

Adiponectin promotes preadipocyte differentiation via the PPAR γ pathway

WENKAI YANG^{1*}, CHUNLI YANG^{2*}, JUN LUO¹, YUTAO WEI³, WENYING WANG² and YINGMEI ZHONG²

¹Department of Cardio-Thoracic Surgery, Ganzhou People's Hospital, Ganzhou, Jiangxi 341000; ²Department of Intensive Care, Jiangxi People's Hospital, Nanchang, Jiangxi 330006; ³Department of Cardio-Thoracic Surgery, The First Affiliated Hospital of Shihezi University Medical College, Shihezi, Xinjiang 832008, P.R. China

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Abstract. According to the results of a preliminary study, it was hypothesized that the effects of adiponectin (APN) on the improvement of atherosclerosis may be associated with adipocyte differentiation and peroxisome proliferator-activated receptor γ (PPAR γ). The present study simulated the inflammatory environment of epicardial adipose tissue by stimulating mature adipocytes with lipopolysaccharide (LPS); subsequently, the differentiation of 3T3-L1 preadipocytes was observed. 3T3-L1 preadipocytes were infected with an adenovirus containing the human adiponectin gene apM1 (Ad-apM1) and were co-cultured with mature adipocytes stimulated with LPS. Differentiation into mature adipocytes was initiated using differentiation medium. After 8 days, an MTT assay was used to examine cell viability and oil red O staining was used to observe preadipocyte differentiation. In addition, the mRNA expression levels of monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-6, IL-8 and tumor necrosis factor α (TNF- α) were examined by quantitative polymerase chain reaction, and the protein expression levels of PPAR γ , CCAAT/enhancer binding protein α (C/EBP α) and preadipocyte factor-1 (Pref-1) were measured by western blotting. The results indicated that APN overexpression significantly increased preadipocyte differentiation and cell viability, inhibited MCP-1, IL-6, IL-8 and TNF- α expression, upregulated PPAR γ and C/EBP α expression, and downregulated Pref-1 under LPS stimulation. In addition, inhibition of PPAR γ activity by T0070907 markedly attenuated the effects of APN overexpression. Taken together, the present study demonstrated that the effects of APN on the promotion of

preadipocyte differentiation under inflammatory conditions may involve the PPAR γ signaling pathway, and at least partly depends on upregulation of PPAR γ expression.

Introduction

Adipose tissue was originally thought to only store fat; however, it is now known to act as an active endocrine organ, which secretes various types of bioactive molecules, including leptin, adiponectin (APN), tumor necrosis factor α (TNF- α) and interleukin (IL)-6 (1-4). Ridker and Silvertown (5) reported that aberrant secretion of endocrine substances by epicardial adipose tissue (EAT) results in the induction of inflammation (6), which is closely associated with the occurrence and development of coronary artery disease (CAD), particularly atherosclerosis (7,8). In addition, there is a close association between APN and atherosclerosis. It has been reported that APN protects against atherosclerosis by inhibiting neointimal thickening, proliferation and migration of smooth muscle cells, and the expression of vascular adhesion molecules, as well as reducing the lipid plaque area (9-11). However, the specific underlying mechanisms remain unclear.

In a preliminary study, quantitative polymerase chain reaction (qPCR) was used to detect peroxisome proliferator-activated receptor γ (PPAR γ) and preadipocyte factor-1 (Pref-1) gene expression in EAT from patients with atherosclerosis; compared with the gene expression in individuals without CAD, Pref-1 was significantly increased and PPAR γ was decreased. It is well known that PPAR γ and Pref-1 are closely associated with proliferation and adipocyte differentiation (12,13). However, the association between the proliferation and differentiation of adipocytes and atherosclerosis remains to be determined, as does the role of APN in atherosclerosis. Therefore, the present study simulated the inflammatory physiological environment in the EAT of patients with atherosclerosis by stimulating mature adipocytes with lipopolysaccharide (LPS); subsequently, the effects of APN on preadipocyte differentiation in this environment were determined.

Correspondence to: Dr Wenkai Yang, Department of Cardio-Thoracic Surgery, Ganzhou People's Hospital, 17 Hongqi Street, Ganzhou, Jiangxi 341000, P.R. China
E-mail: yangwenkai111@163.com

*Contributed equally

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Materials and methods

Reagents. LPS was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and was diluted in pyrogen-free

0.9% saline. Insulin was also purchased from Sigma-Aldrich (Merck KGaA), T0070907 was purchased from Selleck Chemicals (Houston, TX, USA). TRIzol reagent was obtained from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Transcriptor First Strand cDNA Synthesis kit was purchased from Roche Diagnostics (Indianapolis, IN, USA). Anti-APN (ab22554), anti-PPAR γ (ab45036), anti-Pref-1 (ab21682) and anti-CCAAT/enhancer binding protein α (C/EBP α) (ab40764) antibodies were purchased from Abcam (Cambridge, MA, USA); anti- β -actin antibody (KL002) was obtained from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China); and horseradish peroxidase-labeled immunoglobulin G secondary antibody (goat anti-mouse IgG; SA00001-1 and goat anti-rabbit IgG; SA00001-2) was purchased from ProteinTech Group, Inc. (Chicago, IL, USA).

