR detection system (Bio-Rad, Hercules, CA).

Western blot analysis

Distal PA or PSMCs were homogenized in TPER lysis buffer (Pierce, Rockford, IL) containing 5% protease inhibitor cocktail (Sigma, St. Louis, MO), 1 mM EDTA, and 200 M4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride. Western blotting was conducted with the method as we previously described [11].

Luciferase reporter assay

A dual reporter gene assay for studying Hif-1 α -dependent gene regulation was performed as described by literature [20], Firefly and Renilla luciferase activity was measured independently in cell extracts with a bioluminometer (Biozym, Oldendorf, Germany).

Statistical analysis

Statistical data are expressed as mean $\pm$ SEM. When appropriate, t-test and one-way ANOVA were applied. In all cases, $p < 0.05$ was considered statistically significant. All experiments were repeated at least three times.

Results

RSG inhibited CHPH pathogenesis and suppressed chronic hypoxia induced TRPC expression in rats

Consistent with our previous studies [11, 21], rats exposed to chronic hypoxia (10% O₂ for 21 days) successfully developed CHPH, featuring as: right ventricular systolic pressure (RVSP) increased from 21.64 ± 2.35 mmHg to 50.73 ± 4.98 mmHg and RV mean pressure from 10.85 ± 0.40 mmHg in normoxic control rats to 22.77 ± 0.94 mmHg in hypoxic rats, respectively (Fig. 1A–1B); Fulton index [weight ratio of right ventricle to left ventricle plus septum (RV/LV+S)] from 0.22 ± 0.078 (Normoxia) to 0.37 ± 0.013 (Hypoxia; $p < 0.05$ versus Normoxia; Fig. 1C); right ventricle to body weight (RV/Body weight) from 0.53 ± 0.012 (Normoxia) to 0.78 ± 0.024 (Hypoxia; $p < 0.001$ versus Normoxia; Fig. 1D). Intragastric administration of RSG (10 mg/Kg/day) significantly decreased RVSP and RV mean pressure ($p < 0.01$ versus Hypoxia; Fig. 1A–1B), without significantly affecting the RV/(LV+S) ($p = 0.33$ versus Hypoxia; Figure 1C) and RV/Body weight ratios ($p = 0.32$ versus Hypoxia; Fig. 1D). Immunohistochemistry examinations demonstrated that after 21-day chronic hypoxic exposure, the media wall of the distal PA was markedly thickened (Fig. 1E). RSG obviously relieved the hypoxia-induced PA wall thickening. The ratio of vascular lumen area ($2 \times$ medial wall thickness/external diameter) in chronic hypoxic rats ($36.4 \pm 2.4\%$) was statistically greater than that in normoxic controls ($15.1 \pm 3.5\%$, $P < 0.01$), and the ratio in Hypoxia+RSG rats drop to $27.9 \pm 4.7\%$ ($P < 0.05$ vs Hypoxic control rats). In parallel, our data showed a marked decrease in PPAR γ and increase in TRPC1, TRPC6

protein expression in the isolated rat distal PA from the chronic hypoxic group, which was attenuated by RSG (Fig. 1F–I).

PPAR γ exerted roles of anti-proliferation, anti-migration and pro-apoptosis in hypoxic PSMCs

Hypoxia enhances PSMCs proliferation [10]. Accumulating evidence implicated that PPAR γ activation elicits anti-proliferative, pro-apoptotic and vasodilatory effects [17, 18]. We found that GW1929, a high potent PPAR γ agonist, attenuated hypoxia-induced proliferation in a dose and time dependent manner (Online Figure 2). To confirm these results, siRNA targeting PPAR γ (siPPAR γ) and adenovirus harboring PPAR γ (Ad-PPAR γ) were synthesized/constructed to knockdown or overexpress PPAR γ in PSMCs, respectively. Non-targeting siRandom was used as a negative control for siPPAR γ and Ad-Shuttle served as a mock-vehicle control for Ad-PPAR γ . As seen in Fig. 2A and 2B, prolonged hypoxia (4% O₂, 60 h) enhanced proliferation was aggravated by siR-PPAR γ treatment, in contrast, attenuated by Ad-PPAR γ treatment. Likewise, similar effects existed in the migration assay (Fig. 2C and 2D) and measurement of apoptosis, as reflected by the expression level of cleaved caspase-3, which is well recognized as a key element in the signal cascade leading to apoptosis (Fig. 2E and 2F) of PSMCs. These results indicated PPAR γ inhibits hypoxia-induced proliferation and migration, rescues hypoxia-suppressed apoptosis of PSMCs.

