Effect of peroxisome proliferator-activated receptor γ on the cholesterol efflux of peritoneal macrophages in inflammation

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Abstract. Atherosclerosis, a chronic inflammatory disorder characterized by lipid and cholesterol accumulation, is the principal contributing factor to the pathology of cardiovascular disease. Macrophages contribute to plaque development by internalizing native and modified lipoproteins that convert them into cholesterol-rich foam cells. With multiple factors, including hypercholesterolemia and inflammation, promoting atherosclerosis, it is of great significance to elucidate how the mechanism of cholesterol efflux from the macrophages changes and the role of peroxisome proliferator-activated receptor γ (PPARγ) in these situations. Following isolation and culture of peritoneal macrophages from C57BL/6 mice in the present study, the cells were divided into three groups: The control group, the ciglitazone group and the PPARγ antisense oligonucleotide group. The expression of PPARγ and nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α (IκBα) in each group was observed through the levels of protein and mRNA, and then the cholesterol efflux of each group was investigated. In addition, the same experiments were repeated following stimulation of each group with lipopolysaccharide (LPS). No significant difference in the expression levels of PPARγ between the control group and ciglitazone group was observed. The expression levels of PPARγ in the PPARγ antisense oligonucleotide group were evidently lower than those in the control group. Subsequent to stimulation with LPS, the expression levels of PPARγ in the three groups were higher than those of each group prior to stimulation. The cholesterol efflux of the PPARγ antisense oligonucleotide group was clearly suppressed following stimulation with LPS in comparison with that of the other groups. PPARγ contributes to anti-inflammation by protecting IκBα from being phosphorylated and degraded and promoting cholesterol efflux from peritoneal macrophages in inflammation.

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Introduction

Atherosclerosis, a chronic inflammatory disease of blood vessels, is one of the main causes of cardiovascular disease, which is the most common cause of mortality in industrialized societies and is increasingly becoming the leading cause of mortality worldwide (1). Atherosclerosis is induced by multiple factors and regulated by a number of genes (2). Macrophages, which are known to reside within atherosclerotic plaques, contribute to the pathology of atherosclerosis by internalizing native or modified lipoproteins or lipoprotein remnants that have invaded the vessel wall to form cholesterol-rich foam cells (3). As macrophages are one of the precursors of foam cells, their cholesterol counter transport system (cholesterol efflux) is important to maintain the balance of cholesterol in cells and influence the formation of foam cells (4). The conversion of macrophages into foam cells is orchestrated by disruption of the normal cholesterol homeostatic mechanism that controls the uptake, intracellular metabolism and efflux of cholesterol (5).

The peroxisome proliferator-activated receptor γ (PPARγ), a member of a superfamily of ligand-dependent transcription factors that regulate immunity and inflammation, is one of the nuclear receptors expressed in macrophages (6,7). Numerous studies have indicated that PPARγ and its ligands promote cholesterol efflux from macrophages through the PPARγ-liver X receptor α-ATP-binding cassette, sub-family A, member 1 signaling pathway (8), and this process may downregulate the expression of pro-inflammatory genes in macrophages that may be associated with the transrepression of the transcription factor nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) (9,10). A study concerning the anti-inflammatory effects of PPARγ has shown that its agonists markedly inhibit the secretion of pro-inflammatory mediators, including tumor necrosis factor-α and interleukin-1 and -6 in activated macrophages (11). However, the effect of PPARγ on the cholesterol efflux of macrophages in inflammation remains unclear. Pretreating wild-type mice with PPARγ ligands may reduce the expression of pro-inflammatory cytokines and alleviate injury of local and distant tissues (12), which

has a therapeutic effect in numerous inflammatory diseases, including acute myocarditis, autoimmune encephalitis and multiple sclerosis (13).

Atherosclerosis has been acknowledged as a consequence of lipid metabolism disorder and chronic inflammation (14). Thus, with multiple factors, including hypercholesterolemia and inflammation, promoting atherosclerosis either individually or in combination, it is of great significance to clarify how the mechanism of cholesterol efflux from macrophages changes and the role of PPARγ in these situations. This will help to explain the formation of foam cells and provide novel methods of preventing and curing atherosclerosis.

In view of the action of PPARγ on various key transcriptional factors, we proposed the hypothesis that PPARγ is the primary regulator of macrophage cholesterol efflux and suppressor of the inflammatory response. The present study aimed to provide evidence to elucidate the possible mechanism of PPARγ on the cholesterol efflux of peritoneal macrophages in inflammation and the role of PPARγ in maintaining the balance between the cholesterol efflux and anti-inflammatory response.

