Research Article

Effect of PGC-1α on Proliferation, Migration, and Transdifferentiation of Rat Vascular Smooth Muscle Cells Induced by High Glucose

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We assessed the role of PGC-1α (PPARγ coactivator-1 alpha) in glucose-induced proliferation, migration, and inflammatory gene expression of vascular smooth muscle cells (VSMCs). We carried out phagocytosis studies to assess the role of PGC-1α in transdifferentiation of VSMCs by flow cytometry. We found that high glucose stimulated proliferation, migration and inflammatory gene expression of VSMCs, but overexpression of PGC-1α attenuated the effects of glucose. In addition, overexpression of PGC-1α decreased mRNA and protein level of VSMCs-related genes, and induced macrophage-related gene expression, as well as phagocytosis of VSMCs. Therefore, PGC-1α inhibited glucose-induced proliferation, migration and inflammatory gene expression of VSMCs, which are key features in the pathology of atherosclerosis. More importantly, PGC-1α transdifferentiated VSMCs to a macrophage-like state. Such transdifferentiation possibly increased the portion of VSMCs-derived foam cells in the plaque and favored plaque stability.

1. Introduction

Activation of vascular smooth muscle cells (VSMCs) including proliferation, migration, and inflammatory gene expression plays an important role in the development of atherosclerosis. In early atherosclerosis, VSMCs may contribute to the development of the atheroma through the production of proinflammatory mediators such as monocyte chemoattractant protein-1 (MCP-1) [1]. VSMC-derived foam cells have been demonstrated in vitro [2] and in vivo atherosclerotic plaques [3]. These foam cells assume a macrophage-like state [4].

Hyperglycemia has been implicated as a major contributor to several diabetes complications by inducing key factors including oxidant stress and inflammatory gene expression [5]. Furthermore, these factors may also lead to abnormal proliferation, migration and inflammatory gene expression of VSMCs, which are key features in the pathology of atherosclerosis. Recent studies have demonstrated that treatment of cultured VSMCs in vitro with diabetogenic agents such as high glucose can increase the production of various proinflammatory cytokines and chemokines, which are all associated with vascular inflammation [6].

PPARγ coactivator-1 alpha (PGC-1α) is originally identified as a transcriptional coactivator of PPARγ [7]. PGC-1α can coactivate many nuclear receptors such as liver X receptor [8], and nonnuclear receptors such as Sry-related HMG box-9 [9]. As a metabolic regulator, PGC-1α functions in adaptive thermogenesis in brown fat [7], gluconeogenesis in liver [10], and insulin secretion in islets [11]. Our group has demonstrated that PGC-1α is an important negative regulator for VSMC proliferation and migration under pathophysiological conditions, such as oleic acid stimulation [12]. Additionally, PGC-1α participates in the differentiation procedure including adipocyte differentiation [7] and chondrogenesis [9].

In the present study, we assessed the role of PGC-1α in glucose-induced proliferation, migration, and inflammatory gene expression of VSMCs. We also carried out phagocytosis studies to assess the role of PGC-1α in transdifferentiation of
2. Materials and Methods

2.1. Materials. Carboxylate-modified microspheres (F8821) with red fluorescent were purchased from Molecular Probes (Eugene, OR). RNeasy kit (cat. no.74104) and RNase-free DNase I (cat. no 79254) were from QIAGEN. SYBR green PCR master mix (Part no.: 4309155) was from Applied Biosystems. 

2.2. Cell Culture. Rat aortic VSMCs were isolated from the thoracic aortas of 3- to 4-week-old male Sprague-Dawley rats as described previously [13]. Isolated VSMCs were cultured in DMEM with 25 mmol/L or 5.5 mmol/L D-glucose (Gibco-Invitrogen, Carlsbad, USA), supplemented with 10% FCS (GBICO BRL, Rockville, MD), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Cells after the 4th to 8th passages were used in experiments.

2.3. Adenovirus Infection. Recombinant adenoviruses expressing PGC-1α-GFP (Green Fluorescence Protein) fusion protein and GFP alone were provided by Dr. Dan Kelly (Washington University, Saint Louis, MO, USA). Purified virus stocks were prepared through CsCl density gradient centrifugation [14]. Cells were grown to subconfluence, deprived of serum for 24 hours, and followed by infection with adenovirus at multiplicities of infection (moi) of 50 for 48 hours. The infection efficiency was determined by fluorescence intensity of GFP.

2.4. RNA Analysis. Total RNA of VSMCs was isolated using RNeasy kit and RNase-free DNase I. RNA was reverse transcribed into cDNA with oligo (dT) 18 primers and AMV reverse transcriptase at 42°C for 1 hour in standard buffer (Table 1).

