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PGC-1-related coactivator (PRC) negatively regulates endothelial adhesion of monocytes via inhibition of NF κB activity

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ABSTRACT

PGC-1-related coactivator (PRC) is a growth-regulated transcriptional cofactor known to activate many of the nuclear genes specifying mitochondrial respiratory function. Endothelial dysfunction is a prominent feature found in many inflammatory diseases. Adhesion molecules, such as VCAM-1, mediate the attachment of monocytes to endothelial cells, thereby playing an important role in endothelial inflammation. The effects of PRC in regards to endothelial inflammation remain unknown. In this study, our findings show that PRC can be inhibited by the inflammatory cytokine LPS in cultured human umbilical vein endothelial cells (HUVECs). In the presence of LPS, the expression of endothelial cell adhesion molecular, such as VCAM1 and E-selectin, is found to be increased. These effects can be negated by overexpression of PRC. Importantly, monocyte adhesion to endothelial cells caused by LPS is significantly attenuated by PRC. In addition, overexpression of PRC protects mitochondrial metabolic function and suppresses the rate of glycolysis against LPS. It is also found that overexpression of PRC decreases the transcriptional activity of NF-κB. These findings suggest that PRC is a negative regulator of endothelial inflammation.

1. Introduction

Inflammation is now considered a major underlying condition associated with many diseases, including vascular disease [1]. Endothelial dysfunction has also been associated with certain chronic vascular inflammatory diseases. Inflammatory cytokines are responsible for the activation of endothelial cells, a condition characterized by the expression of endothelial cell surface adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, which promote the attachment of circulating monocytes to the endothelium [2] as well as their migration and differentiation in the vascular intima-media layer [3]. It is well documented that increased circulating cytokines, including interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNFα), and Lipopolysaccharide (LPS), are also associated with such cardiovascular events [4]. Adhesion molecules, such as VCAM-1, mediate monocyte attachment to endothelial cells, thus playing an important role in the development and progression of atherosclerosis. Further understanding the mechanism by which inflammation regulates endothelial function is beneficial in reducing or preventing the high incidence of morbidity and mortality associated with vascular complications.

PGC-1-related coactivator (PRC) is a member of the PGC-1α family, which plays an important role in metabolic regulation [5] as well as the regulation of multiple physiological activities, including mitochondrial biogenesis, fatty acid oxidation, thermogenesis, gluconeogenesis, and cell proliferation. It has been found that homozygous PRC knockout embryos develop to the blastocyst stage but perish at the time of implantation [6], thereby suggesting the importance of PRC in maintaining cell survival. Previous studies have shown that many of these PRC stress genes are common factors of chronic inflammation associated with multiple age-related diseases [7]. PGC-1α has been demonstrated to be capable of regulating inflammation by suppressing the expressions of IL-6 and TNF-α mRNA levels in human skeletal muscle tissue [8]. Moreover, another study reports that PGC-1α suppresses proinflammatory effects by inhibiting the activation of NF-κB in skeletal muscle cells [9]. More importantly, NF-κB activity, MCP-1, and VCAM-1 induced by TNF-α are suppressed by overexpression of PGC-1α in human aortic endothelial cells (HAECs) [10]. However, whether PRC plays a role in regulating inflammation, especially in endothelial cells, is still unknown. In this study, the effects of PRC on endothelial inflammation in human umbilical vein endothelial cell (HUVEC) lines were investigated. Firstly, we found that the endotoxin lipopolysaccharide (LPS) leads to downregulation of PRC expression. Secondly, it was found that overexpression of PRC inhibits the expression of endothelial adhesion molecules and attenuates the adhesion of THP-1 cells to the surface of endothelial cells. Finally, our results also demonstrate that PRC exerts its anti-inflammatory effects via NF-κB.

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2. Materials and methods

2.1. Cell culture and Treatment

Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza, USA. Cells were cultured in EBM-2 media with supplemental growth factors according to the manufacturer's instructions [11]. Cells were treated with Lipopolysaccharide (LPS) at various doses for various periods of time. Human monocytic leukemia cell line THP-1 cells were purchased from the ATCC, USA. Cells were maintained in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), antibiotic–antimycotic, and L-glutamine (Life Technologies).