Materials and methods

Reagents and kits. LPS (*Escherichia coli*, O111:B4) was purchased from Sigma (St. Louis, MO, USA) and reconstituted in phosphate-buffered saline (PBS). PPARγ antibody (rabbit anti-mouse) and PPARγ antibody (goat anti-rabbit) were purchased from Sigma. Phosphor-nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α (IκBα; Ser32) was purchased from Youyizhonglian Bio-Corporation (Beijing, China). [H^3] cholesterol and apolipoprotein AI (ApoAI) were purchased from Sigma. Ciglitazone was purchased from Sigma, and the final concentration of ciglitazone dissolved in dimethylsulfoxide (DMSO) was 3 μmol/ml. The sequences of the PPARγ antisense and missense oligonucleotides were 5'-CATGAGGCTTATTGTAGAGCTGA-3' and 5'-GCCAGGTACCACTCACTCTGCAGT-3', respectively. The procedure of synthesis, purification and subpackage of the sequence was operated by Shenggong Bio-Corporation (Shanghai, China).

Animals. Fifteen C57BL/6 mice (8-10 weeks old, males, weighing 20-26 g) were obtained from the Laboratory Animal Centre of Chongqing Medical University (Chongqing, China). These mice were housed in an animal room and fed a standard diet. All experimental protocols described in this study were approved by the Ethics Review Committee for Animal Experimentation of Chongqing Medical University.

Experimental protocol. The 15 mice were randomly divided into three groups. Proceeding from isolation and culture of peritoneal macrophages from the C57BL/6 mice, the cells were divided into three groups: The control group, the ciglitazone group and the PPARγ antisense oligonucleotide group. The expression of PPARγ and IκBα in each group was observed through the levels of protein and mRNA, and then the cholesterol efflux of each group was investigated. The intraperitoneal injection of LPS into mice is a widely used method of constructing inflammatory animal models (15). In addition, the same experiment was repeated subsequent to stimulation of each group with LPS.

Table I. Primer sequences for RT-PCR.

DNA amplified	Primer sequence (5'→3')	Size (bp)
PPARγ		476
Sense	CAATCCGAATTTTTCAAGGGTGCCA	
Antisense	GAGCACCTTGGCGAACAGCTGAGAG	
β-actin		355
Sense	GAGAAGAGCTATGAACTTCCTGACG	
Antisense	TTTGCTGGAAGGTGGACAGAGAGGC	

RT-PCR, reverse transcription-polymerase chain reaction; PPARγ, peroxisome proliferator-activated receptor γ.

Isolation and treatment of peritoneal macrophages. Pre-cooled PBS (2 ml) was injected into the abdominal cavity of the mice, whilst the abdomen was kneaded softly for 2 min. The PBS was drawn out and collected, and then centrifuged for 10 min at 2,000 x g. The supernatant liquid was discarded and placed in RPMI-1640, which regulated the concentration of the cells at 3-5x10^6cells/ml. The cells were cultivated in 24-well plates at 37˚C for 2 h until they had adhered, then the cultivation holes were washed with pre-cooled PBS. The adherent cells were peritoneal macrophages. The peritoneal macrophages were cultivated for 24 h and then randomly divided into three groups: The control group (RMPI-1640+25 μl DMSO), the ciglitazone group (RMPI-1640+25 μl DMSO+ciglitazone; final concentration, 10 μmol/l), and the PPARγ antisense oligonucleotide group (RMPI-1640+25 μl DMSO+PPARγ antisense oligonucleotide; final concentration, 400 nmol/l). The final concentration of LPS was 80 ng/ml.

Immunocytochemical staining analysis of peritoneal macrophages. The peritoneal macrophages were cultured on a chamber slide, which was washed with PBS and air-dried. The slide was fixed with methanol for 30 min at -20˚C and then stained with PPARγ antibody (rabbit anti-mouse; Abcam, Cambridge, MA, USA) and PPARγ antibody (goat anti-rabbit; Abcam) for 24 h at room temperature. The cells that were stained purple were considered positive.

