

## OX-LDL PLAYS DUAL EFFECT IN MODULATING EXPRESSION OF INFLAMMATORY MOLECULES THROUGH LOX-1 PATHWAY IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

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### 1. ABSTRACT

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) has been recognized to be the major endothelial receptor for oxidized low-density lipoprotein (ox-LDL). Ox-LDL has been reported to induce the expression of inflammatory adhesive molecules from vascular endothelium. However, the mechanism of this action has not been fully elucidated. Peroxisome proliferation-activated receptor-gamma (PPARgamma) regulates the expression of inflammatory adhesive molecules. The present study was carried out to investigate the role of LOX-1-PPARgamma pathway in regulating expression of adhesion molecules, ICAM-1 and E-selectin in HUVECs. Ox-LDL increased the expression of ICAM-1 and E-selectin in a concentration (10-50 microgram/ml)-and time (6-36 hours)-dependent manners. These effects were significantly inhibited by pretreatment of HUVECs with polyinosonic acid or carrageenan. Preincubating HUVECs with 15-deoxy-Delta(12,14)-PGJ2 (15d-PGJ2) attenuated the expression of ICAM-1 and E-selectin in response to ox-LDL, although ox-LDL stimulated the expression of PPARgamma. Upregulation of ICAM-1 and E-selectin mediated by ox-LDL were inhibited more significantly by the combination of 15d-PGJ2 and polyinosonic acid as compared to either 15d-PGJ2 or polyinosonic acid alone. The results suggested that ox-LDL through its receptor LOX-1 promotes pro-inflammation response by increasing expression of ICAM-1 and E-selectin, simultaneously activates PPARgamma triggering cellular anti-inflammation response in protection from the inflammation lesions in HUVECs.

### 2. INTRODUCTION

It has been widely accepted that inflammation plays a central role in the development, progression, and outcomes of atherosclerosis (1-2). Atherosclerosis development begins with endothelial cell activation, including overexpression of leukocyte adhesion proteins. Triggering of this inflammatory response include oxidized low density lipoproteins (Ox-LDL). Several lines indicated that uptake of ox-LDL by vascular endothelial cells has been demonstrated to be a critical step for the endothelial activation, initiation and development of atherosclerotic processes (3-4). The studies have evidenced that ox-LDL enhanced the expression of adhesive molecules, such as E-selectin, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule (ICAM-1) and monocyte chemotactic protein-1 (MCP-1) in endothelial cells (5-6). However, the mechanism by which ox-LDL increases the expression of adhesive molecules in vascular endothelium remains to be elucidated.

Lectin-like receptor for ox-LDL (LOX-1) is a novel receptor for ox-LDL. Previous studies have suggested that the endothelial uptake of ox-LDL seems to depend on this cell-surface receptort (7). Recently, studies have suggested the possibility that LOX-1 may transmit some intracellular biological signals after uptake of its ligand ox-LDL and result in a series of intracellular transcription including secreting adhesion molecules (8). LOX-1 can be upregulated by pro-athromatous stimuli, such as TNF-alpha, angiotensin II, ox-LDL and fluid shear stress (9-10). It was evidenced that overexpression of endothelial LOX-1 occurred during the

early stage of atherosclerosis before intima accumulation of macrophage foam cells (11). This indicated that LOX-1 might be involved in the pathogenesis of endothelial activation. Furthermore, ox-LDL inducing the overexpression of adhesive molecules of endothelium may be related to the mediation of LOX-1. If LOX-1 plays a determinant role in ox-LDL inducing the expression of adhesive molecules from endothelium as upstream regulation, what is the downstream pathway of the endothelial activation induced by ox-LDL?

Peroxisome proliferator-activated receptors (PPARs) are member of the nuclear receptor superfamily of ligand-activated transcription factors. Recently, it has been considered that PPARs play an important role in inflammatory response. Beyond the improvement of insulin resistance in syndrome X (diabetes mellitus, hypertension, obesity and so on), PPARs have been involved in a variety of pathological conditions, including atherosclerosis, cancer and chronic inflammation, and have multiple metabolic functions increasingly recognized cardiovascular effects (12-13). PPAR $\gamma$  is an important member of PPARs superfamily, and expressed by macrophages, vascular endothelial cells, and smooth muscle cells (14). It has recently been demonstrated that PPAR $\gamma$  negatively modulate nuclear factor (NF)- $\kappa$ B signaling pathways and downregulate inflammatory response in monocyte/macrophages, smooth muscle cells and endothelial cells (15). Treatment of human vascular endothelium with 15d-PGJ2 or its synthetic PPAR $\gamma$  ligand reduced the expression of inducible inflammatory molecules (16). In general, PPAR $\gamma$  ligand exerts anti-atherosclerotic effects, and its effect was considered to be involved in the inhibition of LOX-1 and the adhesion of monocytes to endothelium (17). This indicated that PPAR $\gamma$  might participate in the vascular endothelial activation mediated by LOX-1 receptor.

The present study is to determine whether LOX-1 mediates the vascular endothelial activation response, or mediates the adhesive molecules expression from endothelial cells treated by ox-LDL; and further to investigate the possible role of LOX-1-PPAR $\gamma$  signaling pathway in this process.

### 3. Materials and Methods

#### 3.1. Endothelial cells isolation and culture

The normal placental umbilical cords were obtained from the First Affiliated hospital of Sun Yat-sen University (Guangzhou). The cords were warmed to 37°C in incubator prior to use. Areas with varicosity and clamp marks in the vein were removed. The vein was cannulated with sterile blunt needles. The needle was fixed over the cord with hemostat. The vein was perfused with sterile PBS (0.01mmol/L, pH7.6) until the filtrate was achromatous. Umbilical vein was filled with trypsin-EDTA (0.25% trypsin:0.02% EDTA=1:1 (V/V)) and incubated for 15 min at 37°C. The cord was massaged and squeezed gently to help cells detachment and trypsin-EDTA solution was flushed from the cord by perfusion with 20 ml of PBS. The harvest cells were cultured in a humidified incubator at 37°C and 5% CO<sub>2</sub> with PRMI-1640 supplement with 20% fetal calf serum

(FCS), 2 mM L-glutamine, 100U/ml penicillin, 100 microgram/ml streptomycin and 2g/L bicarbonate. Medium was first replaced at 12 hours and every 2 days following until confluence (4 days). HUVECs was identified by its cobblestone morphology under phase-different microscopy, its staining for factor VIII and presenting of W-P bodies under electron-microscopy. The cells were serially passaged and the forth- or fifth-generation of HUVECs were used in the following experiment.

