Egr-1 mediates leptin-induced PPARγ reduction and proliferation of pulmonary artery smooth muscle cells

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INTRODUCTION

Pulmonary hypertension (PH) is a life-threatening disease characterized by increased pulmonary vascular resistance and pressure, which finally leads to right ventricle failure and death (Bazan and Fares, 2015). Despite various treatments have been used during the past few decades, PH is still incurable (Humbert et al., 2010). Different types of PH share a common pathogenesis including vasoconstriction, pulmonary vascular remodeling, and thrombosis in situ (Humbert et al., 2004). The essential pathological characteristics of PH are excessive proliferation of pulmonary arterial smooth muscle cells (PASMC), leading to medial hypertrophy and vascular remodeling. However, the molecular mechanisms underlying this process are still not well understood.

Leptin is a 16-kDa, 146-amino-acid residue nonglycosylated protein encoded by obese (ob) gene and mainly synthesized and secreted by adipocytes (Zhang et al., 1994) and exerts its actions through its specific receptors present in a variety of tissues (Fruhbeck, 2006). Leptin is primarily known for its role as a hypothalamic modulator of food intake, body weight, and fat stores (Akther et al., 2009). In addition, leptin is also implicated in the modulation of other physiological processes, such as angiogenesis, wound healing, central and peripheral endocrine actions, and renal and pulmonary functions (Mantzoros et al., 2011). Recently, leptin signaling has been found to play an important role in the development of PH by stimulating PASMC proliferation (Schroeter et al., 2013; Chai et al., 2015; Huertas et al., 2015, 2016). However, the exact mechanisms underlying leptin-induced PASMC proliferation are still largely unknown.

ABSTRACT Loss of peroxisome proliferator-activated receptor γ (PPARγ) has been found to contribute to pulmonary artery smooth muscle cell (PASMC) proliferation and pulmonary arterial remodeling therefore the development of pulmonary hypertension (PH). Yet, the molecular mechanisms underlying PPARγ reduction in PASMC remain poorly understood. Here, we demonstrated that leptin dose- and time-dependently induced PPARγ down-regulation and proliferation of primary cultured rat PASMC, this was accompanied with the activation of extracellular regulated kinase1/2 (ERK1/2) signaling pathway and subsequent induction of early growth response-1 (Egr-1) expression. The presence of MEK inhibitors U0126 or PD98059, or prior silencing Egr-1 with small interfering RNA suppressed leptin-induced PPARγ reduction. In addition, activation of PPARγ by pioglitazone or targeting ERK1/2/Egr-1 suppressed leptin-induced PASMC proliferation. Taken together, our study indicates that ERK1/2 signaling pathway-mediated leptin-induced PPARγ reduction and PASMC proliferation through up-regulation of Egr-1 and suggests that targeting leptin/ERK1/2/Egr-1 pathway might have potential value in ameliorating vascular remodeling and benefit PH.
Peroxisome proliferator-activated receptor γ (PPARγ) is ubiquitously expressed in pulmonary vascular endothelial and smooth muscle cells and belongs to the nuclear hormone receptor superfamily with increasingly diverse functions as a transcriptional regulator (Tian et al., 2009; Gong et al., 2011). Mounting evidence has shown that activation of PPARγ attenuates PASMC proliferation and suppresses the development of PH in several animal models (Crossno et al., 2007; Li et al., 2010; Zhang et al., 2014; Xie et al., 2015), while PPARγ expression is reduced in the lungs and pulmonary vascular tissue of patients with PH and in several experimental models of PH (Ameshima et al., 2003; Tian et al., 2009; Gong et al., 2011; Lu et al., 2013). Several lines of evidence indicate that the increased leptin level is associated with the down-regulation of PPARγ and promotes cell proliferation in various cell types of nonpulmonary artery smooth muscle cell (Zhou et al., 2009a; Jain et al., 2011; Wang et al., 2012). However, it is still unclear whether leptin also causes the down-regulation of PPARγ and implicates in PASMC proliferation. To clarify this, primary cultured PASMC were stimulated with leptin, the expression of PPARγ and phosphorylation of ERK1/2 were determined, and the molecular mechanisms underlying these changes were further investigated.