Assessment of gene expression of PPARγ by quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA samples of the peritoneal macrophages were extracted using an RNA extraction kit Takara Bio Inc. (Shiga, Japan) according to the manufacturer's instructions. Total RNA was quantified with the ratio of absorption values of RNA samples at 260 and 280 nm. Each total RNA sample was reversely transcribed to complementary DNA using an RT-PCR kit and stored at -70˚C. All PCR products were electrophoresed on 2% agarose gels. The RT-PCR was performed using the sense and antisense primers for PPARγ or β-actin (Table I). The relative expression of mRNAs were assessed by taking the ratio of the intensity of the DNA bands of PPARγ

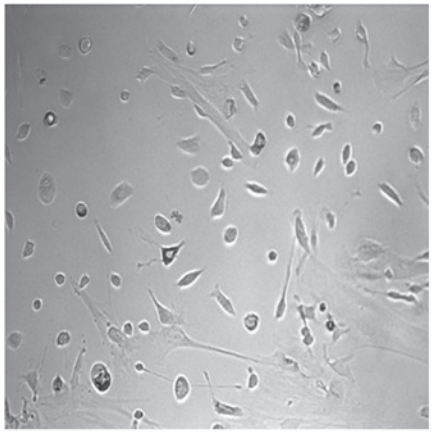

Figure 1. Normal peritoneal macrophages. Magnification, x200.

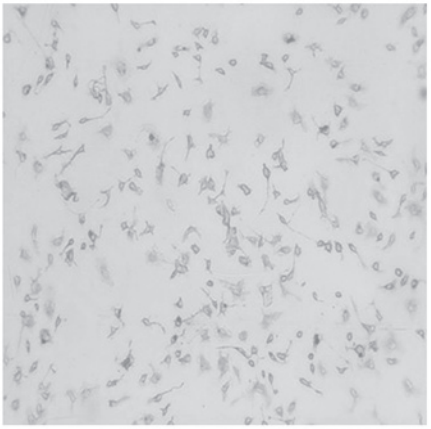

Figure 2. Immunocytochemical staining of PPARγ in peritoneal macrophages prior to stimulation by LPS. Magnification, x200. PPARγ, peroxisome proliferator-activated receptor γ; LPS, lipopolysaccharide.

to the β-actin band using the Bio-Image analysis system (Gel Doc 2000; Bio-Rad, Hercules, CA, USA) and expressed as arbitrary units.

Western blotting analysis of PPARγ and IκBα. Total protein of the peritoneal macrophages was extracted by homogenizing the macrophages in a cell lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China), then by two cycles of centrifugation at 12,000 x g for 15 min. Protein concentration was determined using a Bradford assay kit (Beyotime Institute of Biotechnology). The total protein was separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes, which were then incubated with rabbit anti-mouse PPARγ polyclonal antibody (diluted 1:1000, IMG-441; Sigma) and horseradish peroxidase-conjugated goat anti-rabbit IgG (diluted 1:2000; Zhongshan Jinqiao, Beijing, China). The immune complexes were developed with an Enhanced Chemiluminescence Detection kit (Pierce Biotechnology, Inc., Rockford, IL, USA) and the membranes were then immediately exposed to autoradiographic film (Kodak, Rochester, NY, USA). The relative amount of PPARγ protein was quantified from the optical density of the corresponding band by Bio-Image analysis system (Gel Doc 2000; Bio-Rad).

Determination of peritoneal macrophage cholesterol efflux. The concentration of macrophages was regulated at $3.0x10^9$ cells/l and the macrophages were transferred into RPMI-1640 containing fetal bovine serum, penicillin and streptomycin, and [^{3}H] cholesterol. After 24 h, the cells were cultivated in new medium containing 50 μg/ml ApoAI for 12 h. The [^{3}H]cholesterol in the culture solution and cells was detected by liquid scintillation counting. The effluxion of cholesterol was calculated using the following formula: [^{3}H] (culture solution)/[^{3}H] (culture solution and cells) x 100.

Statistical analysis. Data are reported as the mean ± standard deviation and were analyzed using one-way analysis of variance with Tukey's multiple comparison test, and the statistical program SPSS, version 11.0 (SPSS, Inc., Chicago, IL, USA). $P \leq 0.05$ was considered to indicate a statistically significant difference.