PPAR γ abolished hypoxia-elevated basal [Ca²⁺]_i and SOCE in PSMCs

Cultured PSMCs were divided into four groups: Normoxia+siR-NC, Normoxia+siR-PPAR γ , Hypoxia+siR-NC, Hypoxia+siR-PPAR γ . Consistent with previous data [2], SOCE determined by Mn²⁺ quenching was significant higher in hypoxic PSMCs ($45.3 \pm 3.05\%$, $n=4$; $P < 0.001$ versus Normoxia) than in normoxic cells ($33.7 \pm 2.06\%$, $n=3$). Knockdown of PPAR γ increased the Mn²⁺ quenching rate to $53.3 \pm 1.52\%$ in hypoxic cells ($P < 0.05$ vs. Hy+siR-NC control; Fig. 3A and 3B), but not in normoxic cells ($34 \pm 1.5\%$ in Nor+siR-PPAR γ group vs. $33 \pm 3.7\%$ in Nor+siR-NC control, $P = 0.89$). Whereas, Ad-PPAR γ infection decreased the Mn²⁺ quenching rate to $42 \pm 4.1\%$ in hypoxic cells ($P < 0.05$ vs. $55 \pm 2.64\%$ Hy+Ad-shuttle control; Fig. 3C and 3D), but not in normoxic cells. As shown in Fig. 3E and 3F, PSMCs exposed to prolonged hypoxia showed significantly higher resting [Ca²⁺]_i level (Fig. 3E, 0.644 ± 0.013 in normoxia vs 0.886 ± 0.064 in hypoxia, $P < 0.05$; Fig. 3F, 0.679 ± 0.021 in normoxia vs 1.084 ± 0.079 in hypoxia, $P < 0.01$), which was enhanced by siR-PPAR γ treatment (1.127 ± 0.034 ; $P < 0.05$ vs. Hypoxic control cells; Fig. 3E) and attenuated by infection with Ad-PPAR γ (0.696 ± 0.014 ; $P < 0.05$ vs. Hypoxic control cells; Fig. 3F). These results suggested that PPAR γ may exert its anti-proliferative effect by inhibiting SOCE and resting [Ca²⁺]_i.

PPAR γ inhibited hypoxia-upregulated TRPC expression in PSMCs

Similar to the previous finding [22], our results showed similar pattern of hypoxia increased TRPC1 and TRPC6, decreased PPAR γ mRNA level (Fig. 4A and 4B), as well as protein level (Fig. 4C and 4D). Transfection with siR-PPAR γ (25 nM) significantly aggravated hypoxia increased mRNA and protein of TRPC1 and TRPC6 (Fig. 4A, 4C and 4E). On the

other side, Ad-PPAR γ remarkably decreased both mRNA and protein level of TRPC1 and TRPC6 under hypoxic condition, but not under normoxic condition (Fig 4B, 4D and 4F).

Analysis of rat TRPC1/6 promoter area and determine the effect of PPAR γ on hypoxic activation of TRPC1/6 promoter

To further determine whether PPAR γ could regulate hypoxia triggered TRPC1 and TRPC6 promoter activity, potential homologous transcription factor binding sites in the TRPC1 and TRPC6 promoter were assessed (Online Figure 3) and we contrast reporter constructs for TRPC1 and TRPC6 promoter. The effect of hypoxia and PPAR γ on TRPC1 and TRPC6 promoter activity was then measured by transfecting these reporter constructs into cultured PSMCs for luciferase reporter assay. As demonstrated in Fig. 5A and 5B, hypoxic exposure significantly enhanced the promoter activity of TRPC1 and TRPC6 (Fig. 5A, Fig. 5B). Since that hypoxia elevated the Hif-1 α accumulation and triggered the expression of hypoxia-responsive element (HRE) targeted genes, the up-regulation of TRPC may be stimulated by Hif-1 α . We then treated the cells with GW1929 during the last 24 h of hypoxia exposure and found that the enhanced promoter activity of TRPC1 (Fig. 5B, 0.1928 ± 0.0230 vs Hypoxia Control, $P < 0.05$), especially TRPC6 (Fig. 5A, 0.1570 ± 0.0316 vs Hypoxia Control, $P < 0.05$) was significantly recovered. On the contrary, inhibition of PPAR γ with antagonist T0070907 stimulates hypoxia-driven TRPC promoter activity. These results collectively suggested a recovery effect of PPAR γ on hypoxia-induced activation of TRPC6 and TRPC1 promoter.