2.2. Adenoviral methods

The plasmid Ad-PRC was constructed using the AdEasybasic kit ATCC according to the manufacturer's instructions following the methods previously described [12]. PRC/pAdTrack-CMV was generated by cloning the XhoI/NotI fragment of NmycPRC/pBSII into SalI/Not-digested pAdTrack-CMV. PRC/pAdTrack-CMV was then used in a recombination reaction with pAdEasy-1 to produce Ad-PRC. HEK-293 was used to linearize and transfect with the adenoviral plasmids by suing Lipofectamine 2000 (Invitrogen). High titer viral stock was then collected. HUVECs were infected, and the infection efficiency (95–100%) was determined by GFP expression 24 h after infection.

2.3. Luciferase reporter assays

HEK 293 cells were infected with Ad-PRC and transfected with pNF-κB-Luc using Lipofectamine 2000 (Invitrogen). Transfected cells were treated with LPS. After 24 h, the cells were lysed and luciferase activity was determined by use of a dual luciferase kit (Promega, USA) and TD-20/20 luminometer (TurnerDesigns, USA). Luciferase activity was normalized to the Renilla luciferase activity, which served as an internal control for transfection efficiency.

2.4. Real time polymerase chain reaction (PCR)

Total RNA was extracted from HUVECs using Trizol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized using reverse transcription PCR with 2 μg total RNA. cDNA was then used to perform quantitative real-time PCR analysis using the step one plus real time PCR system (Applied Biosystems, USA) with the SYBR Green detection method. The following primers were used in this study: human GAPDH (internal control), 5′-GGAGAAGGCTGGGGCTCAT-3′ (forward) and 5′-TGATGGCATGGACTGTGGTC-3′ (reverse); human PRC: 5′-AGTGGTTGGGGAAGTCGAAG-3′ (forward); 5′-CCTGCCGAGAGAGACTGAC-3′ (reverse); VCAM-1: 5′-CTTAAAATGCCTGGGAAGATGGT-3′ (forward); 5′-GTCAATGAGACGGAGTCACCAAT-3′ (reverse); ICAM-1: 5′-CGATGACCATCTACAGCTTTCCGG-3′ (forward); 5′-GCTGCTACCACAGTGATGATGACAA-3 (reverse); E-selectin: 5′-CCGTCCGCCAGCCTCA GAAT-3′ (forward); 5′-TAGCCTCGCTCGGGGTTGGAC-3′ (reverse).

2.5. Western blot

Cells were lysed with cell lysis buffer (Cell signaling, USA). Proteins were separated by SDS–PAGE and electroblotted onto Immobilon-P membranes according to the manufacturer's instructions. Western blot analysis was performed as previously described [13]. The following antibodies were used in this study: anti-β-actin (Sigma), anti-ICAM-1 (Cell Signaling Technology), anti-VCAM-1 (Santa Cruz Biotechnology), anti-E-selectin (Santa Cruz Biotechnology); anti-PRC (Santa Cruz Biotechnology).

2.6. Immunofluorescence

After the indicated transfection or treatment, HUVECs were fixed in 4% paraformaldehyde for 10 min at RT, followed by permeabilization with 0.4% Triton X-100 and blocked with 5% BSA and 2.5% FBS in PBST. Cells were then incubated with anti-NF-κB for 2 h at RT, followed by Alexa-594 conjugated secondary antibodies for 1 h at RT.

2.7. Adhesion assay

HUVECs were infected for 24 h with PRC or control adenoviruses. THP-1 cells were labeled with 0.2 mg/L calcein red AM for 30 min at 37 °C and labeled cells were seeded onto confluent HUVECs. After 2 h incubation, co-cultured cells were washed with 1X PBS containing 1% bovine serum albumin (BSA). Images were captured by an inverse optical microscope (Axiovert 25) and Axio Vision Release 4.7 software (Carl Zeiss MicroImaging GmbH, Germany).