#### 3.2. Lipoprotein preparations

Human LDL (1.01 to 1.06 mg/ml) was isolated from the plasma of healthy human subjects by sequential ultracentrifugation at 4°C. LDL was dialyzed against 3 changes of LDL buffer (150mmol/L NaCl; 0.24mmol/L EDTA, pH 7.4) for 36 hours at 4°C, filtered through 0.45 micron filters and stored at 4 °C. After extensive dialysis against 3 changes of PBS for 36 hours at 4°C, LDL was modified by incubating with 5.0 micromol/L CuSO<sub>4</sub> at 37 °C for 18 hours. And dialyzed against 3 changes of LDL buffer for 36 hours at 4 °C. Oxidation of LDL was determined by the amount of lipid peroxides by measuring the thiobarbituric acid-reactive substances (TBARS). Values for TBARS in native LDL and ox-LDL were 0.86 nmol MDA /100 microgram protein and 9.66 nmol MDA /100 microgram protein respectively. Protein concentrations were determined by the method of Lowry's method.

#### 3.3. Measurement of LDH release and evaluation of cell viability

Lactate dehydrogenase activity was measured in endothelial cell cultures treated with ascending doses of ox-LDL (0, 10, 20, 50, 100, 200 microgram/ml) for 24 hours according to a spectrophotometric enzyme activity method based on the oxidation of lactate was used (Sigma). LDH activity was expressed as units per milligram of protein (16).

A small of aliquote of cells was incubated in 0.1% trypan blue for a few minutes, and the cells were viewed under a phase-different microscope. Dead cells are permeable to trypan blue and thus become colored, whereas viable cells do not take up the dye. By counting 100 cells, the percentage of viable cells was calculated (16).

#### 3.4. RNA extraction and cDNA synthesis

Total cellular RNA was isolated from HUVECs by using TRIzol reagents (GIBCO-BRL). The amount of RNA was determined by measuring the absorbance at 260 nm in a spectrophotometer (BECKMAN COULTER). The integrity of RNA was confirmed by electrophoresis on a 0.1% agarose gel containing formaldehyde. Complementary DNA (cDNA) was reverse transcribed from 2 microgram total RNA. The total RNA was reverse-transcribed with oligodT and using SuperScript™ reverse transcriptase (Invitrogen life technologies) primed with radom hexamers.

#### 3.5. Real time PCR assay for PPAR $\gamma$ and LOX-1

Oligonucleotide primers and dye-labeled probes for PPAR $\gamma$  and LOX-1 were designed according to the guidelines of Applied Biosystems with help of Primer express Software, version 2.0 (Perkin-Elmer/PE Applied Biosystems, Foster City, CA) and sequences from the GenBank database

(accession no. X90563 for PPARgamma and NM\_002543 for LOX-1). The sequences were as follows: PPARgamma forward primer, 5'-ATGCCACAGGCCGAGAAG-3'; Reverse Primer: 5'-GGATTACAGTGGTCGATATCACT-3'. Probe: 5'-FAM-AGAAGCTGTTGGCGGAG-MGB-3'. LOX-1 forward Primer: 5'-ACCACCAGAATCTGAATCTCCAA-3' Reverse Primer: 5'-TTGCGGACAAGGAGCTGAAC-3' Probe: 5'-FAM-AAACACTGAAGAGAGTAGC-MGB-3'. Primers and probe were custom synthesized by Daan Gene Co., Ltd (Guangzhou, China, <http://www.daangene.com/english>). The probe was labeled at the 5' end with the reporter dye FAM and at the 3' end with the quencher MGB. PCR was carried out on an ABI 7700 sequence detection system (Perkin-Elmer/PE Applied Biosystems). Each 50 microliters reaction contained 5 microliters of first strand cDNA, 10 microliters of 5×PCR master mix (ABI), 1 microliters of 25 micromol/L of each forward and reverse primer, 1 microliters of 20 micromol/L FAM labeled probe, 1 microliters of 10 mM deoxynucleoside triphosphates dATP, dCTP, dGTP, and dUTP dNTP, 4U Taq DNA polymerase and 29 microliters of ddH<sub>2</sub>O. All reactions were carried out under the amplification profile of an initial denaturation at 93°C for 2 min followed by 40 cycles of 93°C for 1 min and 55°C for 1 min. At the end of each extension phase, fluorescence was observed and used for quantitative measurements within the linear range of amplification. Exact quantification was achieved by a serial dilution with cDNA produced from endothelial total RNA extracts using 1:5 dilution steps. Gene expression levels were then given as the ratio of the gene of interest (beta-actin) versus a stable expressed housekeeping gene.

### 3.6. PCR for ICAM-1, and E-selectin

The primers specific to ICAM-1 were: forward: 5'-GTCCCCCTCAAAAGTCATCC-3', reverse: 5'-AACCCCATTCAGCGTCACCT-3'. E-selectin primer were: forward: 5'-GGGACAACGAGAAGCCAACA-3', reverse: 5'-CCGAAGCCAGAGGAGAAATG-3'. We used beta-actin as the internal control. Its primers were: forward: 5'-GGACTCCTATGTGGGTGACGAGG-3', reverse: 5'-GGGAGAGCATAGCCCTCGTAGAT-3'. Every 25 microliters PCR reaction contained 2.5 microliters of 10×PCR buffer, 0.75 microliters of 50 mM MgCl<sub>2</sub>, 0.5 microliters of 10 mM dNTP mix, 2 microliters cDNA, 1 microliters of each forward and reverse primer, 1 unit of Taq DNA polymerase, 17.05 microliters of autoclaved distilled water. 5 microliters aliquot of each PCR reaction mixture was electrophoresed on a 1.5% agarose gel with ethidium bromide staining. Gels were photographed on a transilluminator (VILBER LOURMAT).

