Biochemistry and Biophysics Reports 27 (2021) 101091

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Deficiency of peroxisome proliferator-activated receptor α attenuates apoptosis and promotes migration of vascular smooth muscle cells

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ARTICLE INFO

Keywords:
Angiotensin II
PPARα
Vascular remodeling
Vascular smooth muscle cell

ABSTRACT

Peroxisome proliferator-activated receptor (PPAR) α is widely expressed in the vasculature and has pleiotropic and lipid-lowering independent effects, but its role in the growth and function of vascular smooth muscle cells (VSMCs) during vascular pathophysiology is still unclear. Herein, VSMC-specific PPARα-deficient mice (*Ppar-α*$^{\Delta SMC}$) were generated by Cre-LoxP site-specific recombinase technology and VSMCs were isolated from mice aorta. PPARα deficiency attenuated VSMC apoptosis induced by angiotensin (Ang) II and hydrogen peroxide, and increased the migration of Ang II-challenged cells.

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors at the crossroads of key cellular functions. Among the three PPAR subtypes (PPARα, β/δ, and γ), PPARα is ubiquitously expressed especially in tissues with a high capacity for fatty acid oxidation, including the liver, renal cortex, brown adipose tissue, myocardium and the majority of cell types, including endothelial cells, VSMCs and macrophages, in the vasculature [1].

Vascular smooth muscle cell (VSMC) growth can influence vascular structure. Injurious insults and environmental cues lead to the reorganization of extracellular matrix (ECM) by affecting the dedifferentiation and proliferation of VSMCs, which play a pivotal in the development of intimal hyperplasia [2,3]. On the other hand, VSMC apoptosis is a hallmark of vascular injury and repair, and has been established as an essential process that regulates tissue architecture and large vessel integrity [4]. The role of PPARα in cell growth, including that of VSMC, seems to be dependent on the specific cell type, species and the relative context. In addition, existing data concerning the role of PPARα in cell survival and function are mainly derived from experiments investigating its role using known ligands [5–14]. In *vitro* cell culture models, PPARα activation by docosahexaenoic acid induces apoptosis of VSMCs from Sprague-Dawley rats in a p38-dependent manner [8]. However, PPARα activation by fenofibrate inhibits cell apoptosis and cell cycle arrest in rat vascular adventitial fibroblasts partly through SIRT1-mediated deacetylation of FoxO1 [10]. Similar to the findings exploring the role of PPARα in VSMC apoptosis, the reported role of PPARα in VSMC migration is also inconsistent, *in vitro* studies indicate that PPARα can inhibit tumor growth factor (TGF)-β–induced β5 integrin transcription and VSMC migration [11], but PPARα activation by gemfibrozil fails to affect migration in low or high glucose media [12]. In *vivo* animal models, WY-14643 was shown to diminish oxidative stress and inhibit

Abbreviations: Ang II, angiotensin II; EC, endothelial cell; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; SM22α, smooth muscle 22α; TGF, tumor growth factor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; VSMC, vascular smooth muscle cell.

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https://doi.org/10.1016/j.bbrep.2021.101091
Received 5 May 2021; Received in revised form 23 July 2021; Accepted 26 July 2021

cardiomyocyte apoptosis in a rabbit model of ischemia/reperfusion injury [14]. In addition, PPARα suppresses apoptosis and induces proliferation of mouse hepatocytes, resulting in severe hepatomegaly [15]. While the disagreement in data may be partly explained by cell type specific mechanistic differences for the role of PPARα and heterogeneity between studies, a more plausible explanation for this discrepancy could be due to "off-target" mechanisms of PPARα ligands via ligand-specific interaction with other receptors and unspecified molecular targets [16,17]. In addition, evidence has been provided showing non-genomic signaling induced by PPAR ligands can trigger the activation of mitogen-activated protein kinases, resulting in the phosphorylation of PPARs themselves and an alteration in their regulatory ability on target gene expression [18]. Importantly, PPAR-independent actions have also been implicated in the anti-inflammatory action of PPAR ligands [19].

Clinically, fibrates are well-known synthetic PPARα agonists, among which fenofibrate has been shown in preclinical trials to protect against cardiovascular diseases, especially in the presence of diabetes and insulin resistance [20]. However, as the largest fibrate trial to date, the FIELD (Fenofibrate Intervention and Event Lowering in Diabetes) study failed to detect a significant benefit of daily fenofibrate use on the incidence of coronary heart disease [21]. Notably, it has been recognized that PPARα ligand Wy-14,643 can elicit proinflammatory response via PPARα-independent activation of extracellular signal-regulated kinase (ERK)1/2 mitogen-activated protein kinase (MAPK) pathway [22], whereas 15-deoxy-delta12,14-prostaglandin J2 (15d-PGJ2), a PPARγ ligand, has been shown to possess PPARγ-independent anti-inflammatory activity on human peripheral blood monocytes *in vitro* [23]. In addition, 15d-PGJ2 and ciglitazone, another PPARγ ligand, can both PPARγ-independently induce apoptosis in normal and malignant human B lymphocytes [24]. These results indicate that PPAR-independent pharmacological effects can potentially confound the clinical interpretation and lead to erroneous therapeutic considerations. Therefore, it may be more relevant to investigate PPARα function by genetic rather than pharmacological means.

The renin-angiotensin system (RAAS) represents one of the major mechanisms contributing to the regulation of vascular resistance and pathophysiology. Renin is released primarily by the kidneys in response to glomerular underperfusion or a reduced salt intake. Renin acts upon its substrate angiotensinogen and stimulates the formation of angiotensin I (Ang I), an inactive substance which is cleaved by angiotensin-converting enzyme (ACE) to generate physiologically active angiotensin II (Ang II) [25]. Ang II is the primary active product of the RAAS and regulates vascular tone and pathophysiology by multiple mechanisms including vasoconstriction, stimulation of aldosterone release from the adrenal cortex, retention of sodium and fluid, and modulation of cell growth and proliferation [26]. In this study, VSMCs were isolated from smooth muscle cell (SMC)-specific *Ppara* knockout mice ($Ppara^{\Delta SMC}$ mice) and the role of PPARα in VSMC effector function and survival including apoptosis and migration was examined in the presence of Ang II. The results demonstrated that PPARα deficiency had dual effects on VSMC action. On one hand, PPARα deficiency is protective in terms of VSMC apoptosis, but on the other hand, its deficiency may increase VSMC migration in response to Ang II stimulation.