Quantitative PCR was performed with the ABI Prism 7000 sequence detection system (ABI, Foster City, CA). SYBR green PCR master mix was used. The reactions were amplified for 30 s at 95°C and 1 min at 60°C for 40 cycles. The thermal denaturation protocol was run at the end of the PCR to determine the number of products that were present in the reaction. All reactions were run in triplicate and included no template and no reverse transcription controls for each gene. The relative amount of each mRNA to β-actin RNA was described using the equation 2-ΔCT where ΔCT = (Ct mRNA – Ctβ-actin) [15]. Relative gene expression was multiplied by 10^ΔCt in order to simplify the presentation of the data. The primers used in this study were listed in Table 1.

2.5. Western Blotting Analysis. To obtain total proteins, VSMCs were lysed in a buffer containing 10 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl₂, 10 mmol/L KCl, and 0.5% NP-40, and cell lysates were centrifuged at 13000 g for 5 min. Supernatants were collected as cytosolic extracts for Western blot. Total proteins were applied to SDS-PAGE gel electrophoresis for proteins detection. The used primary antibodies included anti-pgc-1α (sc-13067) and anti-CD68 (sc-9139) from Santa Cruz Biotechnologies, anti-β-actin (#4967) from Cell Signaling, and anti-α-actin (product no. A2547) from Sigma. After incubation with primary antibodies for 2 h and washing, membranes were incubated with corresponding horseradish-peroxidase (HRP-) conjugated secondary antibody and detected with the ECL Plus Kit (Amersham).

2.6. Migration Assays. Migration assays were performed by the modified Boyden chamber [16]. VSMCs were seeded in 6-well plates (1.5 x 10⁵ cells/well) and grew to subconfluence. After 24 h serum deprivation, VSMCs were treated with adenovirus infection for 48h. Boyden chamber cell migration assay was performed using transwell chambers with fibronectin- (Sigma)-coated 8 μm pore size polycarbonate membranes (BD Biosciences). VSMCs treated were then suspended in low-glucose (5.5 mmol/L) DMEM-0.5% FBS to a concentration of 4 x 10⁵ cells/mL. Low glucose (5.5 mmol/L) or high glucose (25 mmol/L) DMEM (0.6 mL) supplemented with 10% FCS were added to the lower compartment. A 0.1 mL cell suspension (final concentration, 4 x 10⁴ cells/well; diameter, 6.5 μm) was added to the upper compartment, and cells were then incubated at 37°C (95%
Figure 1: High glucose stimulated the proliferation, migration, and inflammatory gene expression of VSMCs. VSMCs cultured in low-glucose (5.5 mmol/L) or high-glucose (25 mmol/L) complete media were deprived of serum for 24 h. In proliferation and inflammatory gene expression assays, VSMCs were stimulated by complete media for 6 h, respectively. In migration assays, VSMCs were suspended, and then low-glucose or high-glucose complete media were added to the lower compartment respectively. (a) proliferation of VSMCs, (b) migration of VSMCs, and (c) inflammatory gene expression of VSMCs (*P < 0.05).

2.7. Proliferation and Phagocytosis Assays by Flow Cytometry. Click-iT EdU Flow Cytometry Assay Kits (invitrogen, cat. no. A10202) was used to detect VSMCs proliferation. Cells seeded in 6-well plates (1.5 × 10⁵ cells/well) were cultured up to subconfluence, and the medium was replaced by fresh serum-free medium. The cells were then treated with adenovirus infection for 48 h. Following this, low-glucose (5.5 mmol/L) or high-glucose (25 mmol/L) DMEM supplemented with 10% FCS and 10 μM EdU were added. After 6 h, cells were suspended at 1 × 10⁷ cells/mL in 1% BSA in PBS. 100 μL of cell suspension and 100 μL Click-iT fixative were added to flow tubes, incubated for 15 minutes at room temperature, and protected from light. Cells were washed once with 3 mL 1% BSA in PBS. 100 μL of the 1X saponin-based permeabilization and wash buffer was added, incubated for the 20 minutes at 4°C temperature, and protected from light. Each tube was washed. 0.5 mL Click-iT reaction cocktail were added, mixed well, incubated for 30 minutes at room temperature, and protected from light. Cells were washed once. 0.5 mL 1X saponin-based permeabilization and wash reagent was added. For the detection of EdU, 633/635 nm excitation with red emission filter (i.e., 660/20 nm or similar) was used.

