Original Paper

PTPRO Promotes Oxidized Low-Density Lipoprotein Induced Oxidative Stress and Cell Apoptosis through Toll-Like Receptor 4/Nuclear Factor κ B Pathway

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Key Words

Atherosclerosis • Ox-LDL • Macrophage • Protein tyrosine phosphatase receptor type O

Abstract

Background/Aims: Critical roles of phosphatase receptor type O (PTPRO) and toll-like receptor 4 (TLR4) have been implicated in inflammation. However, little is known about their functional effects on atherosclerosis (AS). We aim to study their potential function in AS. **Methods:** An oxidized low-density lipoprotein (ox-LDL) induced AS model constructed with PTPRO over-expressing RAW264.7 cells and PTPRO knockout macrophages. Cell apoptosis was assayed by flow cytometry and fatty accumulation was evaluated by oil red staining. The production of ROS (reactive oxygen species), SOD (superoxide dismutase), MDA (malondialdehyde), TC (Triglyceride), and TG (total cholesterol) was evaluated. Western blot was performed to detect the expression of CD36, TLR4 and nuclear factor κ B (NF- κ B). **Results:** PTPRO expression was promoted in a dose-dependent and time-dependent manner following ox-LDL challenging. In PTPRO-over-expressing cells, CD36 expression and the level of oil-red staining, TC and TG were increased; ROS production, MDA and level of cell apoptosis were improved, but SOD was reduced. However, in PTPRO knockout cells opposite results were found. TLR4 and NF- κ B/p65 phosphorylation was significantly enhanced in PTPRO over-expressing cells, while significantly down-regulated in PTPRO knockout cells. **Conclusion:** PTPRO plays important roles in AS via promoting ox-LDL induced oxidative stress and cell apoptosis through TLR4/NF- κ B pathway.

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Introduction

Atherosclerosis (AS) is the leading cause of diverse infaust vascular events, including coronary artery disease, peripheral artery disease and stroke. With the increasing worldwide prevalence, it accounts for the most cardiovascular morbidity and mortality [1, 2]. It has been generally established that AS is an inflammatory disease involving a chronic persistent state. AS commonly initiates within the arterial wall primarily as a result of the destruction occurring in endogenously structures, which can be attributed to several matters, such as hypertension, smoking, oxidized low-density lipoprotein (ox-LDL) and so forth [3-5]. Ox-LDL, as the most important factor contributing to the development of AS, can activate both the innate and adaptive immune responses with the differentiation from monocytes to macrophages and the unregulated phagocytic activity of macrophages [6-8], subsequently induce the accumulation of lipid and the formation of foam cells, which is the hallmark event of AS [9, 10].

Previous studies have provided abundant evidence for the pivotal roles of ox-LDL and macrophages in the genesis of AS [11-13]. Generally, overloaded ox-LDL in plasma would be scavenged by macrophages via scavenger receptors, notably the type A scavenger receptor (SRA) and a type B family member, CD36 [14], which results in the activation of macrophages and leading to the production of reactive oxygen species (ROS). Excessive ROS can cause the cellular damage through the detrimental modification of many vital macromolecules, including DNA, RNA, protein and lipids, which may ultimately result in cell metabolic disorder and macrophage apoptosis. Therefore, treatments focusing on the inhibition of the production of ROS induced by ox-LDL may be beneficial for the protection of macrophage activity and thus prevent and suppress the development of AS.

Phosphatase receptor type O (PTPRO) is one of the receptor types of phosphor tyrosine phosphatases (PTPs) [15]. As an integral membrane protein with its full isoform (PTPRO) expressed in many parenchymal cells (including lung, liver, and breast) and truncated isoform identified in inflammatory cells, such as macrophages and lymphocytes, PTPRO has been verified as a functional participator involved in the development of several tumors and inflammatory diseases [16-19]. Former studies suggested that PTPRO played critical roles in acute inflammation mediated by T lymphocytes [20] and macrophages [17] via activation of NF- κ B signaling pathway. AS is a complicated inflammation associated disease, and in the occurrence and progression of AS, many vital molecules and inflammatory signaling pathways are activated, among which NF- κ B pathway is of great significance.

Toll-like receptor 4 (TLR4) was formerly demonstrated to interact with PTPRO in diverse diseases [17, 18]. TLR4 is a most intensively studied member of TLRs family, which is extensively expressed on macrophages. Previous studies have demonstrated that TLR4 was involved in the regulation of macrophage lipid accumulation [21-25], and it is essential for ox-LDL-induced lipid uptake in macrophages [2, 26].

Up to date, the role of PTPRO in AS has not been elucidated. Considering the significant role of TLR4 in AS, in combination with the interaction of TLR4 and PTPRO, we hypothesizing that PTPRO, as an important regulator of macrophage activity, may act as a fine turner in macrophage mediated ox-LDL induced AS.