RESULTS

Leptin stimulates PASMC proliferation

To examine whether leptin induces PASMC proliferation, time course and dose–response of leptin on cells proliferation were investigated. Cell proliferation was determined using the BrdU incorporation assay. As shown in Figure 1A, leptin dose-dependently stimulated PASMC proliferation at 24 h, and the maximal BrdU incorporation was a 2.30-fold increase over control at 100 ng/ml leptin (p < 0.01). Figure 1B demonstrates that leptin induced PASMC proliferation in a time-dependent manner; 100 ng/ml leptin caused a significant increase in BrdU incorporation over control after 24 h, and BrdU incorporation was a 2.81-fold increase compared with control at 48 h (p < 0.01).

Leptin down-regulates PPARγ expression in PASMC

It has been shown that leptin down-regulates PPARγ expression in several types of nonPASMC (Zhou et al., 2009a; Jain et al., 2011; Wang et al., 2012). To clarify whether leptin also reduces PPARγ expression in PASMC, cells were treated with different concentrations of leptin over different time periods, and the expression of PPARγ was determined using quantitative real-time reverse transcription PCR (qRT-PCR) and immunoblotting. As shown in Figure 2, A and B, leptin down-regulated PPARγ expression in PASMC in a dose-dependent manner at 24 h; 100 ng/ml leptin reduced PPARγ mRNA and protein levels to 0.46- and 0.37-fold compared with control, respectively (both p < 0.05). Figure 2, C and D, shows that leptin down-regulated PPARγ expression in PASMC in a time-dependent manner after 6 h treatment, and 100 ng/ml leptin for 24 h incubation reduced PPARγ mRNA and protein levels to 0.45- and 0.42-fold compared with control, respectively (both p < 0.05). These results suggest that leptin also suppresses PPARγ expression in PASMC.

Activation of ERK1/2 signaling mediates leptin-induced PPARγ reduction in PASMC

To investigate the mechanisms of leptin-induced PPARγ reduction, cells were treated with leptin (100 ng/ml) for different times; phosphorylation of ERK1/2 was determined using immunoblotting. As shown in Figure 3A, ERK1/2 phosphorylation was time dependent on 100 ng/ml leptin stimulation. Peak phosphorylation occurred at 5 min, which increased 3.54-fold over control (p < 0.01). To further examine whether ERK1/2 signaling mediated leptin-induced PPARγ down-regulation in PASMC, cells were pretreated with MEK inhibitor U0126 (10 μM) or PD98059 (10 μM) for 30 min followed by leptin (100 ng/ml) stimulation for 5 min or 24 h. The phosphorylation of ERK1/2 was measured after leptin stimulation for 5 min, and mRNA and protein levels of PPARγ were determined at 24 h. Figure 3B indicates that leptin induced a significant ERK1/2 phosphorylation, and this effect was suppressed by either MEK inhibitor U0126 or PD98059, which decreased from a 3.3-fold increase over control in leptin-treated cells to a 1.57- and 2.25-fold increase over control, respectively (both p < 0.05 vs. leptin-treated cells). As shown in Figure 3C, the presence of U0126 or PD98059 dramatically blocked leptin-induced reduction of PPARγ mRNA level, which increased from 0.51-fold over control in leptin-treated cells to 0.88- and 0.73-fold over control, respectively (both p < 0.05). Similarly, pretreatment of cells with U0126 or PD98059 also suppressed leptin-induced reduction of PPARγ protein level, which increased from 0.40-fold over control in leptin stimulated cells to 0.91- and 0.83-fold over control, respectively (both p < 0.05). These results suggest that ERK1/2 signal pathway particularly mediated leptin-induced PPARγ down-regulation in PASMC.