2.8. Measurements of oxygen consumption rates

Real-time measurements of oxygen consumption rates were carried out using an XF24 extracellular flux analyzer according to the previous report [14]. Fluorescence-based optical sensors and custom multi-well plates were used in this device to make repeated oxygen consumption measurements of intact cells growing as monolayers. Drug was added automatically during measurement. Each measurement was continued for 60–80 min. Data were presented from four replicate plates for each sample.

2.9. Measurements of glycolytic rate

Glycolytic rate was measured as previously described [15]. In brief, HUVECs were washed and incubated in Kreb's buffer for 30 min, followed by incubated another 1 h with the addition of 5 μl 5-^{3}H-glucose. Cells were harvested by adding 50 μl trypsin in the reaction system. A 100 μl reaction system was terminated with 50 μl 0.2 N HCl. The ratio of diffused ^{3}H2O and undiffused 5-^{3}H-glucose was used to index the glycolytic rate.

2.10. Statistical analysis

Data are expressed as mean ± SEM. Student's *t*-test was used to determine the difference in the means of the two groups. A value of $P < 0.05$ was considered statistically significant.

3. Results

Firstly, we determined PRC expression in HUVECs incubated with LPS at various doses for various periods of time. LPS treatments administered on HUVECs at the concentrations of 25, 50, 100, and 200 ng/ml for 24 h led to a sustainable downregulation in both messenger RNA (mRNA) levels of PRC (Fig. 1A) and in protein levels (Fig. 1B). HUVECs were incubated with 100 ng/ml LPS for various timespans. Results showed a sustainable reduction at both mRNA levels (Fig. 1C) and protein levels of PRC in a time-dependent manner from 12 to 48 h (Fig. 1D).

In response to inflammatory stimuli, the induction of adhesion molecules, such as vascular cell adhesion molecule (VCAM)-1 and E-selectin, in endothelium is a key event. In order to examine the effects of PRC on endothelial activation in response to

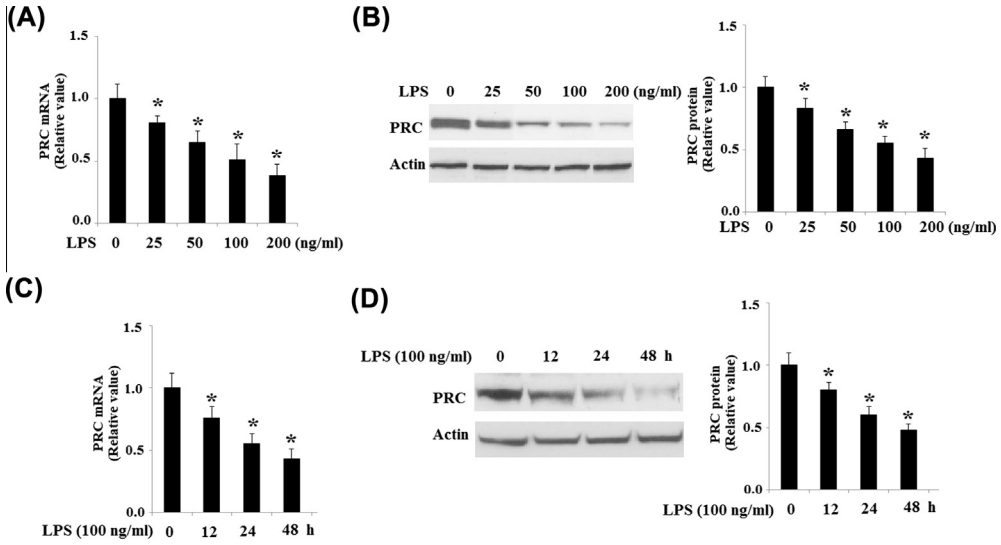

Fig. 1. Expression of PRC in HUVECs after LPS treatment. (A) HUVECs were stimulated with LPS at various concentrations for 48 h, and mRNA levels of PRC were determined by real-time PCR at various concentrations (*$P < 0.01$ vs. nontreated control, $n = 4$). (B) Protein levels of PRC were determined by western blot analysis at various concentrations (*$P < 0.01$ vs. nontreated control, $n = 4$). (C) HUVECs were stimulated with LPS at indicated doses (100 ng/ml) for varying periods of time. mRNA levels of PRC were determined by real-time PCR at varying time periods. The relative values of all results were determined and expressed as the mean ± SEM of three experiments performed in duplicate (*$P < 0.01$ vs. nontreated control, $n = 4$). (D) Protein levels of PRC were determined by western blot analysis at varying time periods (*$P < 0.01$ vs. nontreated control, $n = 4$).

