EXPERIMENTAL STUDY

PPARγ Alleviates Right Ventricular Failure Secondary to Pulmonary Arterial Hypertension in Rats

Ying Xu, MD, Qin Gu, MD, Ning Liu, MD, Yan Yan, MD, Xilan Yang, MD, Yingying Hao, MD and Chen Qu, MD

Summary

Pulmonary arterial hypertension (PAH) is characterized by pulmonary vascular remodeling leading to right ventricular hypertrophy (RVH) and failure. Peroxisome proliferator-activated receptor γ (PPARγ), a member of nuclear receptors, has been proved to ameliorate PAH. However, its effect on PAH-induced right ventricular failure (RVF) remains unknown. Therefore, we investigated the therapeutic potential of PPARγ in preventing monocrotaline (MCT)-induced RV dysfunction. The PAH model was induced by MCT administration. Male rats were administered with MCT to develop PAH and RVF formed by approximately day 30. Significant increase in RV area, RVAW resulted in an ascending RV index. However, the LV function including EF, FS, and LVID did not change significantly. PPARγ agonist prevented PAH-induced RVF by preserving RV index and preventing RVH. PPARγ's beneficial effects seem to result from various factors, including anti-apoptosis, preservation of PPARγ agonist rosiglitazone (Rosi) has been proved to alleviate PAH in rats, including regulating endothelial dysfunction, sustaining angiogenic potential in mature pulmonary microvascular endothelial cells (PMVECs), preventing PASMCs proliferation, etc. However, the mechanisms of PPARγ's effect on PAH-induced RVF remain unknown. As reported, metabolic disorders, chronic inflammation, as well as ROS accumulation has been attributed to myocardial apoptosis, which ultimately resulted in left ventricular failure. In the present study, we detected the changes occurred in RVF secondary to PAH and evaluated the effects of RV remodeling after PPARγ pretreatment in a rat model of monocrotaline (MCT)-induced RVF and explored the possible mechanism.

Methods

Animals and ethics statement: Male Sprague-Dawley (SD) rats weighing 220-260 g were purchased from Beijing WeiTongLiHua Experimental Animal Limited Liability Company (Beijing, China). The protocol of the study was approved by the Institutional Ethics Committee of Nanjing University Health Science Center and compiled

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with the Declaration of the National Institutes of Health Guide for Care and Use of Laboratory Animals (publication no. 85-23, revised 1985).

**Experiment protocol:** Rats were randomly separated into three groups: the negative control (NC) group, which was pretreated and administered with saline, n = 8; the RVF group, which was pretreated with saline and administered with 2% MCT (60 mg/kg, intraperitoneal, Sigma-Aldrich, USA), n = 8; and the RVF+ Rosi group, which was pretreated with Rosiglitazone (1 mg/kg/d, intraperitoneal, Sel-lick, USA), and administered with 2% MCT (60 mg/kg), n = 8. The pretreatment started one week before MCT injection daily and lasted for ten days.

**Echocardiography:** Echocardiography was performed using a Vevo2100 (Visual Sonics, Ontario, Canada) equipped with a 30-MHz transducer. Rats were anesthetized with 2% isoflurane, and two-dimensional guided M-mode tracings were recorded. The internal diameter of the LV in the short-axis plane was measured at end diastole. LV fractional shortening percentage (LVFS) and LV ejection fraction (LVEF), two indexes of LV systolic function, were calculated according to guidelines accompanying the Vevo 2100 UBM system. For RV function, calculation of the global myocardial performance index (MPI) (Tei index) by PW-TDI interval measurements was performed within one cardiac cycle. The Tei index was calculated as: Tei = (isovolumetric contraction time (ICT) + isovolumetric relaxation time (IRT)) / ejection time (ET). Besides, the right ventricular anterior wall (RVAW) and right ventricular area were also detected.