EAT acquisition and detection. Patients provided written informed consent prior to tissue collection, and the present study was approved by the Ethics Committee of Human Investigation of Union Hospital, Huazhong University of Science and Technology (Wuhan, China), and conformed to the Helsinki Declaration. Donation procedures complied with the laws of China, and the specimens obtained were registered with the relevant governmental authorities of Hubei (China). Between March 2013 and August 2013, a total of 34 patients (40-75 years old, 25 male and 9 female) who had been planning cardiac surgery initially underwent selective coronary angiography (CAG). The patients were then divided into a CAD group (n=23) and a non-CAD control group (n=11), according to the results of CAG. EAT biopsy samples (average weight, 0.5-1.0 g) were collected from aortic root near the right coronary artery and were shock-frozen and immediately stored in liquid nitrogen for total RNA extraction. The mRNA expression levels of PPAR γ , Pref-1 and C/EBP α were detected by qPCR.

Cell culture and differentiation. The 3T3-L1 murine preadipocyte cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). 3T3-L1 preadipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM; (Tianjin Hao Yang Biological Products Technology Co., Ltd., Tianjin, China) supplemented with 10% fetal bovine serum (FBS) (TBD) and penicillin (100 IU/ml), and streptomycin (100 mg/ml) (Wuhan Boster Biological Technology, Ltd., Wuhan, China) under an atmosphere of 5% CO $_2$ in air (v/v) at 37°C. The medium was replaced every 2-3 days. Differentiation was induced by replacing the medium with DMEM supplemented with 10% FBS, and 0.5 mM 3-isobutyl-1-methylxanthine, 0.5 mM dexamethasone and 5 mg/ml insulin (MDI; Sigma-Aldrich; Merck KGaA). After 48 h of MDI induction, differentiation medium was replaced with DMEM supplemented with 10% FBS and 5 mg/ml insulin. The medium was then replaced every other day with DMEM containing 10% FBS until day 8. On day 8, the preadipocytes became mature adipocytes (14).

Construction of recombinant adenovirus for human APN overexpression. Cloning of human adiponectin gene from human adipose tissue and an adenovirus expressing apM1 (Ad-apM1) was assembled using pAxCawt [Cyagen Biosciences

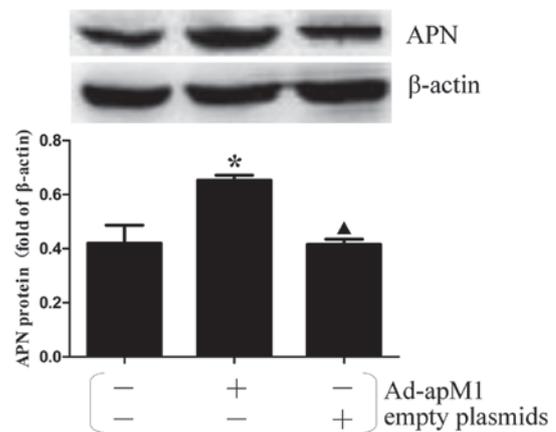


Figure 1. Effects of Ad-apM1 on APN protein expression in 3T3-L1 cells. The 3T3-L1 cell line was used to verify the expression of APN post-infection with a recombinant adenovirus containing the human APN gene apM1. Cells were divided into three experimental groups: The control group; the Ad-apM1 group, in which 3T3-L1 cells were infected with Ad-apM1 (MOI, 100); The negative control group, in which 3T3-L1 cells were infected with an adenovirus containing empty plasmids (MOI, 100). After 48 h, cells were harvested and protein was extracted. Western blotting was used to determine the protein expression levels of APN. Data are presented as the means \pm standard error of the mean for six independent experiments. *P<0.05 vs. the control group; \blacktriangle P<0.05 vs. the Ad-apM1 group. Ad-apM1, adenovirus containing the apM1 gene; APN, adiponectin; MOI, multiplicity of infection.

(Guangzhou) Inc., Guangzhou, China]. The sequence was co-transfected into 293 cells (the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai, China) alongside Ad5DNA-TPC [Cyagen Biosciences (Guangzhou) Inc.] using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. The recombinant adenovirus was produced through homologous recombination. After 48 h, the sample was centrifuged for 20 min at 3,960 x g at 4°C and the viral supernatant was collected. High speed centrifugation was used to concentrate virus particles by ultra-speed centrifuge (Beckman, Fullerton, CA, USA) at 5,000 x g for 2.5 h. Virus was packed and stored at -80°C. Tissue culture infective dose 50 was used to determine virus titer. Western blotting was performed to verify the effects of APN overexpression on protein expression levels following infection of 3T3-L1 preadipocytes with the adenovirus (Fig. 1).