### 3.7. Western blotting experiments

Equal total protein or nuclear protein (50 microgram per lane) extracts from cultured endothelial cells were denaturated at 97 °C for 7 min, followed by sodium dodecylsulfate polyacrylamide gel electrophoresis on 10% polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (PVDF). The membrane was probed overnight at 4°C with 1:200 dilution of goat monoclonal antibody against human LOX-1, PPARgamma, ICAM-1, or E-selectin. After washing with TBST containing 0.1% (V/V) tween20, the membrane was incubated with 1:5000 dilution of horseradish peroxidase-labeled-anti-goat immunoglobulin for 1 hour, washed with TBST containing 0.1% (V/V) Tween 20, and then detected by western blotting luminor reagents

(Santa Cruz Biotechnology). Densitometric scanning was performed to quantify the amounts of target protein using NIH Image.

### 3.8. Data analysis

All data represent the mean results from 3 to 4 independently performed experiments. Data are presented as mean ± SD. Statistical significance was determined by multiple one-way ANOVA followed by a post hoc test using SPSS analysis program (version 10.0). A probability value ≤0.05 was considered significant.

## 4. RESULTS

### 4.1. Cell culture

Cell morphology has not been found altered, but cellular toxicity, as marked by LDH activity, were observed following treatment of cultures with ascending dose of ox-LDL (10 to 200 microgram/ml). Incubation of HUVECs with ox-LDL (10-200 microgram/ml) increased LDH leakage into culture medium. These results were consistent with the change in viability of cells treated with ox-LDL. It was showed that ox-LDL at dose 200 microgram/ml induced significant cell lesion indicating by higher LDH leakage and reduced cell viability. However, ox-LDL below 100 microgram/ml only cause minor cell lesion. Based on the LDH activity and the cellular viability, we used 100 microgram/ml ox-LDL as the highest tolerable noncytotoxic dose in the following experiments.

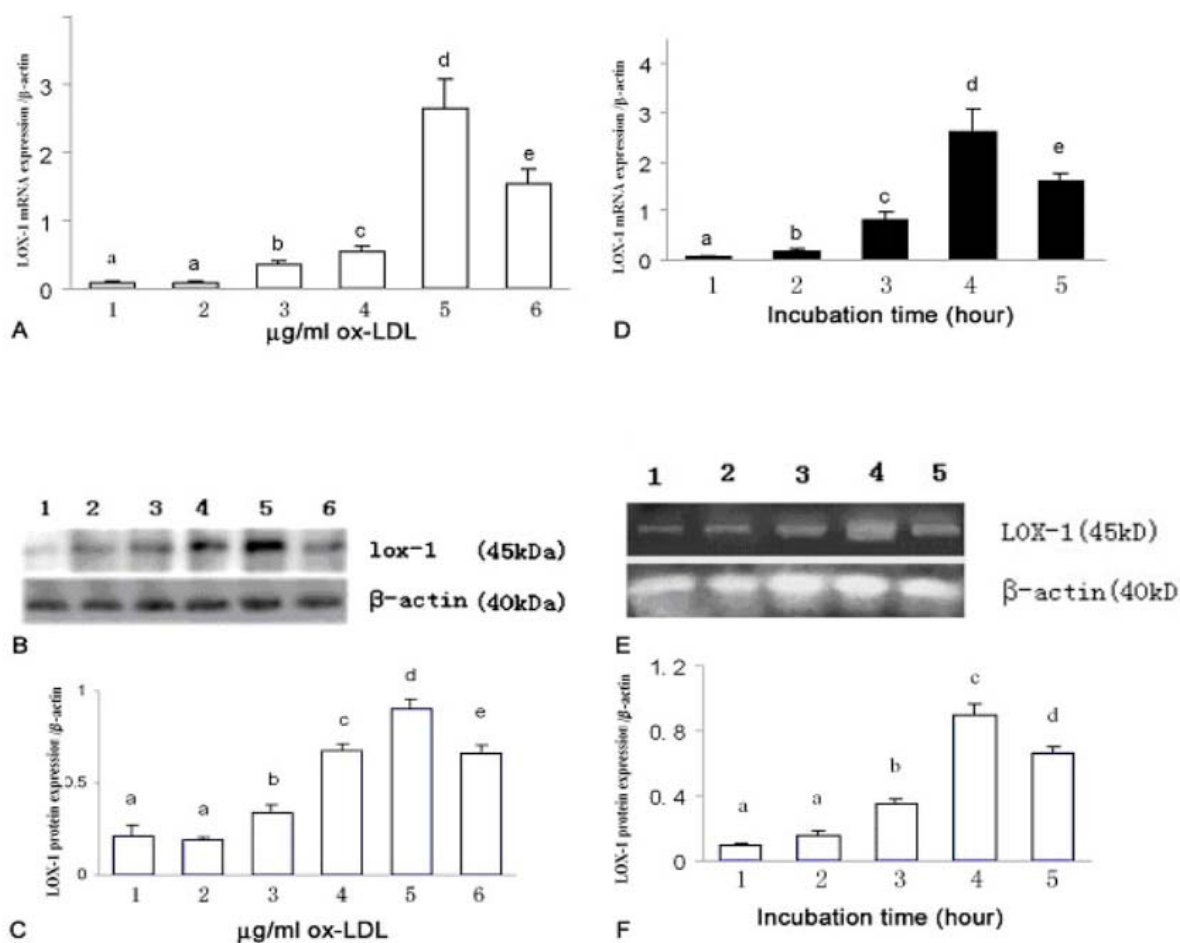
### 4.2. Ox-LDL increase LOX-1 expression in endothelial cells

Incubation of HUVECs with ox-LDL (0, 10, 20, 50, 100 microgram/ml), LOX-1 mRNA and protein were examined by real time RT-PCR and Western blot respectively. Ox-LDL increased the expression of LOX-1 (mRNA and protein) in a concentration-dependent manner (Figure 1). In contrast, native LDL (50 microgram/ml) did not affect the expression of LOX-1. Incubation of HUVECs with 50 microgram/ml ox-LDL in different time (0, 6, 12, 24 and 36 hours) also increased the expression of LOX-1 (mRNA and protein) in a time-dependent manner (Figure 1). The maximal response of LOX-1 expression was found in the cells treated with ox-LDL at 50 microgram/ml for 24 hours.