2. Materials and methods

2.1. Experimental animals

Generation of vascular smooth muscle cell-specific *Ppara* deficient mice. Vascular smooth muscle cell (VSMC)-specific *Ppara* deficient mice were constructed using the Cre-LoxP site-specific recombinase technology [15]. Targeted embryonic stem cells modified by the insertion of two loxP sites that enable the excision of the flanked (floxed) exon 5 *Ppara* allele through Cre-mediated recombination were generated as previously described [15]. Mice with the floxed strain were screened and crossed with flippase recombinase (FLPeR)-expressing transgenic mice (The Jackson Laboratory, Bar Harbor, ME) for removal of FLP recombinase target site-flanked selection cassette [15,27]. $Ppara^{flox-ed/floxed}$ ($Ppara^{fl/fl}$) mice were subsequently crossed with transgenic mice expressing Cre recombinase under the control of smooth muscle-specific SM22 promoter (*SM22Cre*) [28] to produce SMC-specific *Ppara* knockout ($Ppara^{\Delta SMC}$) mouse. The resultant mice were further backcrossed to $Ppara^{fl/fl}$ mouse for at least 10 generations.

Eight-week-old male congenic $Ppara^{\Delta SMC}$ mice and their sex- and age-matched $Ppara^{fl/fl}$ littermates were used for isolation of primary aortic VSMCs. Mice were maintained and experiments were performed in accordance with the NIH guidelines for the Care and Use of Laboratory Animals. The study protocol was approved by the Committee on the Ethics of Animal Experiments of the Capital Medical University. A group of five mice were maintained in individual cages on a 12 h–12 h light-dark cycle at 22–24 °C, 50–60 % humidity, in the Experimental Animal Center of Capital Medical University, China (license No. SYXK (Jing) 2010–0020), and were provided ad libitum access to water and standard chow. The cages were cleaned every 3 days. The disposal of experimental animals was in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, issued by the Ministry of Science and Technology of China [29].

2.2. Isolation and culture of aortic vascular smooth muscle cells

Primary cultures of VSMCs were isolated from the aortas of $Ppara^{fl/fl}$ and $Ppara^{\Delta SMC}$ mice as described [28,30]. In brief, the mouse was euthanized by intraperitoneal injection of tribromoethanol (1.2 %, 0.2 ml/10 g body weight) [31], and the aorta was then harvested, followed by digestion with type II collagenase (37 °C, 30 min) to remove the adventitia. The endothelium was subsequently detached from the intima with a sterile cotton-tipped applicator. The remaining aortic tissue were cut into ~1 mm^3 pieces and incubated with a solution of elastase and collagenase at 37 °C for 30 min. Then the digestion was stopped by the addition of 1 ml smooth muscle cell media (SMCM) (Sciencell, Carlsbad, CA) supplemented with 10 % fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and 1000 U/ml penicillin/streptomycin (Invitrogen), followed by gentle pipetting using a P1000 pipette. The resultant cell suspensions were centrifuged at 2000×*g* at room temperature for 10 min. The supernatant was discarded, cells resuspended and then cultured in SMCM media containing 10 % FBS at 37 °C, 5 % CO_2. The majority of extracted VSMCs were firmly attached to the culture dishes after 24 h. After 3 days, the non-adherent dead cells were washed away with phosphate buffered saline (PBS). The morphology of the adherent VSMCs was gradually apparent in about 5–7 days. The culture medium was refreshed every 3 days, and cells were passaged when they were ~80 % confluent in about 10–15 days (first passage) and then passaged every 7–10 days once they reached ~80 % confluence. $Ppara^{\Delta SMC}$ VSMCs were subjected to real-time polymerase chain reaction (PCR) to assess PPARα mRNA levels before each experiment and only cells with a knockout efficiency of more than 75 % were selected for subsequent use. The VSMCs were used in the experiments between passages 3 and 7. All cells were subjected to immunofluorescent staining of α-SMA (Sigma-Aldrich, St. Louis, MO), which confirmed the purity of VSMCs was over 95 % (Supplementary Fig. 1). Cell maturation and differentiation was also monitored by morphological observations. We have endeavored to use earlier passages between 3 and 5 in all experiments once the cell number meets the study requirements, because sometimes VSMCs would differentiate into other cells and adopt dendritic cell-like appearance after passage 7, and the use of these cells in the experiments was avoided. In addition, efforts were made to use VSMCs from the same passage in each experiment to reduce the potential bias caused by cell passaging and phenotypic change. A final concentration of Ang II at 1 μM was used in all experiments.

2.3. Vascular smooth muscle cell apoptosis

Subconfluent cells were serum-starved in SMCM containing 1 % FBS and 1 % penicillin-streptomycin for 12 h to synchronize cells and exposed to Ang II or hydrogen peroxide (H_2O_2, 300 μM) for 24 h. Apoptotic cells were detected with *in situ* Cell Death Detection Kit (Roche Diagnostics Co. Indianapolis, IN), which allows for detection of apoptosis at single cell level based on labeling of DNA strand breaks (terminal deoxynucleotidyl transferase dUTP nick-end labeling, TUNEL assay) [32,33]. Cell slides were air-dried in a freshly prepared fixation solution containing 4 % paraformaldehyde in PBS for 30 min at 15–25 °C. Slides were then rinsed three times with PBS (5 min each time) and incubated with blocking solution which contains 3 % H_2O_2 in methanol for 10 min at 15–25 °C. The slides were rinsed with PBS for three times (5 min each time) and incubated in permeabilization solution (0.1 % TritonX-100 and 0.1 % sodium citrate in water) on ice for 2 min. Each slide was rinsed with PBS twice (5 min each time), mounted with coverslip to avoid evaporative loss and incubated in 50 μl reaction mixture containing 45 μl label solution (1 × nucleotide mixture including biotin-11-dUTP in reaction buffer), and 5 μl enzyme solution (10 × terminal deoxynucleotidyl transferase in storage buffer) for 60 min at 37 °C under wet conditions. Slides were rinsed 3 × with PBS (5 min each time) and analyzed under a fluorescence microscope. Two negative controls and one positive control were included in each experiment. As negative controls, cells were fixed, permeabilized and then incubated in 50 μl/well label solution (without terminal transferase) instead of TUNEL reaction mixture. As a positive control, fixed and permeabilized cells were incubated with DNAase I recombinant (3000 U/ml in 50 mM Tris-HCL, PH 7.5, 1 mg/ml BSA) for 10 min at 15–25 °C to induce DNA strand breaks prior to labeling procedures.