**Neonatal rat cardiomyocytes isolation, culture, and treatment:** The primary neonatal right ventricular cardiomyocytes were isolated from neonatal SD rats. Briefly, right ventricles were dissected and cut into pieces, and then were digested in trypsin (0.6 mg/mL, Sigma, St. Louis, MO, USA) and collagenase type II (1 mg/mL, Worthington, Lakewood, NJ, USA) at 37°C for 15 min 3-5 times. After filtration and centrifugation, cells were resuspended in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Pasadena, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Pasadena, CA, USA), 5% horse serum (Hyclone, Logan, UT, USA), 1% penicillin-streptomycin, and 0.1 mM 5’-Bromo-2’-Deoxyuridine (BrdU) (Sigma, St. Louis, MO, USA). The cardiomyocytes were then plated in 10 mg/mL gelatin (Sigma, St. Louis, MO, USA)-coated dishes. The primary cardiomyocytes were starved for 8h, and then were treated with 50 μmol/L of phenylephrine (PE, Sigma, St. Louis, MO, USA) for 48 hours to induce hypertrophy in vitro. PPARγ and its control were added 2 hours before PE treatment.

**Cardiac microvascular endothelial cells (CMECs) isolation, culture and treatment:** CMECs were isolated from the right ventricles from neonatal SD rats. Briefly, the right ventricles from neonatal SD rats were cut into pieces and digested in trypsin (0.6 mg/mL, Sigma, St. Louis, MO, USA) and collagenase type II (1 mg/mL, Worthington, Lakewood, NJ, USA) for 10 min × 3 times at 37°C. Dissociated cells were then filtered and centrifuged and resuspended in endothelial cell medium (Sciencell, Carlsbad, CA) consisting of 10% FBS. CMECs were seeded (5 × 10^6) in 6-well plates and were stimulated with lipopolysaccharide (LPS, 10 ng/mL, Sigma-Aldrich, USA) for 24 hours. PPARγ was added 2 hours before LPS treatment.

**Inflammatory cells culture and treatment:** As one of most important inflammatory cells, the rat alveolar macrophage NR8383 cell line (CRL-2192) was obtained from ATCC (USA) and was cultured in Ham’s F12K medium (Sigma-Aldrich, USA) supplemented with 15% FBS. The culture was performed at 37°C in a humidified atmosphere (5% CO2). NR8383 cells were seeded on 12-well plates at a density of 2 × 10^6 cells per well in 1 mL culture medium and left for 3 hours to adhere. The culture supernatant with non-adherent cells was discarded and exchanged for fresh medium alone or fresh medium with LPS for 24 hours. Then the culture medium was collected, centrifuged at 1000 rpm for 5 minutes. The supernatant was used for cytokines measurement.

**Immunofluorescent staining for α-actinin and cell size determination:** The cardiomyocytes size was determined by immunofluorescent staining for α-actinin (Sigma, St. Louis, MO, USA). Briefly, cardiomyocytes were washed with PBS, and fixed in 4% paraformaldehyde (PFA) for 20 minutes, then blocked with 10% goat serum for 1 hour at room temperature. After that, cardiomyocytes were incubated with α-actinin antibody (1:500 dilutions) overnight and then were incubated with Fluorescein isothiocyanate (FITC)-AffiniPure goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) coupled with DAPI(1:200 dilutions). Finally, the cell images were taken in a Nikon eclipse Ti microscope, and the cell surface area was analyzed by software Image J.

**Intracellular reactive oxygen species assay:** Cardiomyocytes were seeded (2 × 10^6) in 96-well plates and were pretreated with PPARγ for 2 hours. The production of intracellular reactive oxygen species (ROS) was measured by DCFH oxidation. Briefly, 10 mM DCFH-DA was dissolved in methanol and diluted in Hank’s Balanced Salt Solution (HBSS, 1:500). Cells then were exposed to DCFH-DA for 1 hour and followed by a 1 hour treatment with PE (50 μmol/L). After incubation, the fluorescent signal from dichlorofluorescein (DCF; excitation 495 nm, emission 529 nm) was registered.

**Concentration of free fatty acid:** Cardiomyocytes were seeded (2 × 10^6) in 12-well plates and were pretreated as described above. Cardiomyocytes were washed with PBS 3 times and then were dissolved in a lysis buffer. Concentrations of free fatty acid in cardiomyocytes were detected by free fatty acid quantitation kit (Sigma-Aldrich, USA) with cell lysates according to the manufacturer’s instructions.