PPAR γ down-regulated Hif-1 α expression in PSMCs

Previous evidence indicated that Hif-1 α might act as a positive regulator of hypoxic induction of TRPC expression and function as an upstream of TRPC proteins [11]. To explore whether PPAR γ affect Hif-1 α expression, primary cultured rat distal PSMCs were transfected with siR-PPAR γ . As seen in Fig. 6, prolonged hypoxia increased Hif-1 α mRNA expression for 32% and increased protein expression for 153.5%. Transfection with siR-PPAR γ significantly aggravated hypoxic increases in Hif-1 α mRNA (Fig. 6A, $123.9 \pm 0.068\%$ vs Hypoxia siR-NC, $P < 0.05$) and protein (Fig. 6C and 6E, $139.7 \pm 0.037\%$ vs Hypoxia siR-NC, $P < 0.05$). In parallel, infection with Ad-PPAR γ attenuated hypoxia-induced mRNA (Fig. 6B, $77.9 \pm 0.116\%$ vs Hypoxia Ad-shuttle, $P < 0.05$) and protein (Fig. 6F and 6H, $51.6 \pm 0.068\%$ vs Hypoxia Ad-shuttle, $P < 0.05$) level of Hif-1 α . However, neither siR-PPAR γ nor Ad-PPAR γ statistically affected Hif-1 α expression under normoxic conditions. These results implied that PPAR γ could inhibit the hypoxic induction of Hif-1 α .

Hif-1 α down-regulated PPAR γ expression in PSMCs

As PPAR γ and Hif-1 α are known both locate in nucleus and can regulate targeted gene expression at transcription level, combined with the aforementioned results, we further examine the effect of Hif-1 α on PPAR γ using siR-Hif1 α and Ad-Hif1 α . PSMCs were exposed to prolonged hypoxia (4% O₂, 60 h) with or without siR-Hif1 α or Ad-Hif1 α treatment. As shown in Fig 7, Hif1 α expression was up-regulated in hypoxia exposed PSMCs as expected. PPAR γ expression are suppressed by Ad-Hif-1 α infection (Fig. 7B, $33.1 \pm 0.041\%$ vs Hypoxia siR-NC, $P < 0.05$) and protein (Fig. 7E and 7F, $64.9 \pm 0.063\%$ vs Hypoxia siR-NC, $P < 0.05$) and promoted by siR-Hif-1 α transfection in mRNA (Fig. 7A,

129.6± 0.093% vs Hypoxia siR-NC, $P<0.05$) and protein (Fig. 7C and 7D, 150.6± 0.063% vs Hypoxia siR-NC, $P<0.05$) levels, which indicates a mutual regulation between PPAR γ and Hif-1 α convincingly.

Hif-1 α and PPAR γ shared mutual inhibition mechanism

Since Hif1 α and PPAR γ inhibited the expression of each other, suggesting a potential mutual inhibition mechanism, we sought to further confirm this hypothesis by more direct evidence. To elucidate this question, firstly, we applied treatment of cobalt chloride (CoCl₂), a well-known hypoxia mimetic agent, to rapidly accumulate Hif1 α protein in normoxic PSMCs. As we expected, CoCl₂ promoted Hif1 α and TRPC expression drastically, and inhibited PPAR γ inversely (Fig. 8A, 8B). Then, combination of ad-PPAR and siR-Hif1 α treatments were performed to measure the signaling transduction activity on TRPC expression. As shown in Fig 8C, 8D, Hif1 α expression was induced in hypoxic PSMCs and attenuated by either siR-Hif1 α transfection or Ad-PPAR γ infection, extremely down-regulated by treatment of Ad-PPAR γ plus siR-Hif1 α . PPAR γ expression are suppressed significantly under hypoxia and promoted by siR-Hif1 α transfection or Ad-PPAR γ infection, and this enhancement also displayed after combination treatment of Ad-PPAR γ and siR-Hif1 α . Similarly, TRPC expression followed the same tendency as Hif1 α expression, whereas, its comparative expression in Ad-PPAR γ solely and combination treatment of Ad-PPAR γ and siR-Hif1 α indicated that HIF-1 is the upstream of PPAR γ and further regulate TRPC expression.