For detection of cleaved-caspase 3 immunofluorescence, the sections were blocked with 10 % goat serum for 30 min, incubated overnight with rabbit primary antibody against cleaved caspase 3 (1:200, Cell Signaling, MA, USA), and further incubated with the secondary antibody (1:100; goat-anti-rabbit, 488 nm, green fluorescence; Zsbio, China) for 1 h at room temperature. Then nuclear DNA staining was conducted using mounting medium for fluorescence with DAPI (VECTOR H-1200, Burlingame, CA). Fluorescence signals were recorded using a Leica digital camera (Leica, Germany).

To substantiate the role of PPARα in Ang II-induced VSMC apoptosis, a parallel assay was performed with the Annexin V-FITC Apoptosis Detection Kit (KeyGEN) as per the manufacturer's recommendations [34]. Briefly, 2×10^5 cells were seeded into 6-well plates, serum-starved in SMCM containing 1 % FBS and 1 % penicillin-streptomycin for 12 h and treated as specified. The cells were then isolated using ethylenediaminetetraacetic acid (EDTA) -free trypsin (Beyotime Institute of Biotechnology, Nanjing, China) and harvested. Then cells were centrifuged at 2000×*g* for 5 min, rinsed with cold PBS for three times and re-suspended in 500 μl binding buffer containing 5 μl annexin V-FITC and 5 μl propidium iodide. Subsequently, cells were cultured in the dark at room temperature for 15 min and 400 μl binding buffer was added to the cell suspension. Apoptotic cells were detected with a flow cytometer within 1 h.

2.4. Quantitative real-time polymerase chain reaction

RNA was extracted from various tissues including heart, liver, spleen, lung, kidney, skeletal muscle, brown adipose tissue, white adipose tissue, aorta and cultured VSMCs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions [35,36]. Briefly, tissue samples were homogenized in 1 ml of TRIzol reagent per 50 mg of tissue using a homogenizer (Union instruments, Suzhou, China). Cultured VSMCs were lysed directly in a 35 mm diameter culture dish by adding 1 ml of TRIzol reagent and passing the cell lysate several times through a pipette. The resultant samples in TRIzol were then mixed thoroughly with 0.2 ml chloroform (Sigma-Aldrich), spun down and the upper fraction was harvested. The fraction was mixed with same volume of isopropanol, and centrifuged at 10,000×*g* for 10 min. The pellet was washed with 0.8 ml of 75 % ethanol, and dissolved in RNase free water (Ambion, Thermo Fisher Scientific, USA). Samples with 260/280 ratio of 1.9–2.0 were used for analysis. A total of 2 μg RNA was obtained to synthesize cDNA using GoScript reverse Transcription System (Promega, Mannheim, Germany). Real-time PCR reactions were performed in duplicate using SYBR Green I (Takara, Shiga, Japan) on an iCyclerIQsystem (Bio-Rad, Hercules, CA, USA) as per the manufacturers' instructions. Primers used were as follows: *Mcp-1* (gene for monocyte chemoattractant protein-1, MCP-1), forward: ATTGGGATCATCTTGCTGGT, reverse: CCTGCTGTTCACAGTTGCC; *Ppara,* forward: TTCGCCGAAAGAAGCCCTTA, reverse: CCCTGAACATCGAGTGCGAA; *Actb*, forward: TTCTTTGCAGCTCCTTCGTT, reverse: ATGGAGGGGAATACAGCCC. mRNA expressions were normalized to β-actin gene (*Actb*) and calculated using the $2^{-\Delta\Delta CT}$ method.

2.5. Wound healing assay

VSMCs were isolated and cultured as indicated above, and cells in passage 3 were harvested and seeded in 6-well plates (5×10^4/well). Cells were allowed to adhere and cultured in SMCM containing 10 % FBS for 24 h until reaching 80–90 % confluence. Then cells were serum-starved for 12 h and a sterile 200 μl pipette tip was adopted to make consistently sized scratches perpendicular to the bottom of the well. Another line was scratched perpendicular to the first line to create a cross in each well. Cells were washed gently with SMCM to remove debris and subsequently incubated with 1 % FBS-containing SMCM supplemented with dimethyl sulfoxide (vehicle) or Ang II for 24 h. Then the culture medium was discarded and cells were washed three times with PBS at 37 °C. Then cells were fixed with 4 % paraformaldehyde at room temperature for 30 min, washed with PBS for 5 min (3 times) and the 0.1 % crystal violet staining solution was applied at room temperature for 30 min. Double-distilled water was used to remove floating color. Cells were observed under a Nikon microscope (Nikon Eclipse Ti–U, Tokyo, Japan) [37–39]).

2.6. Transwell assay

Chemotaxis was measured by transwell assay using Transwell 24-well cell culture inserts with 5 μm pores (Corning Inc. Corning, NY, USA) as we previously reported [40]. Briefly, VSMCs in passage 3 were serum-starved for 12 h, harvested and added to the insert (5×10^4 cells/well in 1 % FBS). The lower chamber was supplemented with 1 % FBS-containing SMCM supplemented with vehicle or Ang II (1 μM) for 4 h at 37 °C and in 5 % CO_2. The culture medium in the upper chamber was discarded and non-migrating cells were carefully removed from upper filter surfaces with cotton swabs. The filter was washed twice with PBS. Residual cells were fixed with 4 % paraformaldehyde for 30 min and stained in 0.1 % crystal violet for 30 min. Five randomly selected fields were photographed under the microscope for counting.