Incubation of HUVECs with 250 microgram/ml of Poly(I) or carrageenan (both are LOX-1 chemical bloker) did not altered the expression of LOX-1 (data not show), however, pre-incubating HUVECs with 250 microgram/ml of Poly(I) or carrageenan and then with 50 microgram/ml of ox-LDL, the increased expression of LOX-1 induced by ox-LDL were significantly decreased (Figure 2).

### 4.3. Effects of ox-LDL on ICAM-1 and E-selectin expression by HUVECs

To investigate the effects of ox-LDL on secretion of adhesive molecules in endothelium, we analyzed the mRNA and protein expression of ICAM-1 and E-selectin in endothelial cells. Incubation of HUVECs with ox-LDL (10-100 microgram/ml) enhanced the expressions of ICAM-1 and E-selectin in a concentration-dependent manner (Figure 3). But on the contrary, ox-LDL did not affect the expression of VCAM-1 by HUVECs.

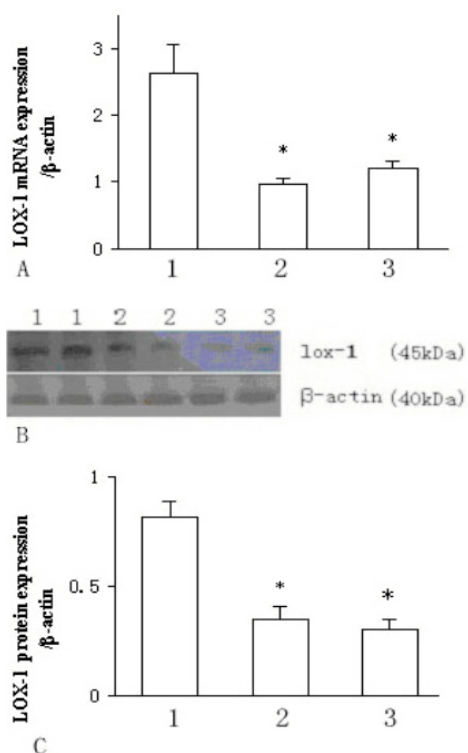


**Figure 1.** The influence of concentration and incubating time of ox-LDL on LOX-1 expression in HUVECs. (A,D) A real-time RT-PCR analysis of LOX-1 mRNA. The amounts of LOX-1 mRNA was indicated by copies of LOX-1 mRNA (per microgram of total RNA)/copies of beta-actin (per microgram of total RNA). (B,C,E,F) Western blot determined expression of LOX-1 protein. Each protein band was normalized with beta-actin band. Ox-LDL increased the expression of LOX-1 mRNA and protein in a concentration-dependent manner. Data from three different experiments in mean  $\pm$  S.D. In the bar chart, different letter on the top of each bar indicates  $P < 0.05$ . In figure 1A, 1B and 1C, the number of 1, 2, 3, 4, 5, 6 indicates group of 50 microgram/ml n-LDL and 0, 10, 20, 50, 100 microgram/ml ox-LDL respectively. In figure 1D, 1E and 1F, the number of 1, 2, 3, 4, 5 indicates group of 0, 6, 12, 24, 36 hours respectively.

#### 4.4. LOX-1/PPARgamma signaling pathway affects the expression of ICAM-1 and E-selectin induced by ox-LDL in HUVECs

To investigate whether the expression of ICAM-1 and E-selectin induced by ox-LDL was mediated by LOX-1, HUVECs were pretreated with 250 microgram/ml poly(I) or 250 microgram/ml carrageenan (both are LOX-1 chemical blocker) for 2 hours, and then incubated with 50 microgram/ml ox-LDL for another 24 hours. Both poly(I) and carrageenan obviously decrease expression of ICAM-1 and E-selectin induced by ox-LDL (Figure 4 and Figure 5). As control, 250 microgram/ml poly(I) or 250 microgram/ml carrageenan did not alter the expression of ICAM-1 and E-selectin (data not show). The results indicated that expression of ICAM-1 and E-selectin induced by ox-LDL maybe partially mediated by LOX-1.

To determine the mechanism of ICAM-1 and E-selectin expression elicited by ox-LDL mediated by LOX-1, we explore the role of PPARgamma pathway. Ox-LDL increased PPARgamma expression by HUVECs. Ox-LDL-induced PPARgamma upregulation was inhibited by pretreatment of HUVECs with LOX-1 blockers (Poly(I) and carrageenan). The results indicated that the expressions of PPARgamma induced by ox-LDL were also mediated by the endothelial receptor-LOX-1 (Figure 6). Pretreatment of HUVECs with 10 mM PPARgamma agonist, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J2 (15d-PGJ2) for 12 hours and then with ox-LDL at 50 microgram/ml for 24 hours. The expression of LOX-1, ICAM-1 and E-selectin were decreased significantly, but PPARgamma expression increased. The results indicated that 15d-PGJ2 attenuated the up-regulation of LOX-1 and adhesive molecules induced by ox-LDL. To



**Figure 2.** Effects of Poly(I) or carrageenan (both are LOX-1 chemical blockers) on expression of LOX-1 induced by ox-LDL. Both 250 microgram/ml Poly(I) and carrageenan did not altered the expression of LOX-1, but significantly reduced the expression of LOX-1(mRNA and protein) induced by ox-LDL. Data from three different experiments in mean  $\pm$  S.D (\* $P < 0.05$  vs group of 50 microgram/ml ox-LDL). The number of 1,2 and 3 indicate group of 50 microgram/ml ox-LDL, 50 microgram/ml ox-LDL+250 microgram/ml Poly(I) and 50 microgram/ml ox-LDL+250 microgram/ml carragenan respectively.