Phagocytic activity was revealed by microspheres. After 24 h serum deprivation, VSMCs were infected by Ad-PGC-1α or Ad-GFP for 48 hours. Then VSMCs were incubated with 1μm carboxylate-modified microspheres with red fluorescent (2.7 × 10⁶ beads per mL) for 10 h. Cells with the above treatments were suspended with 0.05% trypsin and 0.02% EDTA and then washed three times in PBS. Following this, the cells were analyzed using excitation/emission maxima of 580/605 on FACS Calibur (Becton-Dickinson, San Jose, CA, USA).

2.8. Statistical Analysis. Data are expressed as means ± SEM. Data were analyzed using a one-way ANOVA followed by Fisher’s LSD post hoc test. Calculations were performed using SPSS/Windows version 12.5S statistical package (SPSS,
Figure 2: Overexpression of PGC-1α inhibited proliferation, migration, and inflammatory gene expression of VSMCs in high glucose. VSMCs cultured in high-glucose (25 mmol/L) complete media were deprived of serum for 24 h. The cells were then treated with adenovirus infection for 48 h. (a) Proliferation of VSMCs, (b) migration of VSMCs, (c) inflammatory gene expression of VSMCs (* P < 0.05), and (d) Western blotting analysis for PGC-1α in VSMCs.

3. Results

3.1. High Glucose Stimulated the Proliferation, Migration, and Inflammatory Gene Expression of VSMCs. VSMCs cultured in low-glucose (5.5 mmol/L) or high-glucose (25 mmol/L) complete media were deprived of serum for 24 h. In proliferation and inflammatory gene expression assays, VSMCs were stimulated by complete media for 6 h, respectively, (Figures 1(a) and 1(c)). In migration assays, VSMCs were suspended, and then low-glucose or high-glucose complete media were added to the lower compartment, respectively, (Figure 1(b)). Results showed that the VSMCs in high glucose were in a preactivated state.

3.2. Overexpression of PGC-1α Inhibited Proliferation, Migration, and Inflammatory Gene Expression of VSMCs Induced by High Glucose. VSMCs cultured in high glucose (25 mmol/L) complete media were deprived of serum for 24 h. The cells were then treated with adenovirus infection for 48 h. In proliferation and inflammatory gene expression assays, VSMCs were incubated by complete media for 6 h (Figures 2(a) and 2(c)). In migration assays, VSMCs were suspended and then high-glucose complete media was added to the lower compartment (Figure 2(b)). Results showed that proliferation, migration, and inflammatory gene expression of VSMCs were inhibited. Expression of PGC-1α in VSMCs treated with adenovirus infection was showed in Figure 2(d).

3.3. Overexpression of PGC-1α Downregulated the VSMCs-Specific Genes Expression and Upregulated the Macrophage-Specific Genes Expression. To characterize the phenotypic changes after overexpression of PGC-1α, we determined the abundance of mRNA for several VSMCs [17] and macrophage-specific genes at the end of the adenovirus treatment for 48 h. As shown in Figure 3(a), VSMCs infected by Ad-PGC-1α had dramatically decreased in mRNA levels of α-actin (57.7% of control, P < 0.05), SM22α (59.5%, P < 0.05), smooth muscle myosin heavy chain (31.5%, P < 0.05), and calponin H1 (57.8%, P < 0.05). At the same time,
the mRNA levels of the macrophage-related proteins (CD68) increased (569% of control, \( P < 0.05 \)). Another macrophage marker, Mac-2, increased to 373% of controls (\( P < 0.05 \)) (Figure 3(b)). ABCA-1, a key regulator of cholesterol efflux from peripheral cells to high-density lipoprotein [18] and associated predominantly with macrophages in vivo [19], increased to 221% (\( P < 0.05 \)) in VSMCs infected by Ad-PGC-1α (Figure 3(b)).

Western blot also revealed the presence of specific proteins commonly taken as characteristic for VSMC or macrophage phenotype. Results are showed in Figures 3(c) and 3(d).

### 3.4. Phagocytosis of VSMCs

To investigate whether VSMCs infected by Ad-PGC-1α acquired functional aspects of macrophages, VSMCs were incubated in 1 \( \mu \)m microspheres with red fluorescent and then assessed by flow cytometry.

As shown in Figure 4, 27.36% of VSMCs infected by Ad-GFP had phagocytic activity. However, the proportion of VSMCs having phagocytic activity increased to 77.45% after overexpression of PGC-1α. This finding indicated that the acquisition of functional properties of macrophages existed in VSMCs, in addition to the increases of macrophage-specific gene and protein expression in VSMCs.