Hif-1 α acted upstream of PPAR γ in hypoxic PSMCs

To further detect that between Hif-1 α and PPAR γ , which one acts as an upstream factor to regulate the other one in hypoxic PSMCs, the expression dynamics of the two factors within 12 hours of hypoxic exposure were then examined. Data showed that the expression of Hif-1 α was slightly increased 126.6± 0.130% at 0.5 h of hypoxic exposure, significantly elevated 148.7± 0.077% at 2 hours and reached a peak at 4 hours (171.1± 0.073%), started declining at 8 hours and remained detectable until 12 hours of hypoxic exposure (Fig. 8E, 8F). On the other hand, the expression of PPAR γ was sharply reduced 52.4± 0.09% at just 0.5 hour of hypoxic exposure, then recovered weakly until 4 hours (80.8± 0.047%) and next declined until 12 hours of hypoxic exposure (47.7± 0.010%) (Fig. 8E–F). These results indicated that hypoxia rapidly leads to Hif-1 α increase and results in PPAR γ suppression.

DISCUSSION

The present study demonstrated that RSG attenuated the characteristics of CHPH likely through recovering hypoxia-downregulated PPAR γ to: 1) inhibited hypoxia-increased expression of TRPC1 and TRPC6; 2) attenuated hypoxia-triggered SOCE and basal [Ca²⁺]_i; 3) inhibits hypoxia-elevated proliferation, migration and reverses hypoxia-inhibited apoptosis in PSMCs. In addition, we also provided fundamental mechanistic evidence to demonstrate for the first time that targeted rescue of PPAR γ could negatively inhibit Hif-1 α level and reverse hypoxia-upregulated TRPC expression and hypoxia-elevated SOCE in hypoxic PSMCs.

Currently, the group of specific PPAR γ agonists, like RSG, has been proved by several studies to be beneficial in the treatment of PH. By obtaining decreased RVSP and media wall thickening of small pulmonary vasculature in CHPH and MCT-PH models, we confirmed that RSG could markedly attenuated the elevated RVSP in both of the models, which are in line with previous reports [17, 18]. However, we also surprisingly saw an unaltered Fulton index after RSG treatment, which indicated no obvious effect on right ventricle hypertrophy (RVH). There are several potential explanations regarding to this phenomenon: 1) the decrease in RVSP could be attributed to a decompensated RV, as we would predict that RVSP would fall in a decompensated heart; 2) the decrease in RVSP could be caused by a decrease in afterload and is not a consequence of heart fail; 3) RSG treatment may have beneficial effects on the pulmonary vasculature in PH, but no obvious or even negative effects on the heart, which has been previously reported in the diabetic models [23]. Due to the lack of the data representing the cardiac output and the mean pulmonary arterial pressure, which of these potential explanations are correct remains unclear. To figure out this question will be one of our main goals in the future study.

Previous publication indicated that PPAR γ ligands play essential roles in impairing endothelial function and attenuating the development of atherosclerotic lesions in apolipoprotein E (ApoE) or LDL receptor deficient mouse atherosclerosis models [24, 25]. PPAR γ also suppresses the expression and activity of NADPH oxidase through down-regulating Nox4 [26]; inhibits heightened PDGF signaling [27–29]; reduces TGF- β 1-induced extracellular matrix molecule synthesis and growth factor secretion [30]. We and others have previously demonstrated that increased intracellular free Ca²⁺ concentration in PSMCs is a major trigger for pulmonary vasoconstriction and an important stimulus for PSMCs proliferation and migration [31]. Exposure to chronic hypoxia caused enhanced activity of SOCCs other than VDCCs [32]. As a consequence, chronic hypoxia not only elevated and maintained increased resting [Ca²⁺]_i and active tone, but also upregulated expression of the main SOCCs components, TRPC1 and TRPC6 [11]. We then further examined the effect of RSG on hypoxia upregulated TRPC1 and TRPC6 expression in distal PAs and PSMCs. Notably, RSG inhibited hypoxic increases of TRPC1 and TRPC6.