inflammatory cytokines, HUVECs were overexpressed with PRC for 24 h, stimulated with LPS for an additional 4 h, and then assessed for adhesion molecule mRNA and protein abundance. As shown in Fig. 2A, treatment of HUVECs with LPS strongly induced VCAM-1, E-selectin, and ICAM-1 in control virus infected cells. In contrast, overexpressing PRC strongly inhibited the induction of VCAM-1 and E-selectin, but not ICAM-1 mRNA. Consistent with these observations, PRC inhibited VCAM-1 and E-selectin protein expression but did not affect ICAM-1 (Fig. 2B).

These proteins participate in mediating monocyte attachment to endothelial cells [16]. We further determined the functional consequence of PRC's effect on adhesion molecule expression [17]. As shown in Fig. 2C and Fig. 2D, HUVECs infected with control Ad-GFP exhibited robust THP-1 cell attachment. In contrast, THP-1 cell attachment was markedly attenuated in PRC-overexpressing cells. These data demonstrate that PRC can inhibit adhesion molecule expression and monocyte attachment to activated endothelial cells.

The PGC-1 family has been well characterized to have a wide range of metabolic effects due to their ability to amplify transcription factor activity toward target genes. In order to examine the effects of PRC on metabolic changes in response to inflammatory cytokines, we further investigated the rate of respiration and the rate of glycolysis in HUVECs. Our results indicated that LPS treatment caused a significantly reduction in rates of oxygen consumption, an indicator of mitochondrial respiration (Fig. 3A). However, overexpressing PRC could attenuate these effects. It has been reported that some cell types can compensate for reduced mitochon-