attempt to clarify the combined effects of PPARgamma agonist and LOX-1 blocker, HUVECs were pretreated with 15d-PGJ2 and poly(I) and then with ox-LDL. Combination of 15d-PGJ2 and poly(I) inhibited the expression of ICAM-1 and E-selectin more significant than the inhibition responded to 15d-PGJ2 or poly(I) alone.(Figure 4 and Figure 5)LOX-1 and adhesive molecules induced by ox-LDL. To attempt to clarify the combined effects of PPARgamma agonist and LOX-1 blocker, HUVECs were pretreated with 15d-PGJ2 and poly(I) and then with ox-LDL. Combination of 15d-PGJ2 and poly(I) inhibited the expression of ICAM-1 and E-selectin more significant than the inhibition responded to 15d-PGJ2 or poly(I) alone.(Figure 4 and Figure 5)

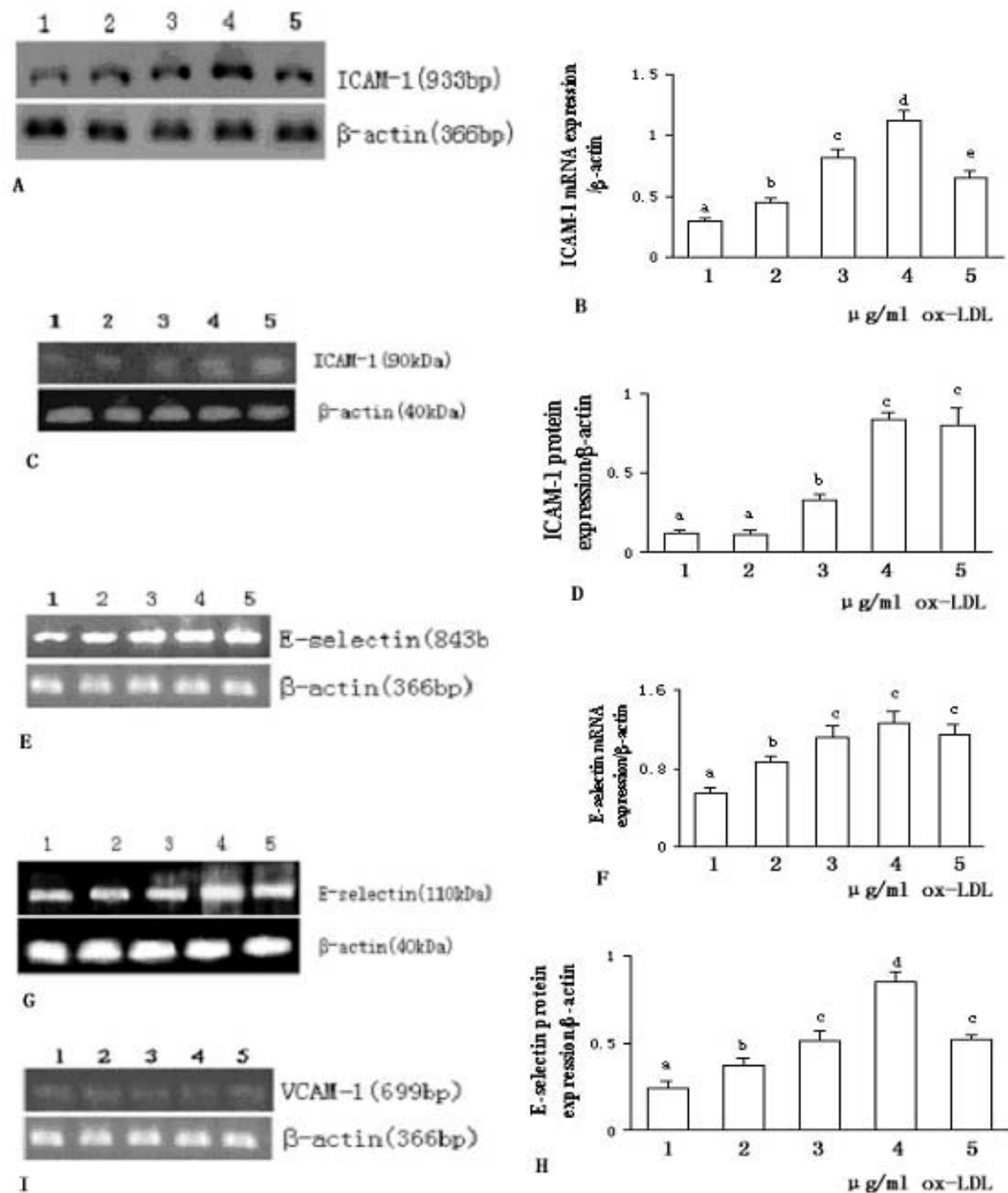
## 5. DISCUSSION

It is suggested that monocyte recruitment into early atherosclerotic lesions depends on the endothelial adhesiveness for monocytes and lymphocytes (19). In vivo and in vitro experiments have identified molecules, such as ICAM-1, VCAM-1, and P-selectin (19). Oxidized LDL,