Up-regulation of Egr-1 by ERK1/2 signaling mediates leptin-induced PPARγ reduction

It has been shown that activation of ERK1/2 signaling up-regulates Egr-1 expression in several types of nonPASMC (Hartney et al., 2011; Lee et al., 2015; Huynh et al., 2016; Simo-Cheyou et al., 2016; Systol et al., 2016; Wang et al., 2016). Previous studies have reported that the PPARγ proximal promoter contains an overlapping binding site for Egr-1, which is involved in the down-regulation of PPARγ (Zhou et al., 2009b; Nebbaki et al., 2012). It is therefore interesting
to examine whether induction of Egr-1 by ERK1/2 activation mediates leptin-induced PPARγ down-regulation in PASMC. Cells were incubated with MEK inhibitor U0126 (10 μM) or PD98059 (10 μM) for 30 min followed by leptin (100 ng/ml) stimulation for 24 h. Figure 4A shows that PASMC treated with 100 ng/ml leptin for 24 h exhibited a 2.11-fold increase in Egr-1 protein level compared with control (p < 0.01), while pretreatment of cells with MEK inhibitor U0126 or PD98059 dramatically suppressed leptin-induced up-regulation of Egr-1, which reduced to a 1.14- and a 1.32-fold increase over control, respectively (p < 0.05).

To verify the involvement of Egr-1 in leptin-induced PPARγ reductin in PASMC, knockdown of Egr-1 was applied. Figure 4B indicates that transfection of 100 nM Egr-1 specific small interfering RNA (siRNA) for 48 h reduced Egr-1 protein level to 21% of control (p < 0.01), while nontargeting siRNA did not affect Egr-1 protein expression. Figure 4C shows that PPARγ protein level decreased to 0.40-fold over control in cells treated with 100 ng/ml leptin for 24 h (p < 0.01 vs. control), while presilencing Egr-1 increased PPARγ protein level to 0.93-fold over control in leptin-stimulated cells (p < 0.05). In addition, PPARγ protein level was elevated in cells loss of Egr-1, which was a 1.70-fold increase over control (p < 0.05), suggesting that Egr-1 also suppressed PPARγ expression in basal condition. The above results indicate that up-regulation of Egr-1 by ERK1/2 signaling pathway specifically mediated leptin-induced PPARγ reduction in primary cultured rat PASMC.

**DISCUSSION**

In the present study, we have provided direct evidence that leptin causes PPARγ reduction in primary cultured PASMC; this effect is coupled to leptin-induced ERK1/2 activation and subsequent induction of Egr-1, which further down-regulates PPARγ expression and results in PASMC proliferation. Our study provides novel molecular mechanisms underlying the down-regulation of PPARγ in pulmonary vasculature in the development of PH.

Leptin has been shown to be involved in the regulation of many pathophysiological processes such as energy balance, hypertension, coronary atherosclerosis, myocardial hypertrophy, diabetes, reproduction, bone homeostasis, and immune function (Mantzoros et al., 2011). Leptin modulates various signaling pathways through the interaction with its receptors, such as janus-activated kinase/signal transducers and activators of transcription (JAK/STAT), mitogen-activated protein kinases (MAPK)/ERK, suppressors of cytokine signaling (SOCS), phosphatidylinositol-3-kinase and insulin receptor substrate proteins (PI3K/IRS) (Sweeney, 2002), and nitric oxide/cyclic GMP/protein kinase G (NO/cGMP/PKG) signal pathways (Rodriguez et al., 2010; Garcia-Juarez et al., 2012). Interestingly, leptin activates both pro-proliferative cascades (Shan et al., 2008; Chavez et al., 2012; Trovati et al., 2014; Yu et al., 2017) and anti-proliferative pathway (Rodriguez et al., 2007, 2010) in vascular smooth muscle cells (VSMC); these conflicting phenomena might be due to cell type specificity, concentration of leptin and cell conditions. Numerous studies have also shown that leptin plays an important role in the pathophysiology of PH (Schroeter et al., 2013; Chai et al., 2015; Huertas et al., 2015, 2016). The results of the present study demonstrated that leptin induced PASMC proliferation and PPARγ down-regulation accompanied with ERK1/2 MAPK phosphorylation. Inhibition of ERK1/2 MAPK markedly suppressed leptin-induced proliferation and PPARγ activator pioglitazone (10 μM) for 30 min and pretransfected with either 100 nM nontargeting siRNA or Egr-1 siRNA for 24 h and then stimulated with 100 ng/ml leptin for 24 h. As shown in Figure 5, prior treatment of cells with MEK inhibitor U0126 or PD98059 significantly suppressed leptin-induced PASMC proliferation, and BrdU incorporation rate decreased from a 1.98-fold increase over control in leptin-treated cells to a 1.19- and a 1.30-fold increase over control (p < 0.05). Furthermore, pretreatment of cells with PPARγ activator pioglitazone dramatically suppressed leptin-induced PASMC proliferation, which reduced from a 1.98-fold increase over control in leptin stimulated cells to a 1.23-fold increase over control (p < 0.05). Collectively, these results suggest that up-regulation of Egr-1 by ERK1/2 signaling cascade and subsequent PPARγ down-regulation mediates the effect of leptin on PASMC proliferation, while activation of PPARγ inhibits leptin-induced proliferation of PASCM.