**The glucose consumption:** Cardiomyocytes were seeded (2 × 10^6) in 12-well plates and were pretreated as described above. The glucose consumption of cardiomyocytes was measured by standard enzyme reagent kit (Sigma-Aldrich, USA) according to the manufacturer’s instructions.

**Measurement of cytokines:** The amounts of TNF-α, IL-6 and IL-1β in the culture medium were measured with commercial ELISA kits from R&D Systems (Minneapolis,
The other half of right ventricular was stored at -80°C fixed in 4% paraformaldehyde overnight, sectioned into 5 μm slices, and stained with hematoxylin and eosin (H&E).

**Immunohistochemistry:** Half of the right ventricular was activated receptor γ (PPARγ), peroxisome proliferator associated X proteins (Bax, 1:1000 dilution; Cell Signaling Technology, Boston, MA, USA).

**Western blotting:** Proteins were isolated from right ventricular and the total protein concentrations were measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Inc., USA). cDNA synthesis was performed with Bio-Rad iScrip™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) in a reaction volume of 20 ml. For quantitative mRNA analysis, a template equivalent to 20 ng of total RNA was subjected to 40 cycles of quantitative PCR using the Takara SYBR Premix Ex Taq™ (TliRNaseH Plus, Takara, Tokyo, Japan) in the 7900HT Fast Real-Time PCR System. Real-time PCR primers were synthesized by GenScript Corporation (Nanjing, China) and were listed in the Table. The relative expression level between treatments was then calculated using the following equation: relative gene expression = 2^(-ΔΔCt control).

**Statistical analysis:** Data are presented as mean ± standard deviation (SD). An independent-samples t test, Chi-squared test or one-way ANOVA was conducted to evaluate the one-way layout data. If a significant difference was observed, Bonferroni’s post hoc test was conducted to identify groups with significant differences. P < 0.05 was considered to indicate a statistically significant difference between groups. All analyses were performed using GraphPad Prism 5.

### Results

PPARγ prevents severe RVF secondary to pulmonary arterial hypertension: Accumulating evidence has suggested that PPARγ agonist could protect the heart from adverse remodeling after ischemia injury which is mainly associated with left ventricular function. In order to distinguish the PPARγ protective effect on RV from LV and gain more insight into the protective effects related to PPARγ, echocardiographic and Doppler flow measurements were carried out for both RV and LV.

RVF was confirmed by qRT-PCR of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) (Figure 1A), which were all significantly increased in RVF rats compared to controls, suggesting that pathological heart remodeling occurred in RVF rats. Echocardiographic and Doppler flow measurements revealed that the LV function including LVFS, LVEF, as well as LVID, did not change significantly between different groups (Figure 1B).