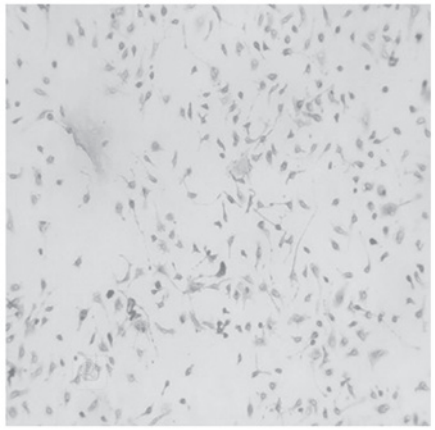

Figure 3. Immunocytochemical staining of PPARγ in peritoneal macrophages following stimulation by LPS. Magnification, x200. PPARγ, peroxisome proliferator-activated receptor γ; LPS, lipopolysaccharide.

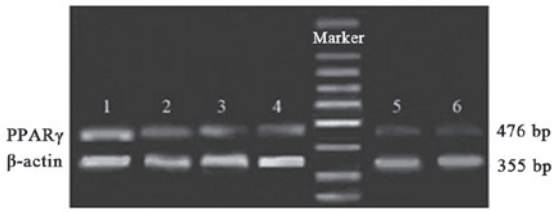

Figure 4. RT-PCR analysis of PPARγ mRNA expression in each group prior to and following stimulation with LPS. Group 1, stimulated ciglitazone group; group 2, unstimulated ciglitazone group; group 3, stimulated control group; group 4, unstimulated control group; group 5, stimulated AODN group; group 6, unstimulated AODN group. PPARγ, peroxisome proliferator-activated receptor γ; RT-PCR, reverse transcription-polymerase chain reaction; LPS, lipopolysaccharide; AODN, antisense oligonucleotide group.

Results

Immunocytochemical staining analysis of peritoneal macrophages. Peritoneal macrophages from C57BL/6 mice were isolated and cultured (Fig. 1). Subsequently, PPARγ in peritoneal macrophages prior to and following stimulation by LPS

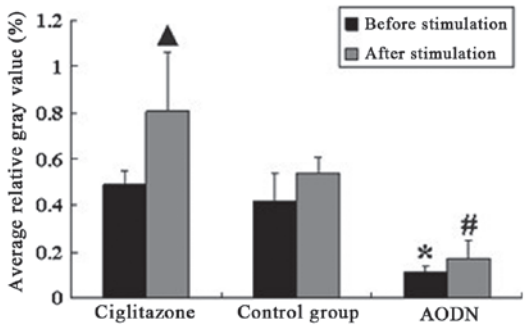

Figure 5. RT-PCR analysis of PPARγ mRNA expression in each group prior to and following stimulation by LPS. (*P<0.01 vs. unstimulated control group, ▲P<0.05 vs. stimulated control group, #P<0.01 vs. stimulated control group). Data are presented as the mean ± standard deviation. AODN, antisense oligonucleotide group; RT-PCR, reverse transcription-polymerase chain reaction; PPARγ, peroxisome proliferator-activated receptor γ; LPS, lipopolysaccharide.

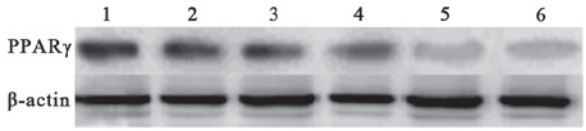

Figure 6. Expression of PPARγ protein in each group prior to and following stimulation with LPS. Group 1, stimulated ciglitazone group; group 2, unstimulated ciglitazone group; group 3, stimulated control group; group 4, unstimulated control group; group 5, stimulated AODN group; group 6, unstimulated AODN group. PPARγ, peroxisome proliferator-activated receptor γ; LPS, lipopolysaccharide; AODN, antisense oligonucleotide group.

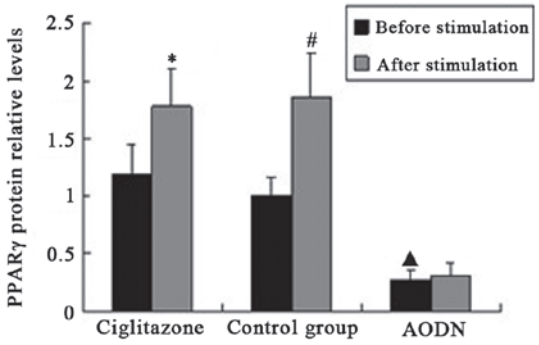

Figure 7. Expression of PPARγ protein in each group prior to and following stimulation with LPS. (*P<0.05 vs. the unstimulated ciglitazone group, #P<0.05 vs. unstimulated control group, ▲P<0.01 vs. unstimulated control group). Data are presented as the mean ± standard deviation. PPARγ, peroxisome proliferator-activated receptor γ; AODN, antisense oligonucleotide group; LPS, lipopolysaccharide.