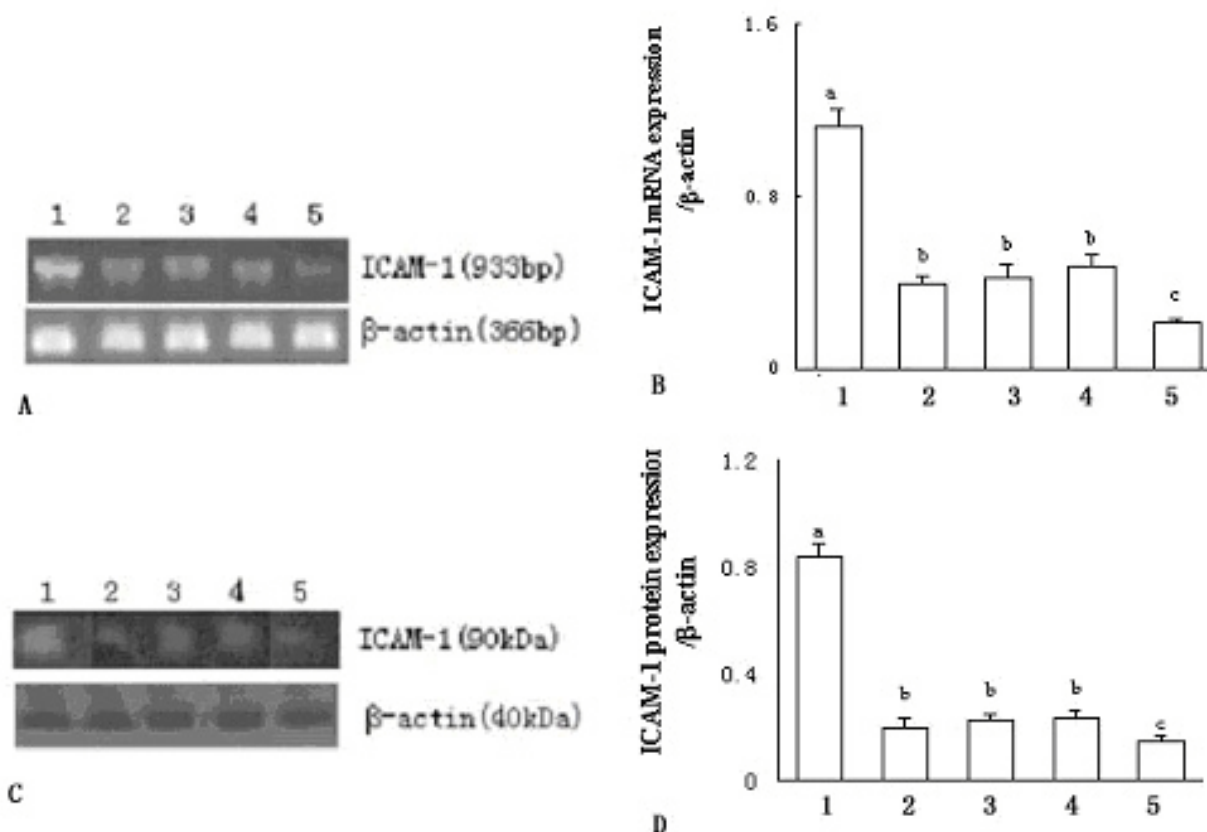
lysophosphatidyl-choline, and oxidized fatty acids induce the expression not only of these adhesion molecules but also of scavenger receptors, such as CD-36, SR-A, and LOX-1 (20). Recent studies (21-22) by several investigators suggested the possibilities that LOX-1 may transmit some intracellular biological signals after the binding or internalization of its ligand ox-LDL. Ox-LDL binding and uptake by LOX-1 in BAEC induces cellular oxidative stress and thereby activates a transcription factor, NF-kappa, which elicits proinflammatory gene transcription (23). Our results confirmed that ox-LDL induced LOX-1 expression, increased the expression of vascular endothelial adhesive molecules, ICAM-1 and E-selectin. The inducible expression of LOX-1 in endothelium by ox-LDL showed a concentration (0-50 microgram/ml) and time (0-24 hr) dependent manner. The use of Poly(I) or carrageenan did not alter the expression of LOX-1 and adhesive molecules in present study. However, exploring specific LOX-1 chemical bloke, Poly(I) or carrageenan, resulted in about 60% inhibition of LOX-1 expression induced by ox-LDL. Furthermore, antagonists of LOX-1 also inhibit (about 70-80%) the expression of ICAM-1 and E-selectin in both protein and mRNA from endothelial cells. It clearly indicated the inducible expression of adhesive molecules, ICAM-1 and E-selectin in the vascular endothelial cell treated by ox-LDL was mediated by LOX-1. As several studies reported that ox-LDL could exerted its atherogenic pathogenesis via several receptor, such as scavenger receptor, CD36 (20). The present study showed that LOX-1 contributed to a major role (more 50%) in mediating the inducible expression of the adhesive molecules from endothelial cells stimulated by ox-LDL.

Although PPARgamma is expressed predominantly in adipose tissue (24), it is also expressed in kidney, intestine and retina (25). More recently, PPARgamma is shown to be expressed in both human monocyte/macrophage and vascular endothelial cells (26) where it appears to participate in the control of the inflammatory response by affecting endothelium function. In this report we show that PPARgamma is remarkably expressed in HUVECs activated by ox-LDL accordingly with the up-regulation of LOX-1 and adhesion molecules, ICAM-1 and E-selectin expression. However LOX-1 inhibitors, poly(I) and carrageenan decreased the up-regulation of PPARgamma simultaneously with the attenuating expression of LOX-1, ICAM-1 and E-selectin.

As a ligand, ox-LDL was taken up by LOX-1 on the surface of endothelial cells. The binding of ox-LDL to LOX-1 led to the internalization and proteolytic degradation inside cytoplasm by various enzymes and then release of 9-HODE and 13-HODE (27). Nagy (28) demonstrated that both of the oxidized metabolites of ox-LDL, 9-HODE and 13-HODE were able to bind and activate PPARgamma, function as pathologic endogenous ligands for PPARgamma. Elevation of both cellular 9-HODE and 13-HODE serve as natural ligand for PPARgamma activation. This pathway might explain that ox-LDL up-regulated PPARgamma expression mediated via LOX-1 receptors.



**Figure 3.** The effects of ox-LDL on intercellular adhesion molecule-1 (ICAM-1) and E-selectin expression by HUVECs. HUVECs were incubated at 37°C for 24 h with medium alone or medium containing different concentration of ox-LDL (10-100 microgram/ml). Ox-LDL increased the expression of ICAM-1 and E-selectin (mRNA and protein) in a concentration-dependent manner. Expression of VCAM-1 was not altered by ox-LDL. In the figures, different letter on the top of each bar indicates  $P < 0.05$  and the same letter means  $P > 0.05$  between them. The number of 1, 2, 3, 4 and 5 indicate group of 0, 10, 20, 50 and 100 microgram/ml ox-LDL respectively.