2.7. Western blot analysis

For Western blot analysis of PPARα expression in VSMCs, nuclear protein was extracted from the cells using KeyGEN Nuclear Protein Extraction Kit (KeyGen Biotech, Nanjing, China) as per the manufacturer's instructions and described [41,42]. Briefly, cells were harvested using EDTA -free trypsin as indicated above, washed twice with 1 ml ice-cold PBS and pelleted by centrifugation at 500*g* for 3 min. The pellet was then resuspended in precooled buffer A [10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1.0 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. The tubes were vortexed for 15 s and then allowed to swell on ice for 15 min. The nuclear pellet was resuspended in 1/20 total volume of ice-cold buffer B [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.4 M

NaCl, 1.0 mM EDTA, 1.0 mM ethylene glycol tetraacetic acid (EGTA), 1.0 mM DTT, and 1.0 mM PMSF]. The tubes were vortexed for 15 s, and then allowed to swell on ice for 1 min and pelleted by centrifugation at 25,186 g for 30 s at 4 °C. Plasma proteins were obtained by collecting the supernatants. 100 μl ice-cold buffer C (each ml contains 1 μl DTT, 5 μl 100 mM PMSF and 1 μl protease inhibitor cocktail) was added onto the precipitate after centrifugation. The tube was placed on ice for 30 min (vortexed for 15 s every 10 min), and then centrifuged at 25,186 g for 10 min at 4 °C. The supernatant containing the nuclear protein was stored at −80 °C for later use. Protein concentrations were determined with the BCA protein assay kit (Thermo). Equal amounts of proteins (30 μg) were then resolved in sodium dodecylsulfate-poly- acrylamide gels and transferred onto polyvinylidene fluoride membranes. The membranes were incubated with primary antibodies against PPARα (1:1000, Abcam, Cambridge, UK) and lamin B1 (1:1000, Abcam) overnight at 4 °C, washed three times with Tris-buffered saline tween, and then incubated with secondary antibodies (1:2000, goat-anti-rabbit and 1:2000, goat-anti-mouse, respectively; Cell Signaling) for 1 h at room temperature. Specific binding was detected with enhanced chemiluminescence reagents. The blots were quantified by Image J software (ImageJ, NIH, Bethesda, MD, USA).

2.8. Statistical analysis

All values are demonstrated as mean ± SEM of at least three independent experiments. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, California, USA). Differences between two groups were analyzed using unpaired Student's *t*-test. Differences between multiple groups were determined using one-way ANOVA followed by Bonferoni's post-hoc test. A p value of less than 0.05 was considered significant.

3. Results

3.1. Generation and characterization of smooth muscle cell-specific *Ppara*-deficient mice

SMC-specific *Ppara* deficient mice with floxed *Ppara* excised from SMCs using a SM22α-Cre transgenic mouse line were generated as described in the METHODS section (Fig. 1A). Disruption of the *Ppara* gene, after recombination of exon 5, was confirmed by PCR analysis (Fig. 1B). Progeny homozygous for *Ppara* deletion was referred to as $Ppara^{\Delta SMC}$ mice. The $Ppara^{\Delta SMC}$ mice and $Ppara^{fl/fl}$ mice had similar survival times, mortality rates, heart/body weight ratio, heart weight/tibia length ratio, left ventricular mass and blood pressure. Meanwhile, $Ppara^{\Delta SMC}$ mice did not exhibit any noticeable sign of hepatomegaly. To