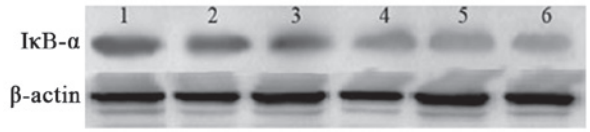

Figure 8. Expression of IκB-α protein in each group prior to and following stimulation with LPS. Group 1, unstimulated control group; group 2, unstimulated ciglitazone group; group 3, unstimulated AODN group; group 4, stimulated control group; group 5, stimulated ciglitazone group; group 6, stimulated AODN group. IκB-α, NF-κB inhibitor; LPS, lipopolysaccharide; AODN, antisense oligonucleotide group.

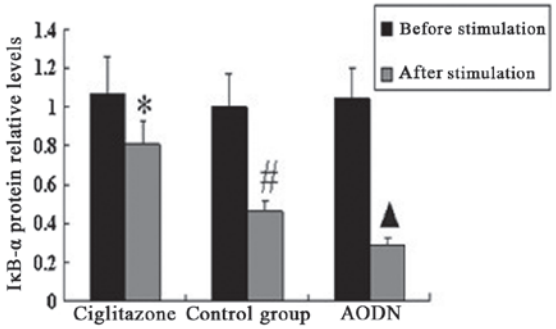

Figure 9. Expression of IκB-α protein in each group prior to and following stimulation with LPS. (*P<0.05 vs. unstimulated ciglitazone group, #P<0.05 vs. unstimulated control group, ▲P<0.01 vs. unstimulated AODN group). Data are presented as the mean ± standard deviation. IκB-α, nuclear factor κ-light-chain-enhancer of activated B cells inhibitor α; AODN, antisense oligonucleotide group; LPS, lipopolysaccharide.

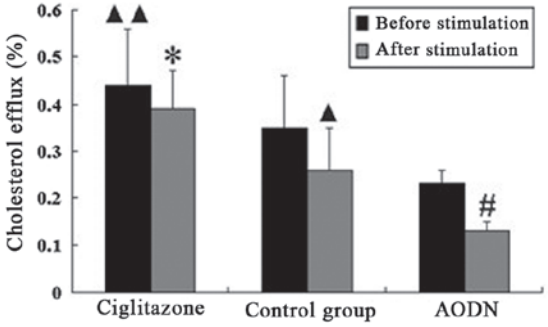

Figure 10. Cholesterol efflux of peritoneal macrophages in each group prior to and following stimulation with LPS. (*P<0.05 vs. unstimulated ciglitazone group, ▲P<0.05 vs. unstimulated control group, #P<0.05 vs. unstimulated AODN group, ▲▲P<0.05 vs. unstimulated control group). Data are presented as the mean ± standard deviation. AODN, antisense oligonucleotide group; LPS, lipopolysaccharide.

was examined using immunocytochemistry (Figs. 2 and 3). It was found that the number of PPARγ-positive cells following stimulation by LPS was greater than that prior to stimulation by LPS. The PPARγ-positive cells were stained purple.

Expression of PPARγ mRNA in peritoneal macrophages pre- and post-LPS stimulation. The results of the RT-PCR indicated that there was no significant difference in the average relative gray value between the control group and ciglitazone group. The average relative gray value of the PPARγ antisense oligonucleotide group was evidently lower than that of control group. Following stimulation with LPS, the expression levels of PPARγ mRNA in the ciglitazone group were higher than those in the control group, while the PPARγ mRNA expression levels of the PPARγ antisense oligonucleotide group were lower than those of the control group (Figs. 4 and 5).

Expression of PPARγ and IκBα protein in peritoneal macrophages pre- and post-LPS stimulation. The results of the western blotting suggested that there was no significant

Table II. Cholesterol efflux of the peritoneal macrophages in each group prior to and following stimulation with LPS (mean ± standard deviation, %).

Measurement	Ciglitazone	Control	AODN
Cholesterol efflux			
Prior to stimulation	0.44±0.12[d]	0.35±0.11	0.23±0.03
Following stimulation	0.39±0.08[a]	0.26±0.09[b]	0.13±0.02[c]
Suppression ratio	11.37	25.72	43.48

[a]P<0.05 vs. unstimulated ciglitazone group; [b]P<0.05 vs. unstimulated control group; [c]P<0.05 vs. unstimulated AODN group; [d]P<0.05 vs. unstimulated control group. LPS, lipopolysaccharide; AODN, antisense oligonucleotide group.

difference in the expression of PPARγ protein between the control group and ciglitazone group. The PPARγ protein expression levels of the PPARγ antisense oligonucleotide group were considerably lower than those of the control group. Subsequent to stimulation with LPS, the expression levels of PPARγ protein in the three groups were higher than those of each group prior to stimulation, and the IκBα protein expression levels of the three groups were lower than those of each group prior to stimulation (Figs. 6-9).