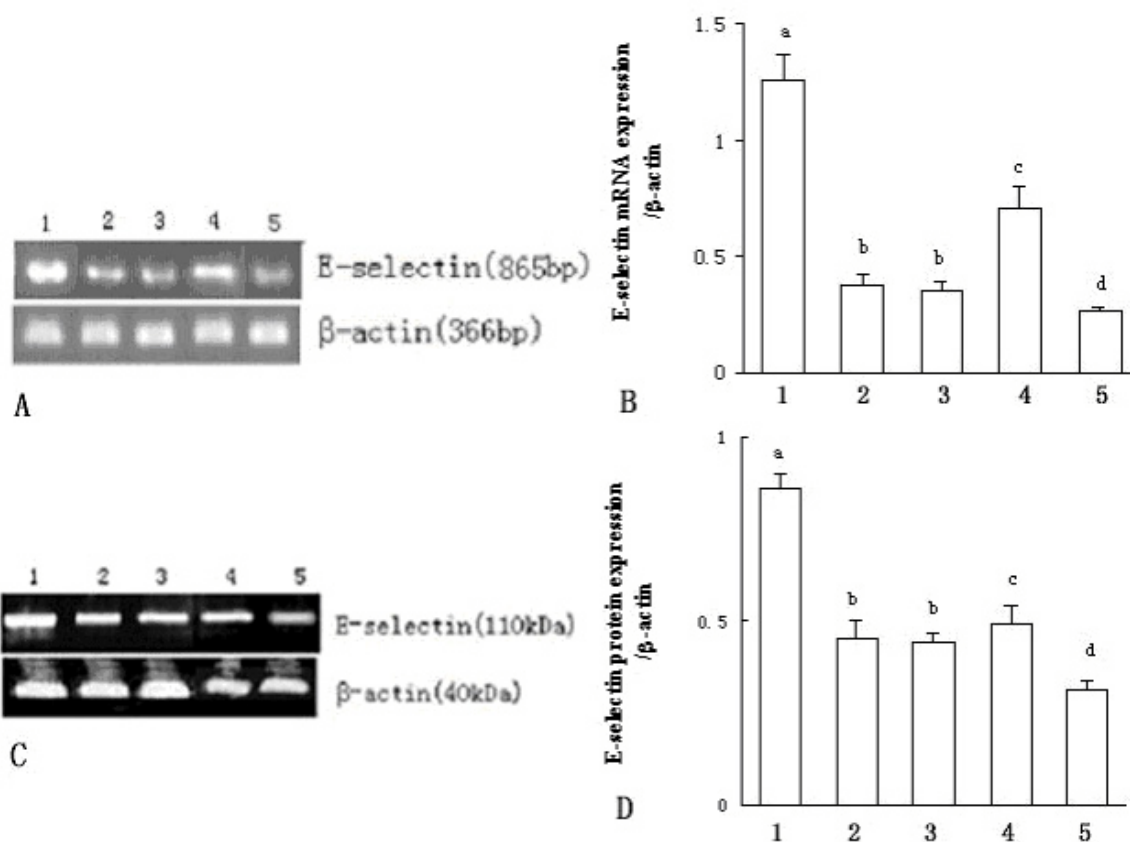
**Figure 4.** The effects of poly(I), carrageenan or 15d-PGJ2 alone and the combination effects of 15d-PGJ2 and poly(I) on the expression of ICAM-1. Pretreatment of HUVECs with 250 microgram/ml poly(I) or 250 microgram/ml carrageenan respectively for 2 h or 10mM 15-deoxy- $\Delta^{12,14}$ -prostaglandin  $J_2$  (15d-PGJ2) for 12 h alone or with 250 microgram/ml of poly(I) prior with 50 microgram/ml of ox-LDL for 24 hours. mRNA and protein expression of ICAM-1 were determined. Poly(I), carrageenan and 15d-PGJ2 inhibited the expression of ICAM-1, but the combined inhibited effects of poly(I) and 15d-PGJ2 were more significant in inhibiting ICAM-1 than that responded to 15d-PGJ2 or poly(I) alone. To normalized signals, beta-actin mRNA and protein levels were compared as an internal control. The means of four independent determination  $\pm$  SD are shown in each column. (Different letter on the top of each bar indicates  $P < 0.01$  between them). The number of 1,2,3,4 and 5 indicate the group of 50 microgram/ml ox-LDL, 50 microgram/ml ox-LDL+250 microgram/ml Poly(I), 50 microgram/ml ox-LDL+250 microgram/ml carrageenan, 50 microgram/ml ox-LDL+10mM15d-PGJ2 and 50 microgram/ml ox-LDL+250 microgram/ml Poly(I)+ 10 mM15d-PGJ2).

PPARGamma participated in various pathophysiological activities. It was reported that activation of PPARGamma receptors inhibits atherosclerosis. The involved mechanism of this action was related to its anti-inflammatory property. PPARGamma ligands inhibit a variety of inflammatory actions in macrophage including expression of inducible nitric oxide synthase (iNOS), superoxide dismutase, gelatinases, matrix-metalloproteinases, and several interleukins (20,30). It was reported that PPARGamma inhibited LOX-1 expression and the adhesion of monocytes to endothelium (31). When PPARGamma expression activated or elevated, the inflammatory gene including LOX-1 could be inhibited consequently. In present studies, agonist of PPARGamma inhibited the expression LOX-1 and adhesive molecules from endothelium, which is consistent with the previous studies that

PPARGamma plays important role in attenuating inflammatory response.

Ox-LDL as a proatherogenic agent stimulated the inflammatory action, indicated by promotion expression of inflammatory agents, such as VCAM-1 (32). On the other hand, ox-LDL could induce protective factor against the lesion caused by itself. As mentioned above, activation of PPARGamma is considered as protective factor against inflammation. Ox-LDL not only caused the vascular damage, but also activated the protective factor, such as PPARGamma, by which body could attenuate the damage caused by ox-LDL. In the present study, ox-LDL stimulated the expression of inflammatory factors via LOX-1 pathway. In addition, ox-LDL promoted the activation of PPARGamma, which consequently reduced the expression of ICAM-1 and E-selectin.