Experimental groups and treatments. In a 6 wells Transwell plate (Corning, Corning, NY, USA), 3T3-L1 preadipocytes (1×10^5 cells) in the inner chamber, were co-cultured with mature adipocytes (2×10^5 cells) in outer chamber, which were differentiated from 3T3-L1 preadipocytes. The cells were divided into the following experimental groups: i) The control group, in which 3T3-L1 preadipocytes were induced to differentiate into adipocytes in the inner chamber, as aforementioned. Mature adipocytes were cultured in the outer chamber, with the addition of PBS, equal in volume to LPS in group ii. ii) The LPS stimulation group, in which mature adipocytes were stimulated with LPS (1 μ g/ml, 18 h) to induce inflammation, as described in previous studies (15,16). The mature adipocytes in the outer chamber were co-cultured with 3T3-L1 preadipocytes in the inner chamber, which were induced to differentiate into mature adipocytes using

MDI, as aforementioned. iii) The human APN recombinant adenovirus group (LPS + Ad-apM1), in which preadipocytes were infected with Ad-apM1 [multiplicity of infection (MOI), 100], as previously described (17). After a 48-h infection with the adenovirus preadipocytes were induced into mature adipocytes and were co-cultured with LPS-stimulated mature adipocytes. iv) The negative control (NC) group, in which preadipocytes were infected with an adenovirus containing empty plasmids (MOI, 100) as an NC; after 48 h, the cells were induced to differentiate into mature adipocytes and were co-cultured with mature adipocytes stimulated with LPS. v) The PPAR γ -inhibited group (LPS + Ad-apM1 + T0070907), in which preadipocytes were infected with Ad-apM1 as aforementioned and were treated with 10 μ M T0070907 24 h prior to being induced to differentiate into mature adipocytes via co-culturing with LPS-treated mature adipocytes. The time point and titer of adenoviruses and the dose of T0070907 used in the present study were determined during preliminary experiments. At day 8, subsequent experiments on the 3T3-L1 cells in the inner chamber were performed.

MTT assay. Cell viability of preadipocytes was determined following differentiation in the inner chamber using an MTT assay (Promega Corporation, Madison, WI, USA). MTT was directly dissolved in cell culture medium and was then incubated with live cells. Briefly, cells in the exponential growth phase were plated at 1×10^4 cells/well in a 96-plate, and were incubated at 37°C with 20 μ l MTT (5 mg/ml) in 100 μ l cell culture medium for 4 h. After 4 h, the supernatant was discarded and 150 μ l of DMSO was added to each well. The vortex was rotated for about 10 min to allow the crystals to dissolve. The absorbance of each well was measured at a wavelength of 490 nm using a Spectra Max Paradigm microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Oil Red O staining. Oil Red O (Sigma-Aldrich; Merck KGaA) staining was performed according to the manufacturer's protocol. A total of 8 days after the induction of adipocyte differentiation, adipocytes in the inner chamber were washed three times with PBS and were fixed with 10% formalin for 1 h at room temperature. The dishes were washed once with 60% isopropanol and were then left to dry completely. Subsequently, the cells were stained with 2 ml Oil Red O for 2 h at 37°C, rinsed with 60% isopropanol, and were thoroughly washed four times with PBS. Finally, images were captured using an inverted microscope (magnification, x400). After extracting Oil Red O with 100% isopropanol, the absorbance of the extracted dye was determined spectrophotometrically at 490 nm wavelength using a Spectra Max Paradigm microplate reader (Molecular Devices, LLC) (18).

RNA preparation and reverse transcription (RT)-qPCR analysis. TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from EAT and adipocytes in the inner chamber after 8 days of differentiation according to the manufacturer's protocol. RT was conducted using the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics) according to the manufacturer's protocol. The oligonucleotide primer sequences, which were designed using Premier Primer 5.0 software (Premier Biosoft International,

Table I. Quantitative polymerase chain reaction primers.

Gene	Primer sequence
β -actin	Forward: TTACAGGAAGTCCCTCACCCCTC Reverse: TCAGGGCATGGACGCGA
MCP-1	Forward: AACTGCATCTGCCCTAAGGT Reverse: ACTGTCACACTGGTCACTCC
IL-6	Forward: ACAAAGCCAGAGTCCTTCAGAG Reverse: GTGACTCCAGCTTATCTCTTGGT
IL-8	Forward: GCACTTGGGAAGTTAACGCA Reverse: GCACTTGGGAAGTTAACGCA
TNF- α	Forward: AGCCGATGGGTTGTACCTTG Reverse: ATAGCAAATCGGCTGACGGT

IL, interleukin; MCP-1, monocyte chemoattractant protein-1; TNF- α , tumor necrosis factor- α .

Palo Alto, CA, USA), are presented in Table I. β -actin was used as an internal control. The synthesized first-strand cDNA samples were subjected to qPCR using SYBR-Green PCR Master mix (Toyobo Life Science, Osaka, Japan) and PCR was performed using an ABI Prism 7700 Sequence Detector (Applied Biosystems; Thermo Fisher Scientific, Inc.). Thermocycling conditions were as follows: 2 min at 95°C followed by 40 cycles of 15 sec at 95°C, 15 sec at 60°C, 20 sec at 72°C, with a final extension step of 60°C for 30 min. Integrity of the PCR products was confirmed by dissociation curve analysis using 7500 Software version 2.0.4 (Applied Biosystems; Thermo Fisher Scientific, Inc.). The quantification cycle (Cq) values were determined and the $2^{-\Delta\Delta Cq}$ method (19) was used to calculate relative gene expression.