Materials and Methods

Animals and cell lines

Male wild-type (WT) C57BL/6 mice were purchased from the Laboratory Animal Center of Nanjing Medical University. The PTPRO-knockout (KO) C57BL/6 mice were gifted by Dr. John L. Bixby (University of Miami, Miami, FL). All mice were maintained and bred under specific pathogen-free facilities in the Laboratory Animal Center of Nanjing Medical University. All experiments were carried out according to the Guide for the Care and Use of Laboratory Animals. RAW264.7 cell line (derived from murine macrophages) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA)

In order to obtain primary macrophages, eight- to ten-week-old mice were intraperitoneally injected with mercaptoethanol (Gibco, Carlsbad, CA, USA) (25mg/kg) for 24h. Then mice were sacrificed and ascites was collected and added to 6-well plates. For cell cultivation, Dulbecco's modified Eagle's medium (DMEM) medium (Invitrogen Corp., Grand Island, NY, USA) added with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin was used. Cells were maintained in 5% CO₂ at 37 °C.

Cell transfection

RAW264.7 cells (0.5–1.0×10⁶) were transfected with PLV-PTPRO-GFP plasmid accompanied with polybrene reagent as previously described [13]. Clone Express™ II One Step Cloning Kit (Vazyme biotech co., Ltd.) was used to construct PLV-PTPRO-GFP plasmids according to the manufacturer's instruction. 293 T cells were used to packaging the PLV-PTPRO-GFP plasmids which were the source of the pressed protein. After 72h, 293 T cell supernatant was collected and applied as the source of the pressed protein (PLV-PTPRO-GFP).

Cell co-culture and cell proliferation assay

RAW264.7 cells were co-cultured with smooth muscle cell line, MOVAS, in an indirect co-culture system as described [27, 28]. Totally 2×10⁵ RAW264.7 cells after 50 mg/L ox-LDL challenging for 24 hours were seeded in the up layer in the Transwell units, and 2×10⁵ MOVAS cells were seeded in the down layer in the well of six-well plates (In Vitro Scientific, CA, USA). After 24 hours cultivation in DMEM medium added with 10% FBS, MOVAS cells were collected and were treated with Cell-Light EdU Apollo567 In Vitro Kit (RiboBio, Guangzhou, China) according to the given instruction. Cell samples were detected with a laser confocal scanning microscopy (Carl Zeiss Jena, Germany).

Real-time polymerase chain reaction (PCR)

Total RNAs were extracted from cells with TRIzol reagent (Invitrogen) and then quantized. The integrity of the isolated RNA was checked using NanoDrop 2000/2000c (Thermo Scientific, MA, USA). In order to get cDNA, reverse transcriptase (TaKaRa, Tokyo, Japan) was used to reverse transcribe total RNAs (500 ng). Real-time PCR was performed using Step one plus machine (Applied Biosystems, Darmstadt, Germany) with SYBR Green Mastermix kit (Takara, Tokyo, Japan) and results were analyzed in triplicate assays. Primers used were listed in Table 1.

Flow cytometry analysis

Cells were incubated with or without 50 mg/L ox-LDL for 24 h after transfection. Then, Annexin V-FITC/ PI Apoptosis Detection Kit (Vazyme Biotech, Nanjing, China) were used to detect cell apoptosis in cells according to the instructions. Briefly, the cells were treated with tyrisin and collected, then washed twice with PBS. Cell pellet was suspended in 1x binding buffer (100 µL), after which annexin APC (5 µL) and PE (5 µL) was added, then cells were gently vortexed and subsequently incubated for 10 min at room temperature in the dark. Flow cytometry analysis was conducted using a FACS Calibur flow cytometer equipped with CellQuest software (BD Biosciences, New York, NY, USA).

Oil red O staining

Cells incubated with or without 50 mg/L ox-LDL (Unionbiol, Beijing, China) for 24 h after transfection were fixed with 4% paraformaldehyde for 10 min. Samples were washed with PBS for twice, then stained with filtered Oil Red O solution (60% Oil Red O dye and 40% water; Sigma, St. Louis, MO, USA) at room temperature for 15 min. Samples were then washed for twice with PBS. Oil red staining was observed under a microscope (Olympus, Tokyo, Japan) and the intensity was measured by Image-Pro Plus 6.0.