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
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</thead>
<tbody>
<tr>
<td>ANP</td>
<td>GCTGCCGTGGCCTGGCTAGGAA</td>
<td>CCGCGTGAGGGTTTAGTG</td>
</tr>
<tr>
<td>BNP</td>
<td>GCTGCCGTGGCCTGGCTAGGAA</td>
<td>CCGCGTGAGGGTTTAGTG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>CACGAAATGGCCTGGGACATATT</td>
<td>GCGATAGGAAATAGCCACCTCT</td>
</tr>
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<td>IL-6</td>
<td>CCAACCTCAATGCTCTCTAATG</td>
<td>TTCAAGTGTTCTCAAGTGTTGG</td>
</tr>
<tr>
<td>TNF-α</td>
<td>GCTGGCCTTGTCAGATGTG</td>
<td>CAGATTGGAAACCACTACATTT</td>
</tr>
<tr>
<td>GADPH</td>
<td>GCGACAGTCAAGGCTAGAGGATG</td>
<td>ATGCGTTGAAAGCAGCGAG</td>
</tr>
<tr>
<td>PPARγ</td>
<td>TACACGCTGGTTACTTTCCT</td>
<td>TTCTCTTTGTGACCATC</td>
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<td>PGCl-α</td>
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<td>TTGCGTTGAAGCTGCTTAT</td>
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</tr>
<tr>
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<td>GCTGCCGCGCTTACACA</td>
<td>TCAGCACGGCTTACAC</td>
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<td>FATP</td>
<td>GCTGCCGCGCTTACACA</td>
<td>TCAGCACGGCTTACAC</td>
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**Table. Summary of Primers Used in qRT-PCR**
Figure 1. PPARγ prevented severe RVF secondary to pulmonary arterial hypertension (PAH). A: The mRNA of ANP and BNP was determined by qRT-PCR. n = 5. B: Echocardiography was performed using a Vevo2100 (Visual Sonics, Ontario, Canada) equipped with a 30-MHz transducer. Left ventricular internal diameter (LVID) was measured at the end diastole. LV fractional shortening percentage (LVFS) and LV ejection fraction (LVEF), two indexes of LV systolic function, were calculated according to guidelines. All the data from LV did not change significantly between groups. n = 5 to 6. C: For RV function, calculation of the global myocardial performance index (MPI) (Tei index) by PW-TDI interval measurements was performed within one cardiac cycle. The Tei index was calculated as: Tei = (isovolumetric contraction time (ICT) + isovolumetric relaxation time (IRT)) / ejection time (ET). Besides, right ventricular anterior wall (RVAW) and right ventricular area were also detected. The PPARγ agonist decreased RV area, RVAW, as well as Tei index. Arrows indicate right ventricle (n = 5 to 6). NC indicates negative control; MCT, monocrotaline; and Rosi, Rosiglitazone. *P < 0.05; **P < 0.01; ***P < 0.001.
The PPARγ decreased apoptosis in RVF rats secondary to PAH. A: PPARγ decreased cardiac apoptosis. Arrows indicate TUNEL staining (n = 3 to 4). B: Bax was determined by western blot at protein level (n = 3). NC indicates negative control; MCT, monocrotaline; Rosi, Rosiglitazone. Scale bar, 50 μm; *P < 0.05; **P < 0.01; ***P < 0.001.

directly toward RV instead of by affecting LV.

PPARγ decreases apoptosis induced by pulmonary arterial hypertension (PAH) in RVF rats: Cardiomyocytes were more inclined to suffering from apoptosis in pressure,19) which might lead to cell loss. As the quantity of cardiomyocytes gets lost to a certain degree, the heart may undergo cardiac compensatory hypertrophy, dysfunction, ventricular remodeling, and ultimately, heart failure.20,21) In order to investigate whether PPARγ protects cardiomyocytes against PAH-induced apoptosis in RVF, TUNEL assay was performed. As a result, cardiac apoptosis was significantly increased in RVF rats. However, PPARγ pretreatment decreased TUNEL positive cells (Figure 2A). The Bcl-2-associated X protein (Bax) is a very important pro-apoptotic protein, which plays important roles in triggering the mitochondrial death cascade,22-24) and PPARγ pretreatment suppressed the over-expression of Bax in our experiment.

PPARγ decreases RV inflammation in RVF rats: Inflammation plays an important role in heart failure. PPARγ was proved to alleviate inflammation in a variety of diseases. In the well-established RVF model, we found that there was an increased infiltration of inflammation cells confirmed by H&E staining (Figure 3A). The elevated pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α were determined by qRT-PCR (Figure 3B). In addition, the pro-inflammatory cytokines in circulation were also elevated and were reversed by PPARγ pretreatment (Figure 3C). All the data above revealed the fact that PPARγ played an important role in anti-inflammation in RVF.

PPARγ improves the metabolism disorders of glucose and lipid: In normal conditions, most of the energy that the heart consumes comes mainly from fatty acid and glucose oxidation. When heart failure occurs, the imbalance of glucolipid metabolism is disrupted.25) In the well-established RVF model, PPARγ and PGC-1α were found down-regulated and were reversed by PPARγ pretreatment (Figure 4A). In addition, glucose transporters (Gluts), which are responsible for transporting glucose into the heart,26) the lipoprotein lipase (LPL) which is associated with the metabolism of core triglycerides of very low density lipoproteins (VLDL) and the fatty acid transporter (FATP), which is involved in long chain fatty acid uptaking across the plasma membrane27) were detected in our experiment. As a result, Glut-4, LPL, and FATP decreased in RVF, whereas the PPARγ agonist reversed the decline (Figures 4B, C). The data above demonstrated PPARγ agonist pretreatment improved the metabolism of fatty acid and glucose.