Western blotting. Following 8 days of differentiation, cells harvested from the inner chamber were lysed with radioimmunoprecipitation assay lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% sodium deoxycholate, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride and 1 mM EDTA. The extracts were then centrifuged at 10,140 x g for 15 min at 4°C to remove insoluble material. Total protein concentrations were determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. An equivalent amount of total protein (40 μ g) per well was diluted with sample buffer containing 100 mM dithiothreitol and was heated at 98°C for 5 min. Subsequently, proteins were separated by 10-15% SDS-PAGE using gel apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and were transferred to polyvinylidene fluoride membranes. The membranes were then soaked in 5% non-fat dry milk for 2 h at room temperature and were incubated overnight at 4°C with primary antibodies (1:500) against β -actin, APN, PPAR γ , Pref-1 and C/EBP α . Subsequently, the membranes were washed with Tris-buffered saline containing 0.05% Tween (TBS-T) and were incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000) with agitation at room temperature for 1 h. The membranes were washed three times with TBS-T (20 min/wash) and immune complexes were visualized by enhanced chemiluminescence

(Tiangen Biotech Co., Ltd., Beijing, China); band intensity was finally measured and semi-quantified (20). The resulting images were analyzed with Quantity One version 4.62 (Bio-Rad Laboratories, Inc.).

Statistical analysis. Data are presented as the mean \pm standard error of the mean. The variance homogeneity test and one-way analysis of variance were performed by SPSS version 20.0 (IBM Corp., Armonk, NY, USA). The least significant difference method was used to compare between groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression levels of PPAR γ , Pref-1 and C/EBP α in EAT. The mRNA expression levels of PPAR γ , Pref-1 and C/EBP α in EAT were detected by qPCR. As presented in Fig. 2, compared with in the non-CAD control group, the mRNA expression levels of PPAR γ and C/EBP α were significantly downregulated in the EAT from patients with CAD, whereas Pref-1 was upregulated. These results suggested that PPAR γ , Pref-1 and C/EBP α may be involved in the occurrence and development of atherosclerosis.

APN increases preadipocyte viability. In order to determine the effects of APN on the viability of 3T3-L1 cells co-cultured with LPS-stimulated adipocytes, an MTT assay was conducted. As presented in Fig. 3, APN overexpression significantly increased 3T3-L1 cell viability, whereas it was decreased in the LPS group. Conversely, the effects of APN on cell viability were reversed by T0070907, which is a selective inhibitor of PPAR γ .

APN promotes preadipocyte differentiation. The differentiation of preadipocytes was examined by Oil Red O staining. As shown in Fig. 4A, cell volume and the number of lipid droplets was reduced in 3T3-L1 cells on day 8 following MDI-induced differentiation and co-culture with LPS-stimulated adipocytes compared with in the control group. However, this situation was markedly reversed in the LPS + Ad-apM1 group. As expected, the effects of APN were markedly attenuated in the NC and LPS + Ad-apM1 + T0070907 groups.

Following extraction of Oil Red O with 100% isopropanol, absorbance of the extracted dye was determined spectrophotometrically. As shown in Fig. 4B, the optical density (OD) value was markedly decreased in the LPS group compared with in the control group ($P < 0.05$), whereas it was significantly increased in the LPS + Ad-apM1 group compared with in the LPS group ($P < 0.05$). Conversely, the OD values were significantly decreased in the NC and LPS + Ad-apM1 + T0070907 groups.

APN suppresses the expression of inflammatory factors. The mRNA expression levels of MCP-1, IL-6, IL-8 and TNF- α were detected in 3T3-L1 preadipocytes by qPCR. As shown in Fig. 5, the mRNA expression levels of MCP-1, IL-6, IL-8 and TNF- α were significantly increased in the LPS group compared with in the control group ($P < 0.05$). Conversely, the mRNA expression levels of MCP-1, IL-6, IL-8 and TNF- α were decreased in the APN overexpression group compared

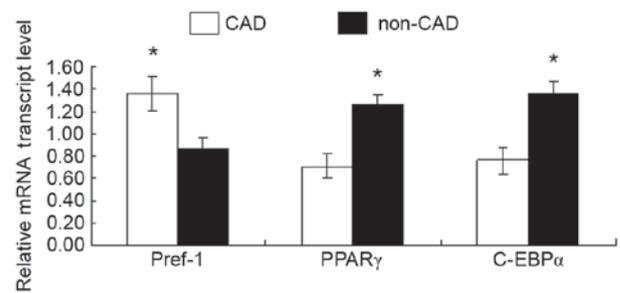


Figure 2. mRNA expression levels of PPAR γ , Pref-1 and C/EBP α in EAT from patients with CAD. mRNA expression levels were detected by quantitative polymerase chain reaction. CAD data are presented as the mean \pm standard error of the mean for 10 independent experiments; non-CAD data are presented as the mean \pm standard error of the mean for three independent experiments. * $P < 0.05$ vs. the non-CAD group. CAD, coronary artery disease; C/EBP α , CCAAT/enhancer binding protein α ; Pref-1, preadipocyte factor-1; PPAR γ , peroxisome proliferator-activated receptor γ .