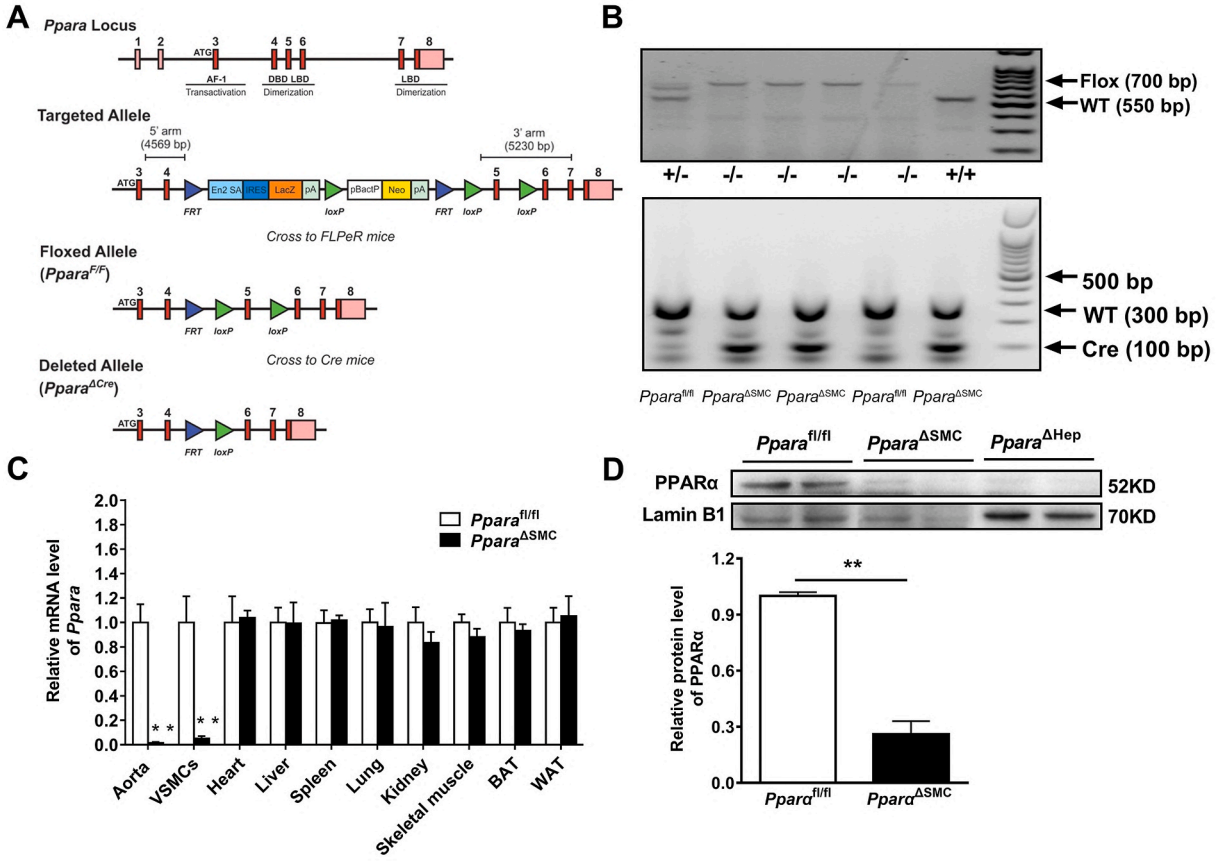

Fig. 1. Generation and characterization of VSMC-specific *Ppara*-deficient mouse. (a) Schematic representation of the targeting construct and deletion strategies. To delete the floxed *Ppara* allele from VSMCs, mice carrying the floxed allele ($Ppara^{flox}$-neo) were crossed to FLPeR mice for excision of the FRT-flanked neo cassette. The resulting floxed ($Ppara^{fl/fl}$) mice were crossed to Cre transgenic mice to excise exons 5, leading to the generation of the *Ppara* null allele ($Ppara^{\Delta cre}$). (b) Polymerase chain reaction (PCR) analysis demonstrates *Ppara-loxp* and *Sm22a-Cre* genes on DNA isolated from mouse tails. Homozygous knockout mice ($Ppara\text{-}loxP^{+/+}/Sm22a\text{-}Cre^{+}$) were designated as $Ppara^{\Delta SMC}$. (c) Real-time PCR for *Ppara* mRNA in various tissues including the heart, liver, spleen, lung, kidney, skeletal muscle, brown adipose tissue (BAT), white adipose tissue (WAT), aorta and cultured VSMCs isolated from $Ppara^{fl/fl}$ mice and $Ppara^{\Delta SMC}$ mice (n = 3–6 for each genotype). (d) Western blot analysis of PPARα protein in VSMCs isolated from the two strains of mouse. Lamin B1 serves as loading control. Liver tissue from hepatocyte-specific *Ppara*-knockout mice ($Ppara^{\Delta Hep}$) serves as a negative control. *$p < 0.05$, **$p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

evaluate the efficiency and tissue specificity of the Cre-mediated deletion of the floxed gene, real-time PCR analyses of *Ppara* expression were performed in various tissues (aorta, heart, liver, spleen, lung, kidney, skeletal muscle, and brown and white adipose tissues) as well as in cultured VSMCs isolated from $Ppara^{\Delta SMC}$ mice as compared to $Ppara^{fl/fl}$ mice (Fig. 1C). The *Ppara* expression was markedly and specifically downregulated in aortic tissue and cultured VSMCs with a knockout efficiency of both more than 90 %. Western blot analysis also confirmed substantially reduced expression of PPARα in VSMCs of $Ppara^{\Delta SMC}$ mice (Fig. 1D).

3.2. PPARα deficiency attenuates vascular smooth muscle cell apoptosis

To determine the role of PPARα in Ang II-induced VSMC apoptosis, VSMCs were isolated from $Ppara^{fl/fl}$ and $Ppara^{\Delta SMC}$ mice and exposed to Ang II (1 μM). Meanwhile, since oxidative stress serves as a major mechanism underlying Ang II-induced vascular damage [43], VSMC apoptosis in response to H_2O_2 (300 μM) stimulation was also examined. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and flow cytometry analysis showed that $Ppara^{\Delta SMC}$ VSMCs were more resistant to Ang II- induced apoptosis as compared to $Ppara^{fl/fl}$ VSMCs (Fig. 2A and B). The possible impact of high concentration of Ang II-induced VSMC apoptosis on the observed results was excluded since neither Ang II incubation at this concentration nor PPARα deficiency was able to trigger marked VSMC necrosis (Supplementary Fig. 2). Meanwhile, apoptosis of $Ppara^{\Delta SMC}$ VSMCs was significantly attenuated in response to H_2O_2 stimulation as compared to $Ppara^{fl/fl}$ VSMCs (Fig. 3A). A subsequent mechanistic investigation demonstrated that cleaved caspase-3, a terminal executor of apoptosis, was induced by H_2O_2 in $Ppara^{fl/fl}$ VSMCs but attenuated in $Ppara^{\Delta SMC}$ cells (Fig. 3B).

3.3. PPARα deficiency enhances vascular smooth muscle cell migration

To further verify the biological role of PPARα in VSMC, isolated cells were subjected to Ang II treatment and cell migration ability was examined. As shown in Fig. 4, PPARα deficiency was unable to significantly affect the migration ability of unstimulated cells as seen in the wound-scratch assay (Fig. 4A) and the transwell migration assay (Fig. 4B). Whereas Ang II significantly increased the migration ability of $Ppara^{fl/fl}$ cells compared to untreated cells, this effect was enhanced by PPARα deficiency.

Because MCP-1 is an established chemoattractant to stimulate migration of VSMCs, the role of PPARα in VSMC MCP-1 expression was investigated. As expected, it was shown that Ang II-induced upregulation of *Mcp-1* expression, and this effect was further enhanced by PPARα deficiency (Fig. 4C). Of note, a higher level of *Mcp-1* was also observed in unstimulated cells (Fig. 4C).

4. Discussion

Over the past few decades, PPARα has been explored extensively as therapeutic targets for cardiovascular disorders [44–46]. Besides its role in the regulation of energy homeostasis, the protective effects of PPARα against myocardial ischemia/reperfusion injury, cardiac fibrosis and hypertrophy, hypertension, vascular inflammation and atherosclerosis have also been documented [45,47–51]. However, the molecular mechanisms mediating these effects are still not fully understood. In this field, diverse approaches, such as transgenic animals with deletion of