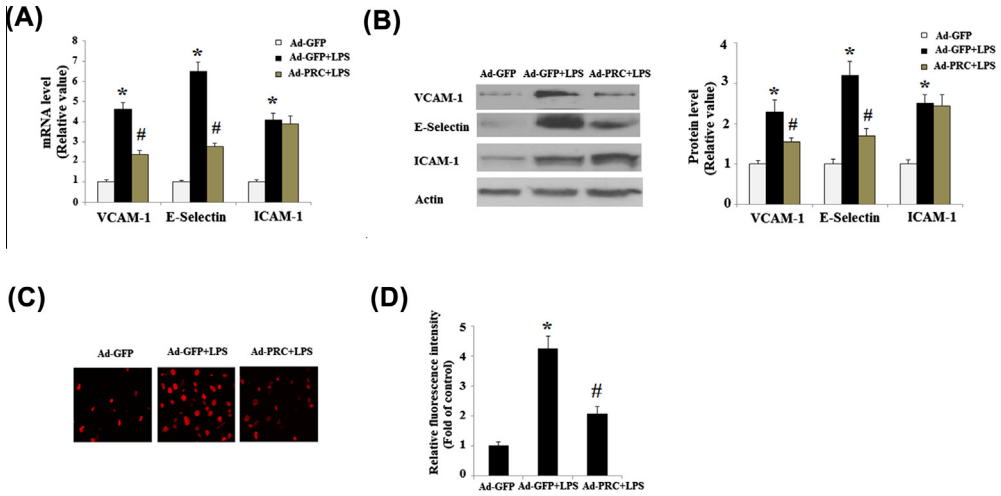

Fig. 2. Effects of PRC on LPS-mediated induction of inflammatory adhesion molecules and THP-1 attachment to endothelial cells. (A) PRC inhibits VCAM-1 and E-selectin, but not ICAM-1 mRNA. HUVECs were infected with the adenovirus at the indicated dose, stimulated with LPS for 4 h, and total RNA was assessed for adhesion molecule expression. In contrast to VCAM-1 and E-selectin, no effect is observed on ICAM-1 expression (*$P < 0.01$ vs. nontreated control, $n = 4$; #$P < 0.01$ vs. Ad-GFP group). (B) PRC inhibits LPS-mediated induction of VCAM-1 and E-selectin protein levels (*$P < 0.01$ vs. nontreated control, $n = 4$; #$P < 0.01$ vs. Ad-GFP group); (C) Representative fluorescence microscopic images of endothelial adhesion of THP-1; (D) quantitative data for corresponding images (*$P < 0.01$ vs. nontreated control, $n = 4$; #$P < 0.01$ vs. Ad-GFP group).

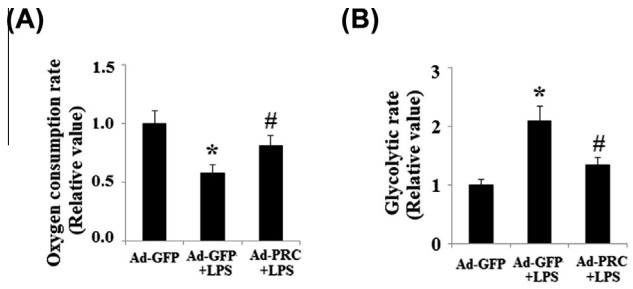

Fig. 3. Effects of PRC on LPS-induced changes in HUVECs metabolism. (A) The reduction of oxygen consumption rates as induced by the administration of LPS was attenuated by PRC ($^{*}P < 0.01$ vs. nontreated control, $n = 4$; $^{\#}P < 0.01$ vs. Ad-GFP group). (B) PRC inhibits LPS-mediated induction of the glycolytic rate ($^{*}P < 0.01$ vs nontreated control, $n = 4$; $^{\#}P < 0.01$ vs. Ad-GFP group).

drial ATP production by increased glycolytic activity through Pasteur effect-like mechanisms [18]. We examined the glycolytic rate in HUVECs after treated with LPS. 5-^{3}H-glucose was incubated with cells and the glycolytic rate was determined by counting $^{3}H2O$ generated from glycolysis. We found that the glycolytic rate was increased to about 2 times in the presence of 100 ng/ml LPS compared to vehicle control. In contrast, the LPS-induced increase in glycolytic rate was significantly attenuated in PRC overexpressing cells.

NF-κB has been considered a critical factor in the activation of both the VCAM-1 and E-selectin promoters in response to inflammatory stimuli [19]. Under normal conditions, this factor exists in the cytoplasm as a heterodimer of the p50 and p65 subunits. Activation of NF-κB is controlled by a family of inhibitors referred to as IκB, which can retain the entire complex in cytoplasm. Inflammatory cytokines are able to induce phosphorylation and subsequent degradation of IκB, thereby leading to the liberation of NF-κB heterodimers, which can then translocate to the nucleus, bind specific DNA sequences, and affect target gene expression.

As a first step, we assessed the inhibitory effect of PRC on NF-κB by performing luciferase reporter assays. As is shown in Fig. 4A, in HUVECs transfected with NF-κB-Luc reporter plasmid, LPS drastically induced NF-κB luciferase activity. This induction was markedly suppressed by PRC. Immunostaining revealed that LPS-induced p65 nuclear translocation was blocked by PRC overexpression, thereby confirming the inhibition effect of PRC in NF-κB activation (Fig. 4B). PRC also blocked LPS-induced degradation of IκBα in cells (Fig. 4C).