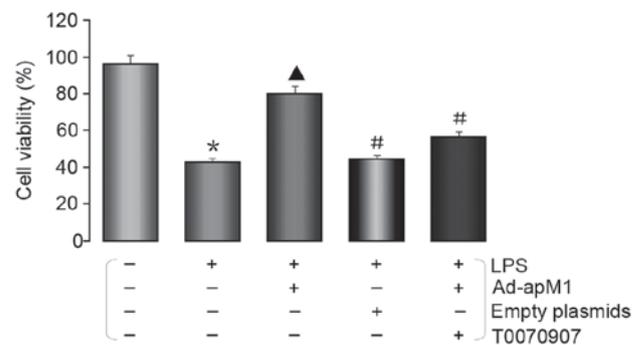


Figure 3. Effects of APN on the viability of 3T3-L1 preadipocytes co-cultured with LPS-stimulated mature adipocytes for 8 days. An MTT assay was performed to measure cell viability. APN overexpression significantly increased 3T3-L1 cell viability after 8 days of MDI-induced differentiation and co-culture with LPS-stimulated mature adipocytes; however, this effect was abrogated by T0070907. * $P < 0.05$ vs. the control group; [▲] $P < 0.05$ vs. the LPS group; [#] $P < 0.05$ vs. the LPS + Ad-apM1 group. Ad-apM1, adenovirus containing the apM1 gene; APN, adiponectin; LPS, lipopolysaccharide; MDI, 3-isobutyl-1-methylxanthine, dexamethasone and insulin.

with in the LPS group ($P < 0.05$). However, the effects of APN overexpression were markedly attenuated in the NC and LPS + Ad-apM1 + T0070907 groups.

APN increases the protein expression levels of PPAR γ . To further investigate whether PPAR γ expression was altered in response to APN overexpression, the protein expression levels of PPAR γ were detected by western blotting. As shown in Fig. 6, the protein expression levels of PPAR γ were significantly increased in the LPS + Ad-apM1 group compared with in the LPS group ($P < 0.05$). However, there was a marked decrease in the expression of PPAR γ in the NC and LPS + Ad-apM1 + T0070907 groups. These results suggested that APN may promote the differentiation of preadipocytes co-cultured with LPS-stimulated mature adipocytes via the upregulation of PPAR γ .

Effects of APN overexpression on the protein expression levels of Pref-1 and C/EBP α . As shown in Fig. 7, the inflammatory environment markedly increased Pref-1 expression compared with in the control group ($P < 0.05$), whereas APN

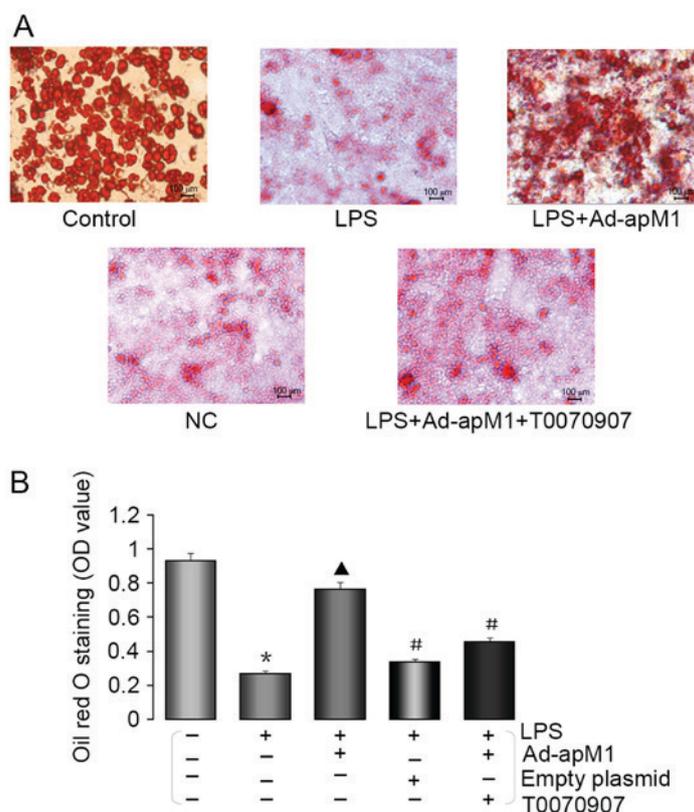


Figure 4. APN promotes the differentiation of preadipocytes co-cultured with LPS-stimulated mature adipocytes. (A) Oil red O staining of 3T3-L1 cells on day 8 of differentiation (magnification, x400). (B) Oil Red O was extracted from cells with 100% isopropanol and absorbance was determined spectrophotometrically at 450 nm. Data are presented as the mean \pm standard error of the mean for six independent experiments. * $P < 0.05$ vs. the control group; $\blacktriangle P < 0.05$ vs. the LPS group; # $P < 0.05$ vs. the LPS + Ad-apM1 group. Ad-apM1, adenovirus containing the apM1 gene; APN, adiponectin; LPS, lipopolysaccharide; NC, negative control; OD, optical density.