Cholesterol efflux of peritoneal macrophages in each group pre- and post-LPS stimulation. The cholesterol efflux of the ciglitazone group was suppressed following stimulation with LPS, and the suppression ratio was lower than that of the control group. However, the cholesterol efflux of the PPARγ antisense oligonucleotide group was greatly suppressed following stimulation with LPS, and the suppression ratio was higher than that of the control group (Table II, Fig. 10).

Discussion

It has been universally acknowledged that atherosclerosis is a disease associated with lipid metabolic disturbance and chronic inflammation (16). Foam cells form from macrophages, in which the cholesterol accumulation is the significant pathological characteristic of atherosclerotic lesions (17). The balance of cholesterol in macrophages depends on coordinated regulation of cholesterol, including intake, storage, *de novo* synthesis and efflux (18). As a thiazolidinedione, ciglitazone is a high-affinity ligand for PPARγ (19,20), and is able to activate PPARγ to suppress the activation of inflammatory cells and delivery of inflammatory mediators (21).

In the present study, measurement of the cholesterol efflux of peritoneal macrophages in each group indicated that pretreating macrophages with ciglitazone increases the cholesterol efflux. However, the cholesterol efflux was weaker in the PPARγ antisense oligonucleotide group compared with that in the control group. This demonstrates that PPARγ, when activated by its ligand ciglitazone, greatly reinforces the cholesterol efflux of peritoneal macrophages. In inflammation, the cholesterol efflux of the three groups was suppressed, but the suppression ratio varied. The cholesterol efflux of the PPARγ antisense oligonucleotide group was evidently suppressed following stimulation with LPS, and the suppression ratio was higher than that of the other two groups. The technique of knockdown using antisense nucleic acids was selected due to its benefits, which include strong specificity to target site, few side-effects and a precise depression effect (22). The results indicate that PPARγ is associated with the suppression of the cholesterol efflux resulting from LPS stimulation. This study demonstrated that stimulation of peritoneal macrophages with LPS suppresses the cholesterol efflux, even when the expression of PPARγ is upregulated, so it was presumed that the activation of PPARγ was affected due to its anti-inflammatory characteristic. When pro-atherosclerotic factors, including inflammation and hypercholesteremia coexist, the anti-inflammatory effect of PPARγ is of great significance.

The activation of NF-κB is an important signal transmission pathway that produces various pro-inflammatory factors (23). NF-κB consists mainly of the heterodimer p50/p65, which is generally bound to IκB, maintaining a state of inactivation in the cytoplasm (24,25). LPS binds to the corresponding receptor in the cytomembrane and leads to phosphorylation and degradation of IκB, and then NF-κB is released into the nucleus to promote the transcription of target genes (26). Certain studies have indicated that PPARγ may suppress several inflammation-correlated signaling pathways, including Janus kinase-signal transducer and activator of transcription, NF-κB, nuclear factor of activated T cell and activator protein 1, to express the anti-inflammatory effect (27-30). In order to explore the association between PPARγ and NF-κB in peritoneal macrophages in inflammation, the three groups in the present study were stimulated with LPS. The results indicated that the expression of IκBα was downregulated in each group by LPS, and the downregulation of IκBα in the PPARγ antisense oligonucleotide group was more significant than that in the other two groups. We consider PPARγ to be closely connected with NF-κB in peritoneal macrophages in inflammation, and PPARγ may produce anti-inflammatory effects by protecting IκBα from being phosphorylated and degraded in order to influence the activation and nuclear translocation of NF-κB.

In conclusion, the present study demonstrates that PPARγ performs a role in anti-inflammation by means of protecting IκBα from being phosphorylated and degraded and promoting cholesterol efflux from peritoneal macrophages in inflammation. As the understanding of the complex association between PPARγ and the cholesterol efflux from peritoneal macrophages in inflammation increases, there will undoubtedly be

an increasing number of opportunities to apply knowledge to the management and ultimately the prevention of atherosclerosis.

Acknowledgements

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