### 4. Discussion

Recent studies showed that VSMCs cultured under high-glucose conditions mimic the diabetic pathophysiological state, which stimulates VSMCs proliferation, migration, and inflammatory genes expression [6, 20]. High glucose stimulates reactive oxygen species production in cultured vascular smooth muscle cells [21]. ROSs have been implicated in all of these above responses including proliferation [22], migration [23], and inflammatory gene expression of VSMCs [24, 25]. PGC-1α is a major transcriptional regulator of the mitochondrial detoxification system [26–29].

In the current study, we found that high-glucose stimulated VSMCs proliferation, migration, and expression of MCP-1 and IL-6. Overexpression of PGC-1α by adenoviruses infection blocked glucose-induced VSMCs proliferation, migration, and expression of MCP-1 and IL-6. These implied that overexpression of PGC-1α could inhibit the preatherogenic responses in VSMCs, which were key features in the pathology of atherosclerosis.

VSMCs maintain considerable plasticity throughout life and can exhibit a diverse range of different phenotypes in response to changes in local environmental cues [17]. In vascular neointimal lesions, VSMCs display diminished expression of a number of proteins that are characteristics
of fully differentiated VSMCs [30]. It is well established that the dedifferentiated VSMCs exhibit a number of properties, including enhanced migration, proliferation, and inflammatory gene expression [31].

Given the fact that overexpression of PGC-1α suppressed the main preatherogenic responses in VSMCs, we examined the expression of VSMCs-related genes. All four smooth muscle markers assessed in the present study are related to the contraction function of VSMCs. Myosin heavy chain and α-actin are essential components of smooth muscle contractile machinery [17]. Surprisingly, our results showed that overexpression of PGC-1α rapidly decreased the genes expression of commonly accepted markers of the phenotype of VSMCs. The results were interpreted as evidences for a de-differentiation process of VSMCs.

In further experiments, we found that the expression of macrophage-related genes was induced in VSMCs by overexpression of PGC-1α, and VSMCs acquired the macrophage-like functions assessed by phagocytic activity. In light of the present results for macrophage-related genes expression of VSMCs, rather than dedifferentiating process, overexpression of PGC-1α in VSMCs may be more appropriately considered as part of a transdifferentiation program that made VSMCs assume macrophage-like states. VSMCs-derived foam cells are usually assumed to occur relatively late in the development of organized atherosclerotic plaque lesion. They retain sufficient phenotypic features to be identifiable by electron microscopy [3]. It has been reported that downregulation of VSMCs-related genes and Up-regulation of macrophage-related genes occurred in VSMCs-derived foam cells [4].

The transdifferentiation was good or bad? Firstly, overexpression of PGC-1α inhibits the expression of MCP-1 and makes it difficult to attract monocytes to bind to VSMCs. Then foam cells derived from macrophages would decrease. Secondly, VSMCs that were transdifferentiated to macrophage-like state compensated for the decrease of macrophage. Thirdly, VSMCs have the relative resistance to cholesterol loading-induced toxicity compared with macrophages, consistent with VSMCs being a more prominent histological feature of the advanced atherosclerotic lesions [32], which reduced the death of VSMCs and then favoured plaque stability.

The mechanisms that overexpression of PGC-1α leads to the conversion of VSMCs to macrophage-like cells remain unknown. Recent studies demonstrated that two transcription factors: Liver X receptor α (LXRA) and PPARγ are strongly regulated by PGC-1α. LXRA is involved in the transcriptional control of a number of genes regulating the transport and catabolism of cholesterol [33]. Identified target genes of LXRA include the ATP-binding cassette transporter A1 (ABCA-1) [34], which is involved in reverse cholesterol transport. In our study, the expression of ABCA-1 is increased by overexpression of PGC-1α in VSMCs. Previous studies have indicated that PPARγ regulates gain of macrophage-like phenotype in primary smooth muscle culture [35] and showed that the uptake of oxidized LDL by macrophages or of cholesterol in endothelial cells could
lead to PPARγ activation [36, 37]. Future experiments will be needed to identify the pathways that are responsible for the transdifferentiation to macrophage-like state in VSMCs.

In summary, we demonstrated that PGC-1α inhibited glucose-induced proliferation, migration, and inflammatory gene expression in VSMCs. In addition, overexpression of PGC-1α decreased VSMCs-related genes expression, while it induced macrophage-related genes expression and phagocytosis in VSMCs. PGC-1α transdifferentiated VSMCs to macrophage-like state. Such transdifferentiation increased the portion of VSMCs-derived foam cells in the plaque and then favored plaque stability. PGC-1α may be a potential target for drug development of atherosclerosis.

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