**FIGURE 2:** Leptin dose- and time-dependently reduces PPARγ expression in PASMC. Cells were treated with different concentrations of leptin ranging from 0 to 300 ng/ml for 24 h, and the levels of PPARγ mRNA (A) and protein (B) were determined using RT-PCR and immunoblotting (n = 5 each group). Cells were treated with 100 ng/ml leptin for the indicated times, and the levels of PPARγ mRNA (C) and protein (D) were determined using RT-PCR and immunoblotting (n = 4 each group). *p < 0.05 vs. control and #p < 0.01 vs. control.
Egr-1 mediates PPARγ reduction in PASM C

PPARγ reduction and PASM C proliferation, suggesting that ERK1/2 signal pathway particularly mediated leptin-induced PPARγ down-regulation and PASM C proliferation. In addition, activation of the ERK1/2 signaling pathway has been shown to cause PPARγ nuclear export, resulting in its transcriptional activity reduction (Burgermeister et al., 2002), and to promote proteasomal-dependent PPARγ degradation (Floyd and Stephens, 2002). Therefore, further studies are still needed to investigate the fully mechanisms responsible for ERK1/2 signaling pathway regulating PPARγ reduction/inactivation in the development of PH. We also detected PKG activity in leptin-treated cells, and the result showed that activity of PKG was slightly but significantly increased compared with control cells (data not shown). We speculated that activation of the PKG pathway was considered a counteracting action to the PASM C proliferation induced by leptin, which was still not sufficient to suppress PASM C proliferation caused by leptin.

Egr-1 is a critical transcriptional factor regulating cell proliferation and differentiation, which is rapidly and transiently induced in response to a heterogenic group of stimuli, such as growth factors (Cao et al., 1999), shear stress (Ni et al., 2010), oxygen deprivation (Chang et al., 2008), and oxidative stress (Zhang et al., 2015). Induction of Egr-1 has been observed in various malignant tumors (Jacob et al., 2016; Park et al., 2016), inflammation (Ho et al., 2016), and cardiovascular diseases (Khachigian, 2006; Shin et al., 2009). Recently, overexpression of Egr-1 has been found in several animal models of PH (van Albada et al., 2010; Dickinson et al., 2011, 2014) and in patients with PH (van der Feen et al., 2016). Mounting evidence suggests that stimuli such as growth factors (i.e., platelet-derived growth factor and brain-derived neurotrophic factor) (Kwapiszewska et al., 2012; Sysol et al., 2016) and hypoxia (Nozik-Grayck et al., 2008; Hartney et al., 2011) induce PASM C proliferation by increasing Egr-1 expression. ERK1/2 signaling cascade has been shown to increase the expression and activity of Egr-1 in several cell types (Zhou et al., 2009b; Hartney et al., 2011). It has been further reported that PPARγ proximal promoter contains a binding site of Egr-1, which is involved in the down-regulation of PPARγ (Zhou et al., 2009b; Nebbaki et al., 2012). The present study confirmed that ERK1/2 signaling cascade mediated leptin-induced PPARγ reduction by up-regulation of Egr-1 in PASM C. We also found that pretreatment with PPARγ activator pioglitazone significantly inhibited leptin-stimulated PASM C proliferation. Taken together, this study provided novel molecular mechanisms by which leptin induced PPARγ down-regulation and PASM C proliferation, suggesting that targeting leptin/ERK1/2/Egr-1/PPARγ pathway might have potential value in ameliorating vascular remodeling and benefit PH.