To further explore the role of PPAR γ in regulating Ca²⁺ homeostasis and functional consequences in PSMCs, we adopted and constructed high potent PPAR γ agonist GW1929, specific siRNA targeting PPAR γ , as well as recombinant adenovirus harboring PPAR γ gene for the following study. All these approaches suggest a protective role of PPAR γ on TRPC1/6 expression, SOCE, basal Ca²⁺ concentration, proliferation and migration in PSMCs. Convincingly, the physiological and pathophysiological benefits of RSG to PH were associated with parallel molecular and functional changes.

To detect how TRPC expression is regulated in transcriptional level by hypoxia, we further analyzed the DNA sequence of TRPC promoter region and strikingly identify PPRE and HRE within the regulatory regions of TRPC gene promoter. By using luciferase assay, PPAR γ agonist GW1929 significantly suppressed TRPC transactivation in PSMCs under hypoxia. On the contrary, inhibition of PPAR γ with antagonist T0070907 aggravated hypoxia-driven TRPC promoter activity. All differences took place under hypoxia condition rather than normoxia. These results suggested that PPAR γ acts indirectly on the TRPC

transcription regulation, and might through influencing Hif-1 α transactivity. This hypothesis was then proved by the inhibition role of PPAR γ on Hif-1 α expression on both mRNA and protein levels. The diversity of regulatory mechanism occurs between PPAR γ and Hif-1 α in various studies. On one hand, Hif-1 α can repress PPAR γ by HIF-1-regulated gene DEC1/Stral3 under hypoxia in adipocyte differentiation [33]. In response to pathologic stress of cardiac metabolism, Hif-1 α directly activates PPAR γ transcription and PPAR γ is a key downstream effector of Hif-1 α -driven TAG accumulation and apoptosis in cardiomyocytes [34]. On the other hand, PPAR γ shifts to the upstream of Hif-1 α . For example, PPAR γ modulates reactive oxygen species generation and activation of NF- κ B and Hif-1 α in allergic airway disease of mice [35]. Additionally, PPAR γ agonist 15d-PGJ2 induced stabilization and accumulation of Hif-1 α in nuclear, without affecting Hif-1 α mRNA levels or proteasome activity in Human Kidney HK-2 cells [36]. Alternatively, whether Hif-1 α interacting with PPAR γ precedes TRPC promoter involvement, remains an area of active investigation in our laboratories. In this event, we supposed that Hif-1 α may bind to HRE sites in PPAR γ promoter area, physically preventing PPAR γ from binding to adjacent sites and thereby inhibiting TRPC promoter activity. To elucidate this issue, we applied CoCl₂ in PSMCs under normoxia for independent of the effects of global hypoxia. The results confirmed that the direct activation of HIF1 α results in decreased PPAR γ expression and increased expression in TRPC1 and TRPC6. Furthermore, combination treatment of ad-PPAR and siR-Hif1 α verified this conclusion convincingly. Finally, the expression dynamic of PPAR γ and Hif-1 α under hypoxic condition was also examined. Data showed that when Hif-1 α reached a peak, PPAR γ subsequently descend, suggesting Hif-1 α expression more sensitive than PPAR γ in response to hypoxia during 24 hours. As a summary, we concluded that PPAR γ and Hif-1 α play mutual antagonistic role in regulating TRPC transcription under hypoxic exposure.

Collectively, an outline was summarized as shown in Figure 8G. Hypoxia exposure led to Hif-1 α accumulation, which enhanced SOCE through activating TRPC expression. As a consequence of hypoxic exposure, the balance between Hif-1 α and PPAR γ signaling becomes dysregulated, resulting in promoted proliferation and migration in PSMCs, elevated vascular remodeling, and PH. However, recovered PPAR γ activity by specific agonist (like RSG) then challenges the Hif-1 α mediated signaling (such as TRPC induction) by directly suppressing Hif-1 α expression, which subsequently prevents the PH pathogenesis. To our knowledge, it is the first report that PPAR γ may regulate TRPC expression through inhibiting Hif-1 α signaling. Our results provide new insights into molecular mechanism basis to explain how PPAR γ inhibits TRPC and intracellular calcium homeostasis in PSMCs, which might be valuable in identifying novel targets to improve the treatment of patients with PH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key messages

1. Rosiglitazone protects PH by normalizing RVSP, but not right ventricle hypotrophy.
2. PPAR γ inhibits PSMCs proliferation via targeting SOCE and TRPC by suppressing Hif-1 α .
3. PPAR γ and Hif-1 α shares mutual inhibitory regulation in PSMCs.
4. PPAR γ restoration might be a beneficial strategy for PH treatment.