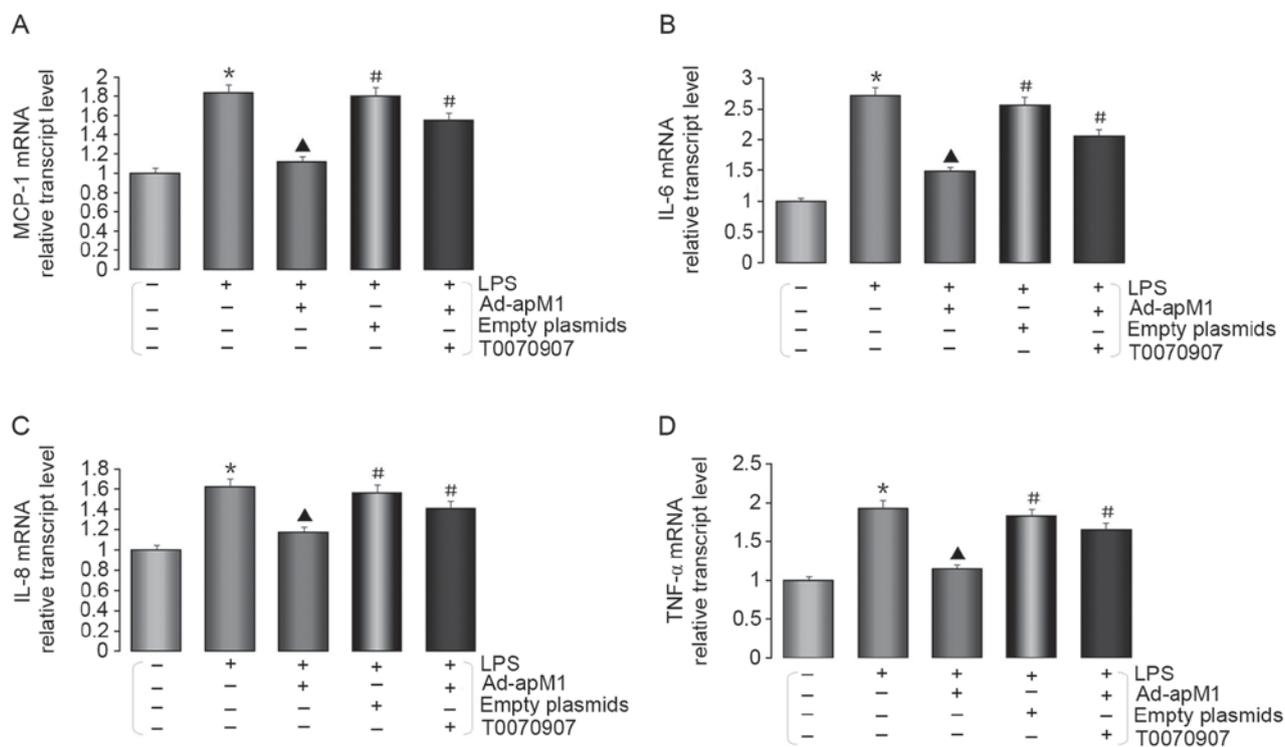


Figure 5. APN suppresses the expression of MCP-1, IL-6, IL-8 and TNF- α in preadipocytes 8 days after co-culturing with LPS-stimulated mature adipocytes. Relative mRNA expression levels of (A) MCP-1, (B) IL-6, (C) IL-8 and (D) TNF- α were detected using quantitative polymerase chain reaction. Data are presented as the mean \pm standard error of the mean for six independent experiments. * $P < 0.05$ vs. the control group; $\blacktriangle P < 0.05$ vs. the LPS group; # $P < 0.05$ vs. the LPS + Ad-apM1 group. Ad-apM1, adenovirus containing the apM1 gene; APN, adiponectin; IL, interleukin; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; TNF- α , tumor necrosis factor- α .