4. Discussion

Inflammatory stimulants, such as bacterial endotoxin (lipopolysaccharide (LPS)), are known to induce tissue damage and endothelial dysfunction [20]. Recent reports highlight an important protective role for PGC-1α in vascular dysfunction. Consistently, a previous study demonstrates that LPS suppresses the expression of PGC-1α [21]. Moreover, activation of PGC-1α exerts beneficial effects on endothelial cell dysfunction [22]. In this study, we report that LPS can possibly decrease PRC expression in HUVECs. To the best of our knowledge, this is the first time the effects of PRC on endothelial dysfunction have been investigated. This result suggests that PRC might potentially play a role in infection induced endothelial dysfunction. In the presence of LPS, there is an increase in the expression of endothelial cell adhesion moleculars, such as VCAM1, E-selectin, and I-CAM1. The induction of VCAM1 and E-selectin is prevented by overexpression of PRC. Importantly, monocyte adhesion to endothelial cells caused by LPS is significantly attenuated by PRC. In addition, overexpression of PRC decreases the transcriptional activity of NF-κB. These findings suggest that PRC is a negative regulator of vascular inflammation. Thus, activation of PRC might be beneficial for many inflammatory diseases associated with endothelial dysfunction.

Like both PGC-1α and PGC-1β, overexpression of PRC results in a significant increase in respiration along with mitochondrial gene expression, activity, and mass [23]. A previous study has reported that LPS-treatment lead to metabolic changes, including the reduced respiration rate and the increased glycolysis [24]. Our results demonstrated that decreased PRC in response to LPS was associated with the reduced rate of respiration and the increased rate of glycolysis in HUVECs. Overexpression of PRC protects mitochondrial metabolic function and suppresses the rate of glycolysis against LPS. These findings indicate targeted expression of PRC in endothelial cells can provide a useful protective effect against inflammatory factors.

Recruitment of immune cells to the surface of the endothelium is a notable feature common to most vascular inflammatory diseases. This process is mediated by endothelial expression of certain adhesion molecules, such as VCAM-1 and E-selectin [25]. The nuclear factor NF-κB is a central regulator of inflammatory processes. NF-κB activation has accordingly been associated with inflammatory stimulants in endothelial cells [26]. It has been demonstrated that NF-κB plays a critical role in the activation of both the VCAM-1 and E-selectin promoters in response to inflammatory stimuli [27]. PGC-1α has been reported to exert many of its protective effects via inhibition of NF-κB activity [9]. Our results suggest that PRC, another member of the PGC-1α family, could prevent endothelial activation through inhibition of NF-κB activity. However, the underlying mechanisms associated with PRC's inhibition of NF-κB activity are still unknown. Further studies will provide a complete picture of the function of PRC in endothelial inflammation and vascular physiology and pathology.

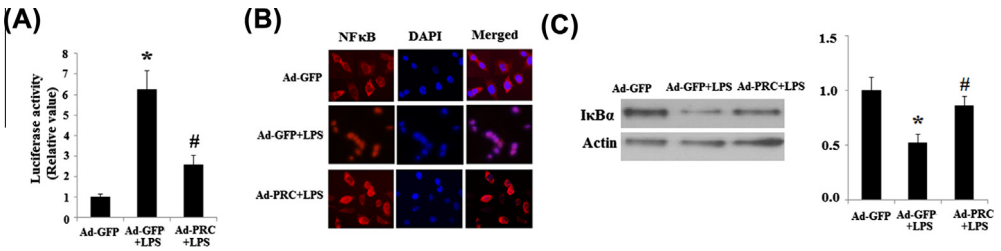

Fig. 4. (A) NF-κB luciferase reporter assays. After indicated transfection, HUVECs transfected with pNF-κB-Luc reporter were treated with LPS (100 ng/ml) as indicated for 24 h before measuring luciferase activity ($^{*}P < 0.01$ vs. nontreated control, $n = 4$; $^{\#}P < 0.01$ vs. Ad-GFP group); (B) Effects of PRC on p65 nuclear translocation. HUVECs were infected with Ad-PRC overnight, followed by 4 h LPS stimulation, as indicated. The cells were immunostaining with anti-p65 antibodies. (C) Western blot and quantification analysis revealed that the reduction of IκBα as induced by the administration of LPS was attenuated by PRC ($^{*}P < 0.01$ vs. nontreated control, $n = 4$; $^{\#}P < 0.01$ vs. Ad-GFP group).

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