Figure 1. Rosiglitazone (RSG) inhibited the characteristic changes and hypoxia induced canonical transient receptor potential (TRPC) expression in chronic hypoxic pulmonary hypertension (CHPH) rats

Rats were exposed to normoxia or chronic hypoxia (10% O₂) for 21 days with or without RSG (i.p., 10 mg/Kg/d) treatment. (A) show the representative traces and bar graphs of RVSP of each group of animals. (B), (C), and (D) are bar graphs showing Right ventricular mean pressure, RV/(LV+S), and RV/body weight, respectively. Bar values are Means $\pm$ SEM, n = 7 in each group. * $P < 0.05$ versus respective normoxic control, and ** $P < 0.05$ versus respective hypoxic control. (E): Lung tissues were fixed in neutral buffered formalin, embedded in paraffin, cross sectioned (50 μ m in thickness), and stained with hematoxylin and eosin (H&E staining). Pictures are representative from four for each group of animals treated with normoxia, normoxia+RSG, hypoxia, or hypoxia+RSG, showing an inhibitory role of RSG on PA wall thickening induced by hypoxia. Arrows indicate PA in each picture. The ratio of vascular lumen area evaluation by 2 x medial wall thickness to external vessel diameter as the bar graph shown. (F) (G) (H) (I) The protein levels of PPAR γ , TRPC1 and TRPC6 were measured by Western blotting, and the intensity of their bands was normalized to that of β -actin. Data were presented as means $\pm$ SEM, n = 3 in each group; * $P < 0.05$ versus normoxia control, and ** $P < 0.05$ versus hypoxia control.

Figure 2. PPAR γ agonist attenuates hypoxia-induced PSMCs proliferation, migration and anti-apoptosis in vitro

PASMCs proliferation was assessed by BrdU assay incorporation rate under normoxic or hypoxic conditions after transfected with siR-PPAR γ (A) or infected with Ad-PPAR γ (B), respectively. The non-targeted siRNA (siR-NC) and shuttle vector (Ad-Shuttle) were used as internal controls. Proliferation was then determined by MTT assay. Values were normalized to normoxia alone and presented as percentages. Each bar represents mean $\pm$ SEM, n=6. * $p < 0.05$ versus respective normoxic control, ** $p < 0.05$ versus respective hypoxic control. (C) and (D): The migration rates of cells cultured under normoxia or hypoxia condition were determined by calculating the ratios of migrated cells (on the lower surface of trans-well membrane) to the total cells (cells on both sides of trans-well membrane) treated with siR-PPAR γ or Ad-PPAR γ respectively. * $p < 0.05$ versus respective normoxic control, ** $p < 0.05$ versus respective hypoxic control. (E) and (F): Protein levels of cleaved Caspase-3 were determined by western blot after knockdown and overexpression of PPAR γ in PSMCs under normoxic or hypoxic conditions, and the band intensity were normalized to that of β -actin. Data were presented as means $\pm$ SEM, n = 4 in each group. * $P < 0.05$ versus respective normoxic control; ** $P < 0.05$ versus respective hypoxic control.

Figure 3. Effects of PPAR γ on hypoxia-induced elevated basal [Ca²⁺]_i and SOCE in PSMCs
 The records of the average quenching of Fura-2 fluorescence by Mn²⁺, and data were expressed as the percentage decrease in fluorescence at time 10 min from time 0, which applied by siR-PPAR γ (A) and (B) and Ad-PPAR γ (C) and (D) in PSMCs treated with normoxia or hypoxia (4% O₂) for 60 h, respectively. (A) and (C) represent the traces of Fura-2 fluorescence by Mn²⁺, and data were analyzed and expressed as the percentage decrease in fluorescence at time 10 min from time 0, as showing in (B) and (D); (E) and (F) represent the statistical data of resting [Ca²⁺]_i in either siR-PPAR γ or AdPPAR γ treated groups. all bar values are Mean $\pm$ SEM, * $P < 0.05$ versus respective normoxic control and ** $P < 0.05$ versus respective hypoxic control.