PPARγ affects the cardiomyocytes from right ventricular directly in vitro: In order to underline the immediate
The effect of PPARγ on right ventricular cardiomyocytes, the experiment was conducted exactly on the cardiomyocytes extracted from the right ventricles. The cells were stimulated by PE. Then the cell surface area, mRNA of ANP and BNP (Figure 5A), intracellular ROS, free fatty acid, and glucose consumption (Figure 5B) were determined. Interestingly, PE-induced cardiomyocyte enlargement and the increase in hypertrophic markers (ANP, BNP) were markedly reversed by pretreatment with PPARγ (Figure 5A), indicating that PPARγ was protective against PE-induced cardiac hypertrophy. Meanwhile, PE-induced accumulation of intracellular ROS and free fatty acid, and the decline of glucose consumption were also reversed by PPARγ pretreatment (Figure 5B), indicating that PPARγ was capable of reducing ROS producing and improving glycolipid metabolism.

The direct effects of PPARγ on CMECs and inflammatory cells: ECs dysfunction leads to expression of adhesion molecules such as ICAM-1 and VCAM-1 for inflammatory cells to migrate and accumulate.28 We have observed the aggregation of inflammatory cells in RV in MCT-induced RVF rats, which could be alleviated by PPARγ pretreatment. Then we aim to verify whether PPARγ plays the protective effect directly on CMECs and inflammatory cells. The results showed that the increased secretion of inflammatory cytokines from NR8383 cells was reversed by PPARγ pretreatment (Figure 6A). In addition, in LPS-stimulated CMECs, the expression of ICAM-1 and VCAM-1 was increased, whereas the PPARγ agonist reduced the expression at protein level (Figure 6B). The data above reveal the fact that PPARγ is able to affect CMECs and inflammatory cells directly, which may be responsible for the reversion of inflammation.

Discussion

Right ventricular hypertrophy (RVH) occurs in response to pressure overload in several kinds of diseases, such as pulmonic stenosis, and in pulmonary arterial hypertension (PAH). Increased pressure may induce changes in wall thickness and geometry in RV remodeling. Peroxisome proliferator-activated receptor γ (PPARγ), a member
of nuclear receptors, has been proved to ameliorate PAH by inhibiting PASMCs proliferation, improving ECs function, alleviating inflammation, etc.\(^{11-14}\) In this experiment, the direct effect of PPAR\(\gamma\) on RV was examined.

RVF is related to contraction and relaxation abnormalities of the RV. RV myocardial performance index, which is also called Tei Index, has been proved to be more effective for analysis of global RV dysfunction than systolic and diastolic measures alone.\(^{29}\) In our study, we found that RVF rats had prolonged MPI of right ventricles compared with normal controls. Besides, the geometry changes of RV also occurred, which was described by RV area and RVAV. PPAR\(\gamma\) pretreatment preserved RV function and geometrical diversion.

The heart is an organ with very high energy demand. It is reported that the stressed RV is more susceptible to metabolic switch. Emerging evidence demonstrated that PPAR\(\gamma\) modulates metabolism in heart failure. In the current study, we found that the MCT-induced decrease of enzymes that was involved in glucose and fatty metabolism was reversed by PPAR\(\gamma\) pretreatment. Meanwhile, PPAR\(\gamma\) decreased PE-induced cardiomyocytes intracellular ROS and free fatty acid accumulation, restored glucose consumption, indicating that PPAR\(\gamma\) was able to affect directly on cardiomyocytes and improve the disorders of glycolipid metabolism. In addition, energy shortage in heart failure has a reduced angiogenic response, and is more likely to activate cell death pathways.\(^{30}\) RV dilatation has been correlated with higher rates of apoptosis. In our study, we demonstrated that PPAR\(\gamma\) reversed the declined apoptosis of cardiomyocytes in vivo, which might be the vital mechanisms in RVF.