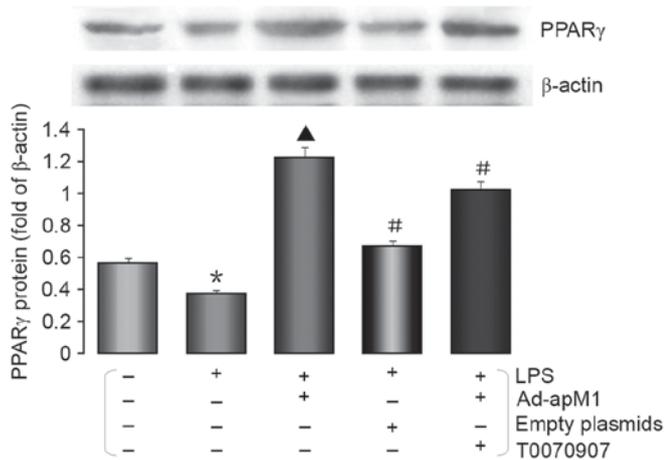


Figure 6. APN increases the protein expression levels of PPAR γ in preadipocytes co-cultured with LPS-stimulated mature adipocytes following 8 days of MDI-induced differentiation. Cell lysates were collected and subjected to western blotting to examine the protein expression levels of PPAR γ . Data are presented as the mean \pm standard error of the mean for six independent experiments. *P<0.05 vs. the control group; [▲]P<0.05 vs. the LPS group; [#]P<0.05 vs. the LPS + Ad-apM1 group. Ad-apM1, adenovirus containing the apM1 gene; APN, adiponectin; LPS, lipopolysaccharide; MDI, 3-isobutyl-1-methylxanthine, dexamethasone and insulin; PPAR γ , peroxisome proliferator-activated receptor γ .

in the LPS group compared with in the control groups (P<0.05), whereas its expression was increased in the LPS + Ad-apM1 group compared with in the LPS group (P<0.05). However, the expression levels of C/EBP α were markedly decreased in the NC and LPS + Ad-apM1 + T0070907 groups.

Discussion

It has previously been reported that elevated proinflammatory cytokine levels mediate insulin resistance, type II diabetes and cardiovascular disease (21). Inflammatory cytokines, including MCP-1, IL-6, IL-8 and TNF- α , which are secreted by adipose tissue, are able to increase infiltration of local immune cells and aggravate chronic inflammation in adipose tissue, thus leading to adipose tissue dysfunction and metabolic disorders (22,23). As adipose tissue, EAT is able to secrete numerous inflammatory factors, which induce inflammation and atherosclerosis (24). In the present study, the differentiation of preadipocytes into mature adipocytes was significantly inhibited, and the expression of inflammatory factors was markedly increased, under inflammatory stimulation.

There are numerous causes of metabolic disorders, of which adipocyte hypertrophy is one of the major risk factors. Under normal conditions, adipose tissue remains metabolically healthy through the constant production of newer and smaller fat cells; preadipocyte differentiation serves an important role in this process (25). APN, which is secreted by adipose tissue, is an adipokine hormone that is closely associated with lipid and carbohydrate metabolism, and is composed of an N-terminal collagenous domain and a C-terminal globular domain (26,27). The effects of APN on adipocyte differentiation remain controversial. It has previously been suggested that APN may promote adipocyte differentiation, insulin sensitivity and lipid accumulation (28). However, Bauche *et al* (29) observed that in a mouse model of obesity, APN overexpression resulted in a marked reduction in energy expenditure and impairment in the differentiation of adipocytes. In the present study, the overexpression of APN significantly improved preadipocyte differentiation under inflammatory conditions, which is consistent with the findings of Fu *et al* (28). Previous studies have reported that APN could suppress the growth of myelomonocytic progenitors and the function of mature macrophages, inhibit macrophage-to-foam cell transformation and promote the secretion of anti-inflammatory cytokines from macrophages (30-32). Furthermore, in adipocytes, APN may suppress LPS-induced nuclear factor- κ B activation, which is closely associated with the downregulation of inflammatory responses, and it has been suggested that there is an inverse relationship between TNF- α and APN (33-37). In the present study, the results of a qPCR analysis indicated that the expression levels of MCP-1, IL-6, IL-8 and TNF- α were suppressed by APN overexpression, thus indicating that the effects of APN on the promotion of preadipocyte differentiation may be associated with suppression of inflammatory factor secretion by adipocytes. These findings suggested that APN may act as an anti-inflammatory factor in adipose tissue metabolism.

The regulatory mechanism underlying adipocyte differentiation is complex. A regulated transcriptional cascade is known to control adipocyte differentiation through activating or suppressing the expression of transcription factors in a