MATERIALS AND METHODS

Cell preparation and culture

Primary PASM C from pulmonary arteries were prepared from 40 male Sprague-Dawley rats (4-wk-old, 70–80 g) according to the method of our previous studies (Wu et al., 2014; Ke et al., 2016). All animal care and experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the Xi’an Jiaotong University Animal Experiment Center. All protocols used in this study were approved by the Laboratory Animal Care Committee of Xi’an Jiaotong University. Briefly, pulmonary arteries were rapidly isolated from killed rats, washed in phosphate-buffered saline (PBS; 4°C), and dipped into DMEM (Life Technologies, Grand Isle, NY) containing 10% fetal bovine serum (FBS; Sijiqing, Hangzhou, China), 100 U/ml penicillin, and 100 μg/ml streptomycin. A thin layer of the adventitia was gently stripped off with a forceps, and the endothelium was carefully removed by scratching the intima surface with an elbow tweezers. The remaining smooth muscle was cut into 1-mm pieces and placed into a culture flask and then incubated in a 37°C, 5% CO2 humidified incubator. PASM C were passaged using 0.25% trypsin (Invitrogen, Carlsbad, CA) until reaching 70–80% confluence. All protocols used in this study were approved by the Laboratory Animal Care Committee of Xi’an Jiaotong University. Primary PASMC from pulmonary arteries were prepared from 40 male Sprague-Dawley rats (4-wk-old, 70–80 g) according to the method of our previous studies (Wu et al., 2014; Ke et al., 2016). All animal care and experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the Xi’an Jiaotong University Animal Experiment Center. All protocols used in this study were approved by the Laboratory Animal Care Committee of Xi’an Jiaotong University. Briefly, pulmonary arteries were rapidly isolated from killed rats, washed in phosphate-buffered saline (PBS; 4°C), and dipped into DMEM (Life Technologies, Grand Isle, NY) containing 10% fetal bovine serum (FBS; Sijiqing, Hangzhou, China), 100 U/ml penicillin, and 100 μg/ml streptomycin. A thin layer of the adventitia was gently stripped off with a forceps, and the endothelium was carefully removed by scratching the intima surface with an elbow tweezers. The remaining smooth muscle was cut into 1-mm pieces and placed into a culture flask and then incubated in a 37°C, 5% CO2 humidified incubator. PASM C were passaged using 0.25% trypsin (Invitrogen, Carlsbad, CA) until reaching 70–80% confluence. All experiments were performed using cells between passages 4 and 6. The purity of PASM C was determined by immunostaining with α-actin as previously described (Wu et al., 2014). Leptin (Peprotech, Rocky Hill, NJ) was used to stimulate PASM C. U0126 (Selleckchem, Houston, TX) or PD98059 (Calbiochem, La Jolla, CA) was applied to inhibit ERK1/2 signaling pathway. PPARγ activator pioglitazone was purchased from Takeda Pharmaceutical Co. (Tianjin, China).
bated with anti-BrdU mAbs conjugated to peroxidase for 90 min at room temperature. After incubation, antibody conjugate was removed and substrate solution was added for reaction for 10 min. Finally, the reaction product was quantified by measuring the absorbance at 370 nm using a microplate reader (Bio-Rad, Richmond, CA). The blank corresponded to 100 μl of culture medium with or without BrdU.