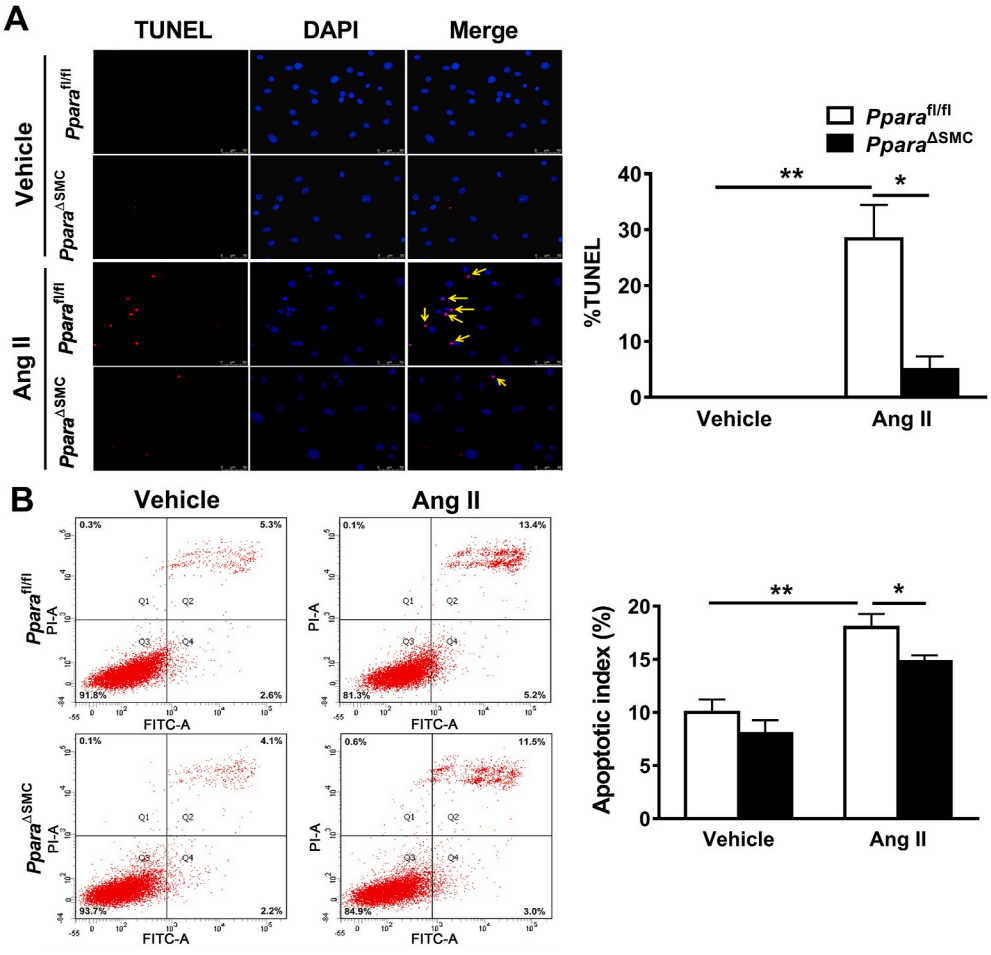

Fig. 2. PPARα deficiency attenuates Ang II-induced VSMC apoptosis. (a) VSMCs were isolated from $Ppara^{fl/fl}$ and $Ppara^{\Delta SMC}$ mice, starved and cultured in the presence of vehicle or Ang II for 24 h. TUNEL (red) and DAPI (blue) double staining were performed to detect apoptotic cells. (b) Cells were incubated with vehicle or Ang II for 24 h, stained with Annexin V-FITC and propidium iodide (PI) and analyzed by flow cytometry. n = 3–4/group. $^*p < 0.05$, $^{**}p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