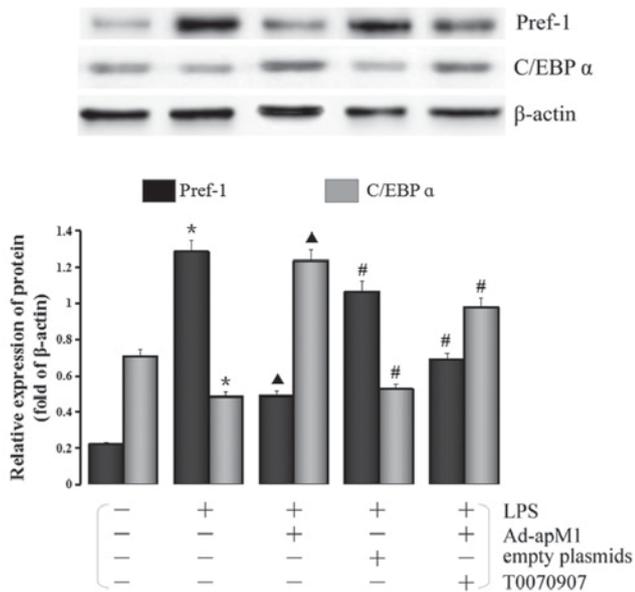


Figure 7. Effects of APN overexpression on the protein expression levels of Pref-1 and C/EBP α . Pref-1 and C/EBP α protein expression was detected in preadipocytes following 8 days of MDI-induced differentiation and co-culture with LPS-stimulated mature adipocytes by western blotting. Data are presented as the mean \pm standard error of the mean for six independent experiments. *P<0.05 vs. the control group; [▲]P<0.05 vs. the LPS group; [#]P<0.05 vs. the LPS + Ad-apM1 group. Ad-apM1, adenovirus containing the apM1 gene; APN, adiponectin; C/EBP α , CCAAT/enhancer binding protein α ; LPS, lipopolysaccharide; MDI, 3-isobutyl-1-methylxanthine, dexamethasone and insulin; Pref-1, preadipocyte factor-1.

overexpression decreased the expression levels of Pref-1 compared with in the LPS group. Conversely, the expression levels of Pref-1 were significantly increased in the NC and LPS + Ad-apM1+T0070907 groups (P<0.05). Furthermore, the expression levels of C/EBP α were significantly downregulated

sequential fashion (38). PPAR γ is a member of the nuclear receptor superfamily of ligand-activated transcription factors (39). *In vivo* and *in vitro* studies have reported that PPAR γ is an essential regulator of adipogenesis (40). PPAR γ is an important transcription factor that initiates the expression of genes that are required to convert precursor cells into mature adipocytes; the activation of PPAR γ is necessary and sufficient for adipocyte differentiation (41). There are two isoforms of PPAR γ , PPAR γ 1 and PPAR γ 2, which are generated from alternate promoter usage and splicing; at the amino-terminus 30 additional amino acids are contained in PPAR γ 2 (42). Both isoforms are specifically activated in the process of adipocyte differentiation; however, only PPAR γ 2 is strictly expressed in adipose tissues. In addition, it has been reported that PPAR γ 2 serves a more central role in adipocyte differentiation (43). A previous study has demonstrated that cytokines that regulating adipocyte differentiation exercise their effects by regulating PPAR γ expression or activity (44). The present study demonstrated that APN overexpression may increase the protein expression levels of PPAR γ in preadipocytes following MDI-induced differentiation and co-culture with LPS-stimulated mature adipocytes. Conversely, the positive effects of APN on preadipocyte differentiation could be antagonized following treatment with T0070907, which is a specific inhibitor of PPAR γ (45). These results suggested that the effects of APN on preadipocytes in an inflammatory environment may be involved with the PPAR γ signaling pathway.

C/EBPs are a basic leucine zipper family of transcription factors, which are crucial for adipogenesis. C/EBP α is a member of the C/EBP family that is specifically required for adipogenesis (38). A previous report indicated that PPAR γ and C/EBP α may promote adipogenesis through modulating the expression of each other. C/EBP α cooperates with PPAR γ by inducing the expression of multiple subsets of adipocyte-specific genes during adipocyte differentiation (46). C/EBP β and δ , which are two other members of the C/EBP family, are also associated with PPAR γ and C/EBP α transcriptional induction (38). The majority of induced genes in the process of adipogenesis are bound by PPAR γ and C/EBP α , thus indicating that the two master regulators may cooperatively upregulate the expression of adipogenic genes (47). The present study demonstrated that APN overexpression could upregulate the protein expression levels of C/EBP α in 3T3-L1 cells following MDI-induced differentiation under inflammatory conditions, whereas its expression was inhibited by T0070907. Furthermore, the expression of Pref-1 was evaluated; Pref-1 is an epidermal growth factor repeat-containing transmembrane protein. The overexpression of Pref-1, or the treatment of preadipocytes with soluble Pref-1, leads to the inhibition of adipocyte differentiation (48). The results of the present study demonstrated that an increase in the expression of inflammatory factors may promote overexpression of Pref-1, which could suppress the differentiation of preadipocytes. Conversely, APN may inhibit Pref-1 expression and promote preadipocyte differentiation, which was attenuated by T0070907. Taken together, these results suggested that APN promotes preadipocyte differentiation under inflammatory conditions, which may be due to upregulation of PPAR γ expression, and the regulation of C/EBP α , Pref-1 and inflammatory factors expression. However, the specific mechanisms remain to be further elucidated.

In conclusion, the present study demonstrated that APN attenuates inflammation-induced inhibition of preadipocyte differentiation, potentially via the PPAR γ signaling pathway. Therefore, it may be hypothesized that APN promotes the differentiation of adipocytes in response to inflammatory stimulation, accelerates the metabolism of visceral adipose tissue and reduces the secretion of inflammatory cytokines in EAT. As a result, atherosclerosis may be improved. However, in the present study, the inflammatory environment of EAT was simulated using LPS-stimulated 3T3-L1 mature adipocytes, which differs from the actual situation in human EAT, which is the most marked limitation of the present research. Although numerous studies have suggested that APN is beneficial to atherosclerosis (9-11), other studies have reported a lack of association between APN levels and atherosclerosis in preclinical rodent models (49). Further research is required *in vivo* and *in vitro* to explore the role of APN in improving atherosclerosis.

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