In addition, inflammation plays an important role in the progression of PAH. Some researchers have shown that PAH can be alleviated by reducing the pro-inflammatory cytokines.\(^{31}\) It has been demonstrated that MPO-positive infiltrates at the RV hypertrophy stage in RV in MCT-treated rats, and continues into the RV failure stage, indicating that inflammation has been involved in RVF ever since the early stage.\(^{30}\) In the current study, we found that PPAR\(\gamma\) pretreatment decreased the infiltration of inflammatory cells, the amount of pro-inflammatory cytokines such as IL-1\(\beta\), IL-6, and TNF-\(\alpha\) both in RV and serum. We also proved that PPAR\(\gamma\) down-regulated LPS-induced pro-inflammatory cytokines in NR8383 cells, indicating that PPAR\(\gamma\) was able to act directly on inflammatory cells so as to alleviate inflammation, which might be another mechanism through which PPAR\(\gamma\) preserved the RV function.

EC dysfunction leads to expression of adhesion molecules for inflammatory cells to adhere and migrate in vascular diseases.\(^{28}\) Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are two of the most important adhesion molecules mainly expressed by ECs. In the LPS-induced ECs dysfunction in vitro, PPAR\(\gamma\) reversed the increased expression of ICAM-1 and VCAM-1, indicating that PPAR\(\gamma\) has a direct impact on ECs, which might be attributed to the inflammatory cells’ migration and accumulation.

Several limitations of the present study should be highlighted. First, as the PPAR\(\gamma\) agonist has been proved to provide the beneficial effects in RVF, the PPAR\(\gamma\) inhibitor should also be taken into the experiment, to verify effectiveness of PPAR\(\gamma\) from the opposite side. Second, as the progression of the pulmonary hypertension can also be affected by PPAR\(\gamma\) pretreatment, it is hard to distinguish the benefits of PPAR\(\gamma\) pretreatment on the right ventricle in vitro from reducing the pulmonary artery hypertension. The PPAR\(\gamma\) specific right ventricular knockout rats will help further clarify their effects in the progress of RVF secondary to PAH.

Many PAH drug therapies target the pulmonary vasculature, providing the benefits on RV by reducing pulmonary artery pressure.\(^{33,34}\) However, the RV is the major determinant of functional state and prognosis in PAH.\(^{35}\) Thus, PPAR\(\gamma\)’s advantage lies in offering the possibility of therapies that treat the RV and pulmonary artery as a whole. As is known to all, PPAR\(\gamma\) agonist provides benefits in PAH. In addition, we determined that the PPAR\(\gamma\) agonist prevented PAH-induced RVF by preserving the RV index and reducing RVH. Despite the limitations mentioned above, this article puts forward that PPAR\(\gamma\) may participate in the progress of RVF secondary to PAH by improving RV apoptosis, fibrosis, inflammation, and glycolipid metabolism, suggesting that PPAR\(\gamma\) has a direct effect on right ventricular cardiomyocytes. In a word, the PPAR\(\gamma\) agonist prevents the development of RVF.
Figure 5. PPARγ affects the cardiomyocytes from right ventricular directly in vitro. A: Rosi reverses PE-induced cardiac hypertrophy. The cell surface of cardiomyocytes was determined using immunolabelling for α-actinin (green). Nucleus was counter-stained with DAPI (blue). Scale bar = 100 μm (n = 5). The mRNA levels of ANP and BNP were assayed with real-time qRT-PCR (n = 5). B: Rosi reverses PE-induced accumulation of intracellular ROS and free fatty acid, preserves glucose consumption (n = 5). NC indicates negative control; PE, Phenylephrine; Rosi, Rosiglitazone. Scale bar, 100 μm; *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 6. The direct effects of PPARγ on CMECs and inflammatory cells. A: PPARγ pretreatment reversed the increased secretion of inflammatory cytokines from NR8383 cells (n = 3). B: PPARγ agonist reduced the expression of ICAM-1 and VCAM-1 in CMECs stimulated by LPS (n = 3). NC indicates negative control; LPS, lipopolysaccharide; and Rosi, Rosiglitazone. *P < 0.05; ***P < 0.001.
Disclosures

Conflicts of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

References