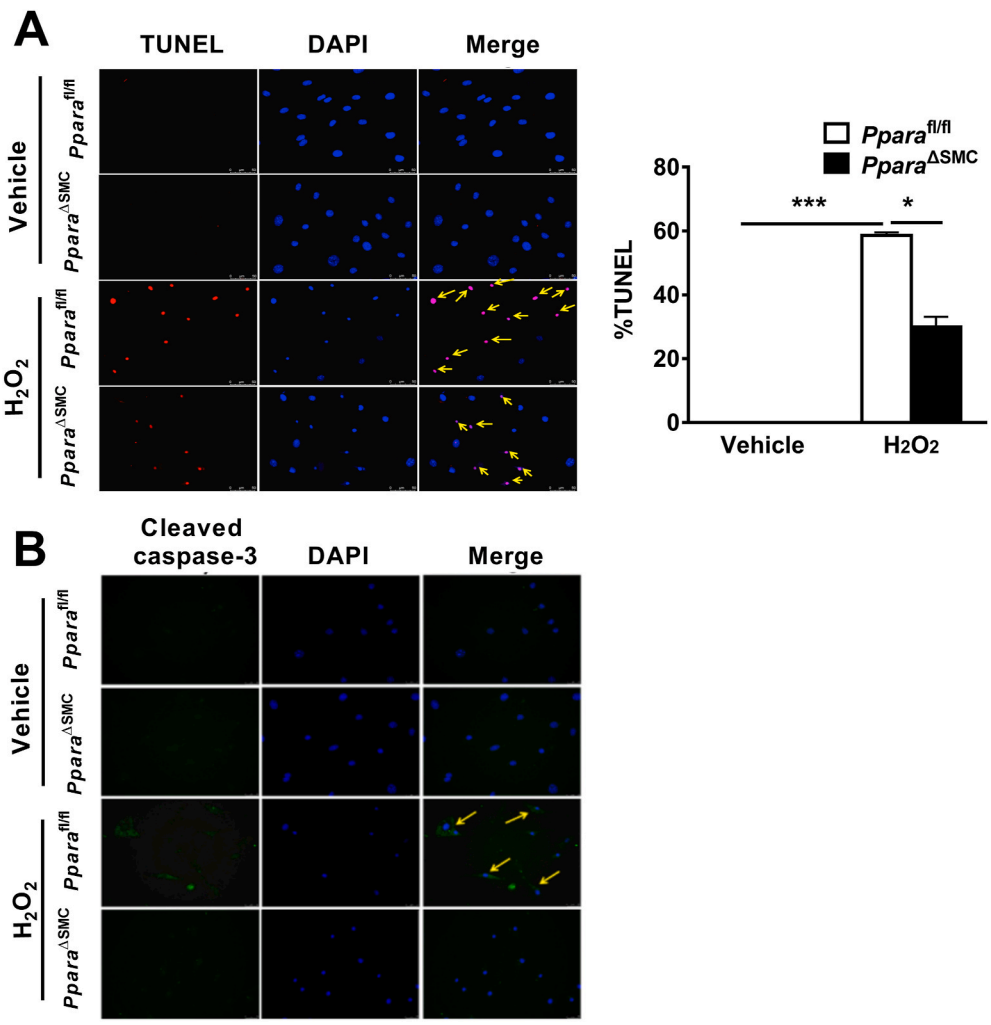

Fig. 3. PPARα deficiency attenuates H_2O_2-induced apoptosis of cultured VSMC. VSMCs were isolated from *Ppara*$^{fl/fl}$ mice and *Ppara*$^{\Delta SMC}$ mice and cultured in the presence of vehicle or H_2O_2 (300 μM) for 24 h. (a) TUNEL (red) and DAPI (blue) double staining were performed to detect apoptotic cells. n = 4–6/group. *$p < 0.05$, ***$p < 0.001$. (b) Cells were double stained with cleaved caspase 3 (green) and DAPI (blue). Arrows indicate double stained cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

PPARα genes [13] or activation of PPARα with specific agonists [52–54] in various models of cardiovascular diseases, have been used to define the role of PPARα in the pathogenesis of cardiovascular disorders. By constructing a SMC-specific PPARα deficient mouse model and isolating VSMCs from these mice, the present study may have substantial advantage over conventional gene knockout studies which may be limited by upregulation of compensatory pathways that would obscure its direct function and over siRNA techniques which also have off-target effects and can induce cell apoptosis in a target-independent fashion [55].

Our results demonstrated that PPARα deficiency did not aggravate but rather protected against Ang II-induced VSMC apoptosis (Figs. 2 and 3), indicating a pro-apoptotic role for PPARα in VSMCs. Signaling mechanisms whereby PPARα promotes VSMC apoptosis are unclear. It was previously demonstrated that PPARα ligand docosahexaenoic acid induced apoptosis via translocation of plasma membrane phosphatidylserine and disruption of mitochondrial transmembrane potential, followed by enhanced expression of bax and activation of caspase 3, a critical proteolytic enzyme involved in the death-signaling pathway [56], which is in line with the present study. There is also possible involvement of the MAPKs, which are regulators of cell growth/apoptosis and include ERK, p38 MAPKs, and c-Jun N-terminal kinase/stress-activated protein kinases [57]. Although ERK activation is characteristically linked with growth and survival signaling [58], the activation of p38 MAPK and c-Jun N-terminal kinase/stress-activated protein kinase has been shown to possess pro-apoptotic effects [59,60] and could be involved in PPARα-induced apoptosis in VSMCs. With reference to the previously reported promoting role of VSMC apoptosis in atherosclerosis acceleration, plaque calcification, medial degeneration and stenosis [61], whether this pro-apoptotic effect contributes to the lack of benefits of fenofibrate in the FIELD study [21] needs further investigation.

Migration of VSMCs is another crucial event involved in the development of post-intervention restenosis and atherosclerosis [62,63]. VSMC migration and proliferation lead to intimal hyperplasia, which is a prominent feature of atherosclerotic plaques [64] and thought to play a fundamental role in the restenosis that accompanies percutaneous coronary interventions, such as coronary artery angioplasty and stenting [65]. The current study, showing that VSMC migration is increased in PPARα deficient VSMCs (Fig. 4), is consistent with previous studies using PPARα ligands WY-14643 and 5,8,11,14-eicosatetranoic acid, which were demonstrated to inhibit TGF-β–induced VSMC migration [11]. The mechanism by which PPARα regulates VSMC migration remains to be fully elucidated. VSMC migration requires the interaction of ECM with cell surface receptors of integrin, a family of heterodimeric transmembrane glycoproteins consisting of noncovalently associated α and β chains [66]. The integrin complexes αvβ3 and αvβ5 are expressed on VSMCs and can modulate their migration via cross-talks with the ECM proteins vitronectin and osteopontin [67]. It was demonstrated that PPARα activators inhibit β5 integrin transcription and VSMC migration through indirect interaction with the TGF-β–regulated Smad4 transcription factors [11]. Notably, β3 integrin promoter is known to contain binding sites for a number of transcription factors that are regulated by PPARα, including Sp1 (specificity protein 1), AP-1