Figure 4. Effects of PPAR γ on hypoxia-induced upregulation of TRPC expression in PASMCs PASMCs were treated with prolonged hypoxia (4% O₂, 60 h) with or without siRNA-PPAR γ or Ad-PPAR γ . The relative mRNA levels (to 18s) for PPAR γ , TRPC1 and TRPC6 were shown as percentage to normoxic control in (A) and (B). The protein levels of PPAR γ , TRPC1 and TRPC6 were represented in (C) and (D), and the band intensity were normalized to that of β -actin (E) and (F). Data were presented as means $\pm$ SEM, n = 4 in each group. * $P < 0.05$ versus respective normoxic control; ** $P < 0.05$ versus respective hypoxic control.

Figure 5. Effects of Hif-1 and PPAR γ on the regulation of TRPC1/6 promoter in PSMCs
 In (A) and (B), cells were exposed for 72 h to control (21% O₂) or hypoxic (4% O₂) conditions, and during the last 24 h of exposure, selected cells were treated with GW1929 (20 μ M) (A) or T0070907 (10 μ M) (B) and an equivalent volume of vehicle as control. Cells were then harvested and subjected to assays for luciferase activity. Each bar represents the means $\pm$ SEM, luciferase activity in each sample relative to Renilla expressed in arbitrary units from 3 experiments performed in triplicate. * P < 0.05 vs. Normoxic PGL-TRPC1; # p <0.05 vs. Normoxic PGL-TRPC6; ** P <0.05 vs. Hypoxic PGL-TRPC1; ### p <0.05 vs. Hypoxic PGL-TRPC6.

Figure 6. Effects of PPAR γ knockdown and overexpression on Hif-1 α expression during prolonged hypoxic exposure in PSMCs *in vivo*
 PSMCs were treated with prolonged hypoxia (4% O₂, 60 h) with or without siRNA-PPAR γ or Ad-PPAR γ . The relative mRNA levels (to 18s) for Hif-1 α were shown as percentage to that of normoxic control in (A) and (B). The protein levels of Hif-1 α and PPAR γ were listed in (C) to (H). (C) and (F) show the blots images and the band intensity were normalized to that of β -actin and summarized as bar graphs in (D), (E), (G) and (H). Data were presented as means $\pm$ SEM, n = 4 in each group. * $P < 0.05$ versus respective normoxic control; ** $P < 0.05$ versus respective hypoxic control.

Figure 7. Hif-1α promoted prolonged hypoxia-induced TRPC expression in PSMCs from normoxic rats, and downregulate PPARγ expression

PASMCs isolated from normoxic rats were treated with prolonged hypoxia (4% O₂, 60h) with or without siR-Hif-1α or Ad-Hif-1α. The relative mRNA levels (to 18s) for PPARγ and Hif-1α were determined by quantitative RT-PCR and the results were shown as percentage to that of normoxia in (A) and (B). The protein levels of PPARγ and Hif-1α were measured by western blotting (C) and (E), and their band intensity were normalized to that of β-actin (D) and (F). Data were presented as means ± SEM, n = 4 in each group. **P* < 0.05 versus respective normoxic control; ***P* < 0.01 versus respective hypoxic control.

Figure 8. Schematic representation of hypoxia-induced TRPC promotion through Hif-1α/PPARγ mechanism

The protein levels of Hif-1α, PPARγ, TRPC1 and TRPC6 after treated with CoCl₂ (100 μM, 60 h) (A) or treated with Ad-PPARγ and siR-Hif1α jointly in PASMCs (C), corresponding intensity of their bands was normalized to that of β-actin (B) or (D). PASMCs isolated from normoxic rats were exposed to hypoxia (4% O₂) for up to 12 h. The protein levels of PPARγ and Hif-1α were measured by Western blotting (E) and their band intensity were normalized to that of β-actin (F). (G) Schematic representation of the hypothesized regulation-signaling axis of hypoxia-induced TRPC upregulation through Hif-1α/PPARγ mechanism. All datas were presented as means ± SEM, n = 4 in each group; * *P* < 0.05, ** *P* < 0.01 versus control.