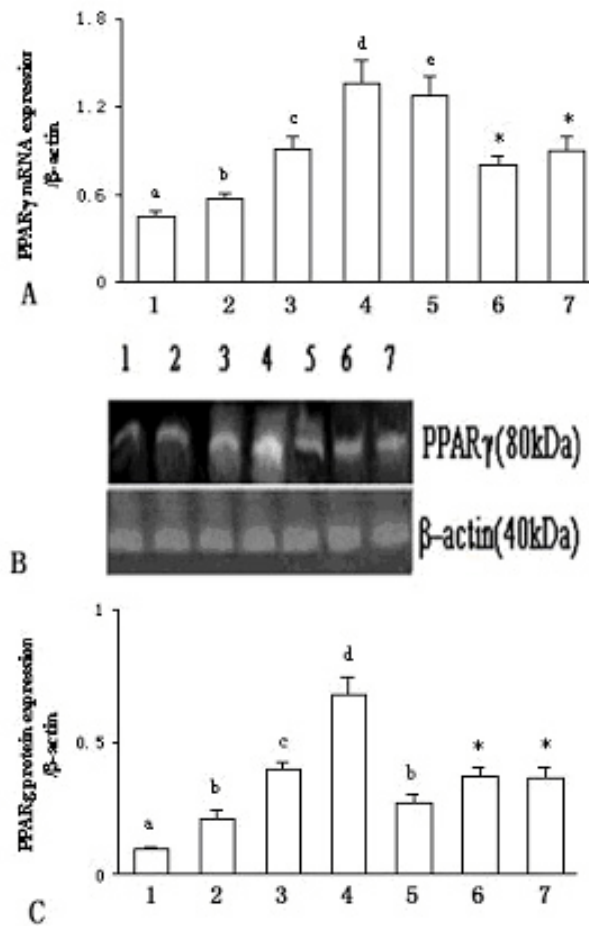


**Figure 5.** The effects of poly(I), carrageenan or 15d-PGJ2 alone and the combination effects of 15d-PGJ2 and poly(I) on the expression of E-selectin. Pretreatment of HUVECs with 250 microgram/ml poly(I) or 250 microgram/ml carrageenan respectively for 2 h or 10mM 15-deoxy- $\Delta^{12,14}$ -prostaglandin J2 (15d-PGJ2) for 12 h alone or with 250 microgram/ml of poly(I) prior with 50 microgram/ml of ox-LDL for 24 hours. mRNA and protein expression of E-selectin were determined. Poly(I), carrageenan and 15d-PGJ2 inhibited the expression of E-selectin, but the combined poly(I) and 15d-PGJ2 were more significant in inhibiting E-selectin than that responded to 15d-PGJ2 or poly(I) alone. To normalized signals, beta-actin mRNA and protein levels were compared as an internal control. The means of four independent determination  $\pm$  SD are shown in each column. (Different letter on the top of each bar indicates  $P < 0.01$  between them). The number of 1, 2, 3, 4 and 5 indicate the group of 50 microgram/ml ox-LDL, 50 microgram/ml ox-LDL+250 microgram/ml Poly(I), 50 microgram/ml ox-LDL+250 microgram/ml carrageenan, 50 microgram/ml ox-LDL+10mM 15d-PGJ2 and 50 microgram/ml ox-LDL+250 microgram/ml Poly(I)+ 10 mM 15d-PGJ2)

There are multiple pathways involved in ox-LDL inducing vascular adhesive molecule expression (33). PPARgamma could be one of the mediator which can be activated by ox-LDL or its metabolites inside cell. After internalized into cells, ox-LDL could affect PPARgamma activation, which further influence the expression of adhesive molecules in endothelial cell. In our study here, we pretreated with the PPARgamma agonist 15d-PGJ2 and then with ox-LDL, with the upregulation of PPARgamma, the expression of LOX-1 and adhesion molecules was significantly inhibited in activated human endothelial cells. It is considered that activated PPARgamma can function to suppress the production of certain inflammatory mediators and cytokines in activated endothelial cells in response to a variety of exogenous agents (34-35). For instance, PPARgamma agonists can inhibit TNF-alpha-induced

VCAM-1 and ICAM-1 expression in cultured endothelial cells (36). However, present study showed that PPARgamma and adhesive molecule expression were all elevated in the endothelial cells treated with ox-LDL, but the combination of LOX-1 blocker and PPARgamma agonist decrease the expression of adhesion molecules more efficiently. These results indicated there may be other pathways more powerfully promoting adhesive molecule expression by ox-LDL. These pathways could be beside of PPARgamma and related to the uptake of ox-LDL into cells by the receptors of LOX-1. Various signal conduction pathways by which ox-LDL stimulate the expression of adhesive molecules possibly function in cells. The integrated influence of ox-LDL on adhesive molecule expression mediated by PPARgamma





**Figure 6.** The effects of Ox-LDL inducing expression of PPAR $\gamma$  were mediated by LOX-1 in HUVECs. Incubating HUVECs with ox-LDL (10-100 microgram/ml) for 24 hours. Realtime RT-PCR and western blotting analysis showed that ox-LDL increased expression of PPAR $\gamma$  (mRNA and protein) in a concentration-dependent manner. Pretreating HUVECs with 250 microgram/ml of poly(I) or carrageenan for 2h and then with 50 microgram/ml of ox-LDL for 24h inhibited the expression of PPAR $\gamma$  duced by ox-LDL. (Different letter on the top of each bar indicates  $P < 0.05$  and the same letter means  $P > 0.05$  between them; \* means  $P < 0.05$  vs group 4). The number of 1, 2, 3, 4 and 5 indicates the group of 0, 10, 20, 50 and 100 microgram/ml ox-LDL respectively. The number of 6 and 7 indicates group of 50 microgram/ml ox-LDL+250 microgram/ml Poly(I) and 50 microgram/ml ox-LDL+250 microgram/ml carrageenan respectively).

and other pathways showed the increased expression of adhesive molecules. Since there is lack of using antagonist of PPAR $\gamma$  in the present study, it is difficult to assess if antagonist of PPAR $\gamma$  could increase adhesive molecules expression include by ox-LDL. Further evidence still needed to prove that PPAR $\gamma$  is a determinant factor mediating the adhesive molecules by ox-LDL.

Taken together, the present studies supported that the PPAR $\gamma$  signaling pathway in vascular endothelial cells may contribute to ox-LDL inducing expression of adhesion molecules mediated by LOX-1. PPAR $\gamma$  agonist or LOX-1 ligand alone can inhibit ox-LDL inducing inflammatory response and their combinative inhibiting effects are stronger. The results indicate that ox-LDL exerts a biphasic effects on inflammatory response. It evokes harmful effects by inflammatory injury on one side and protective effects by triggering the LOX-1/PPAR $\gamma$  signaling pathway on the other hand.

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