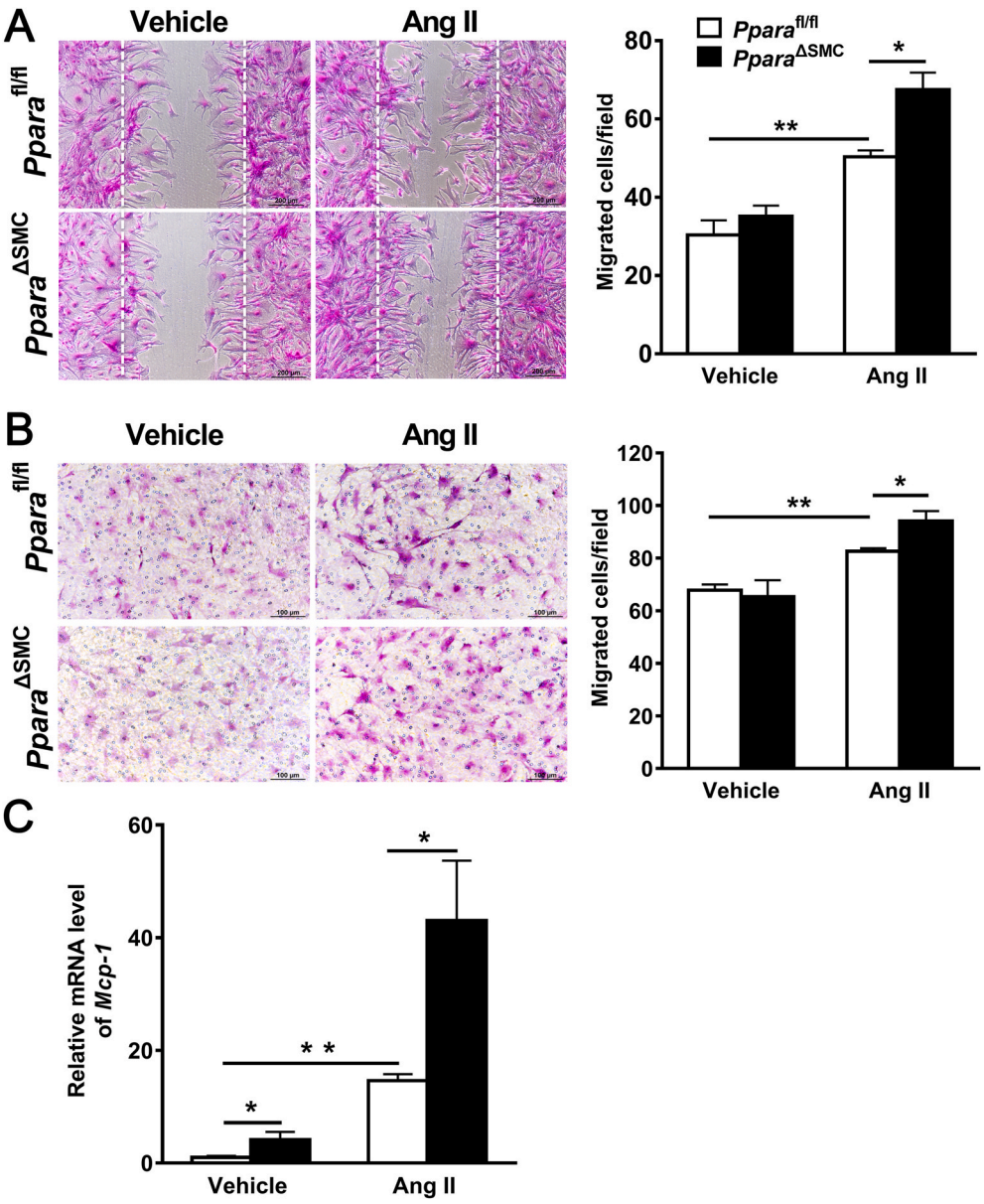

Fig. 4. Deficiency of PPARα increases VSMC migration. (a) VSMCs were isolated from *Ppara*$^{fl/fl}$ mice and *Ppara*$^{\Delta SMC}$ mice, serum-starved and scratches were introduced with a sterile 100 μl pipette tip. Then cells were incubated in the presence of vehicle or Ang II (1 μM) for 24 h, stained with 0.1 % crystal violet staining solution and observed. (b) Migration of VSMCs was detected by trans-well assay after a 4-h incubation of vehicle or Ang II. (c) Cells were exposed to vehicle or Ang II for 24 h, lysed and subjected to real-time PCR analysis for the mRNA expression of monocyte chemoattractant protein 1 (*Mcp-1*). Results shown are the relative expression normalized to β-actin gene (*Actb*). n = 3–5/group. $*p < 0.05$, $**p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(activator protein 1), STAT (signal transducer and activator of transcription) and NF-κB (nuclear factor κB). It was demonstrated that both the mRNA and surface expressions of β3 integrin receptor are elevated in Ang II-treated rat cardiac fibroblasts [68]. Future studies are required to more fully elucidate transcriptional mechanisms involved in PPARα–mediated inhibition of VSMC migration. Nevertheless, the present study adds to the understanding of the protective role of PPARα in atherosclerosis and other vascular diseases featuring the migration of VSMCs in the vascular wall.

The present study has several limitations. First, as discussed above, it does not provide more mechanistic insights into PPARα regulation of VSMC apoptosis and migration. Although these are not the main focus of the current study, a more in-depth investigation would be helpful to more clearly explain PPARα regulation of VSMC pathophysiology. Second, the present study only used VSMCs isolated from mouse aortas to explore the role of PPARα. It would be helpful to further elaborate on this point using other VSMCs, such as those isolated from mouse mesenteric arteries. In addition, we only used cultured VSMCs which represent the cells in isolation, and not an integral part of the vascular tissue. *In vivo* studies using Ang II-infused mouse model of hypertension would be helpful. However, isolated primary VSMCs have the advantage of relatively well-controlled cellular context allowing the investigation of PPARα effects that are difficult to decipher *in vivo* while maintaining the cell identity and property that closely resemble those in the body. Third, as hard as we have tried, we are currently unable to obtain satisfactory commercial antibodies against PPARα, resulting in the somewhat diffuse and blurry bands in the Western blots. Fourth, the concentration of Ang II used in this study (1 μM) was much higher than what has been reported in the body [69]. Nonetheless, the 1 μM concentration used is consistent with other *in vitro* experiments both from our group [40,70] and from many other groups [71–78]. There is a reasonable criticism that Ang II may induce desensitization, down-regulation, and internalization of its receptors [79–81]. However, a high concentration of Ang II at 1 μM was found to be necessary to trigger consistent increases in protein-DNA ratio, a measure of cardiomyocyte growth, reflecting perhaps the importance of receptor recycling rate in the activation of the process coupled to cell growth [71]. Saturation binding isotherms obtained with iodinated Ang II may be useful to more accurately determine total receptor binding and internalization [80].

In conclusion, the present study demonstrates that PPARα can be

both pro-apoptotic which has vascular disrupting effect and anti-migratory which is vascular protective in terms of VSMC pathophysiology. These mixed functions are remarkable given the previously reported lack of concrete protection of fibrates against cardiovascular events or even deleterious effects in clinical trials, such as observed in the FIELD study [21]. In this study, fenofibrate did not significantly reduce the risk of major cardiovascular events in patients with previous cardiovascular disease and in patients older than 65 years of age [21]. Increased apoptosis [82] and decreased migration [83] with age has been observed in previous studies. It is thus of clinical importance and warrants further investigation as to whether aging unleashes the pro-apoptotic effects while blunts the anti-migratory effects of PPARα activation, resulting in a lack of apparent cardiovascular benefits in elderly patients.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

This study was funded by National Natural Science Foundation of China (81370521, 81320157, and 81670400), The Importation and Development of High-Caliber Talents Project of Beijing Municipal Institutions (CIT&TCD20150325), The Key Science and Technology Project of Beijing Municipal Institutions (KZ201610025025 to A. Qu), and The Fok Ying-Tong Education Foundation (151041).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.101091.

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