Quantitative real-time reverse transcription PCR

Total RNA was extracted from PASMC using the RNeasy Micro plus Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. Isolated RNAs were polyadenylated using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Logan, UT). The cDNA synthesized was used to perform quantitative PCR on an IQ5 Real-Time PCR Detection System (Bio-Rad) using the Bio-Rad SsoAdvanced Universal SYBR Green kit. Primers specific for PPARγ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Sangon Biotech (Shanghai, China), and the following primer sets were used: rat PPARγ, 5′-CGGTTGATTTCTCCAGCATT-3′ and 5′-TCGCAC TTTGGTATTCT-TGG-3′; rat GAPDH, 5′-CCTGGAGAAACC-TGCCAAGTAT-3′ and 5′-CTCGGCCGCCT-GCTT-3′. The fold increase relative to control samples was determined by the 2−∆∆Ct method (Livak and Schmittgen, 2001).

siRNA transfection

To silence protein expression, PASMC were transfected with sequence-specific or nontargeting control siRNA (GenePharm, Shanghai, China) using Lipofectamine 2000 reagent (Invitrogen), the following sequences were used: Egr-1 siRNA, sense 5′-CAGGACUUAAAGGCCUCUATT-3′, anti-sense 5′-UAAGAGCCUUUAAGUGUGUATT-3′; NC-siRNA, sense 5′-UUCUCCGAACGUGACACGU5-3′, anti-sense 5′-ACGUAGGACGUUCGGAGAATT-3′. Briefly, cells were cultured until reaching 30–40% confluence; siRNA and Lipofectamine were diluted in serum-free DMEM, separately, and incubated for 5 min at room temperature. Diluted siRNA was mixed with diluted Lipofectamine and incubated at room temperature for 20 min. Then the complex of siRNA and Lipofectamine was added into cells, and cells were cultured for 48 h at 37°C, 5% CO2 in a humidified incubator. The working concentration of siRNA in cell experiments was 100 nM. Effects of siRNA transfection were analyzed using immunoblotting.

Cell proliferation assay

To determine PASMC proliferation, the rate of BrdU incorporation was examined using a BrdU ELISA Kit (Maibio, Shanghai, China) following the established protocol. PASMC were seeded on 96-well plates at 5 × 103 cells per well, allowed to adhere for at least 24 h, and then serum starved overnight (1% FBS in DMEM) before the start of experiments. After different treatments, BrdU labeling reagent was added to the wells and incubated for 2 h at 37°C. Cells were then denatured with FixDenat solution for 30 min and incu-
GAPDH was used as internal control for PPARγ. Amplification was performed at 95°C for 1 min, followed by 40 cycles of 95°C for 5 s, 60°C for 20 s, and 72°C for 30 s.

Immunoblotting

The cultured cells were washed twice with ice-cold PBS and then lysed in radioimmunoprecipitation assay (RIPA) lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.1% SDS, 150 mM NaCl, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na3VO4, 1 mM NaF, and proteinase inhibitors. Lysates were centrifuged at 13,000 rpm at 4°C for 15 min, and the supernatant was collected as total protein. Protein concentration was determined with a BCA protein assay kit (Pierce). Protein was separated on an SDS–PAGE gel and transferred to a nitrocellulose (NC; Bio-Rad) membrane via semidy transfer. The membrane was then blocked with 5% (wt/vol) nonfat dry milk in PBS containing 0.1% (vol/vol) Tween-20. Polyclonal or monoclonal antibodies were used against phosphor-ERK1/2 (p-ERK1/2; Cell Signaling Technology), early growth response-1 (Egr-1; Cell Signaling Technology), total-ERK1/2 (t-ERK1/2; Cell Signaling Technology), cell growth response-1 (Egr-1; Cell Signaling Technology), PPARγ (Proteintech Group, Chicago, IL), and glyceraldehyde-3-phosphate dehydrogenase (Chemicon International, Billerica, MA) (1:1000 dilution) according to the manufacturer’s protocols. Horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit immunoglobulin G was used as the secondary antibodies (Sigma, St. Louis, MO) (1:5000 dilution). Reactions were developed with the SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL) and then exposed to the autoradiographic film. Signaling was quantified from scanned films using Quality One software (Bio-Rad).

Statistical analysis

All values are presented as mean ± SD. Data were analyzed using one-way analysis of variance with Tukey post hoc test by SPSS13.0 software. Probability values of p < 0.05 were considered to represent a statistically significant difference between groups.

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