Table 1. Primers used for real-time PCR

Genes	Forward(5'~3')	Reverse(5'~3')
PTPRO	GAGGTGAACCCTAACGTGGTG	TCATGGAGACTAGCCTCACCC
GAPDH	GCCTCGTCCCGTAGACAAAA	GATGGGCTTCCCGTTGATGA

Triglyceride (TG) and total cholesterol (TC) assay

The TG level was assayed in cell lysates with a TG Quantification Kit (Abcam, Cambridge, MA, USA) and TC level was detected using a Cholesterol Assay Kit (Abcam, Cambridge, MA, USA) according to the manufacturer protocol.

Measurement of intracellular ROS production, malondialdehyde (MDA) and superoxide dismutase (SOD)

Cells were collected and washed with PBS for two times, then were suspended with DCFHDA (10mM) solution. Prepared samples were incubated at 37°C for 20 min. An ROS Assay Kit (Beyotime, Shanghai, China) was used and the measurement of fluorescent intensity was performed with a fluorospectrophotometer (Hitachi, Tokyo, Japan). Cell lysates were collected and centrifuged, subsequently the supernatants were used for measuring MDA and SOD levels using a Lipid Peroxidation MDA Assay Kit (Beyotime, Shanghai, China) and a SOD Activity Assay Kit (BioVision, Milpitas, CA, USA) according to the manual protocols. The absorbance at 450 nm was detected using a Benchmark microplate reader (Bio-Rad, Hercules, CA, USA).

Western blot analysis

Cells lysed with RIPA buffer plus fresh protease and phosphatase inhibitors were collected. Proteins extracted were quantified with the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein samples (30-50µg) were separated by SDS-PAGE and then transferred to a PVDF membrane. Bay11-7082 was used as a specific NF-κB/p65 inhibitor (Sigma-Aldrich, St. Louis, Missouri, USA). Antibodies against PTPRO (Proteintech Group, CA, USA), TLR4 (Santa Cruz, CA, USA), p-NF-κB (nuclear factor-κB)/p65 (CST, Ala, USA), CD36 (CST, Ala, USA) and GAPDH (CST, Ala, USA) were used in immunoblotting. Primary antibodies were diluted 1000 times with Primary Antibody Dilution Buffer (Beyotime, Shanghai, China). Secondary antibodies (anti-rabbit) were diluted 2000 times with TBS (Tris Buffered Saline) (GeneScript, Suzhou, China). The primary antibodies were co-cultured with bands overnight at 4 °C and secondary antibodies for 1 hour at 37 °C. Immunoreactive bands were visualized using the ECL kit (Pierce, Rockford, IL, USA) and integrated density of the bands was quantified by ImageJ software (NIH, Bethesda, MD, USA).

Statistical analysis

Data were presented as mean ± SD. All experiments referred were performed for three times. SPSS software (SPSS Inc., Chicago, IL, USA) was used for analysis. One-way ANOVA analysis followed by LSD test was performed for multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

Ox-LDL induced cell apoptosis and PTPRO expression

RAW264.7 cells were treated with ox-LDL for 24h under the concentration of 0, 25, 50 and 100 mg/L or treated with ox-LDL at 50 mg/L for 0, 12, 24 and 48h. As shown in Fig. 1, we found that cell apoptosis were promoted in a dose-dependent and time-dependent manner following ox-LDL challenging (Fig. 1A and 1B). Additionally, a dose-dependent and time-dependent increase of PTPRO expression was observed (Fig. 1C and 1D). These indicated that PTPRO may be involved in the effective process induced by ox-LDL in macrophages.

PTPRO as a promoter in the formation of ox-LDL-induced macrophage-derived foam cells

To investigate the effect of PTPRO in the formation of ox-LDL-induced macrophage-derived foam cells, PTPRO over-expressing RAW264.7 cells (RAW264.7-PTPRO) (Fig. 2A and 2B), negative control RAW264.7 cells (RAW264.7-NC), macrophages from PTPRO^{-/-} mice (MC-KO) (Fig. 2A and 2B) and WT mice (MC-WT) were separately challenged with 50 mg/L ox-LDL for 24h. The results showed that ox-LDL-induced CD36 was increased when PTPRO was over-expressing and decreased after PTPRO depletion (Fig. 2C and 2D). The level of oil-red staining (Fig. 3A), TC and TG were significantly higher in PTPRO-over-expressing cells and lower in PTPRO-knockout cells (Fig. 3B and 3C). Additionally, assays delineating atherosclerosis events, macrophage-smooth muscle cells crosstalk was also performed using

Fig. 1. Effect of ox-LDL on cell apoptosis and PTPRO expression in RAW264.7 cells. (A, C) RAW264.7 cells were stimulated with ox-LDL for 24 h at 0, 25, 50, and 100 mg/L (B, D) RAW264.7 cells were challenged with 50 mg/L ox-LDL for 0, 12, 24 and 48 h. Cell apoptosis was assayed using flow cytometry. The expression levels of PTPRO and GAPDH were detected with western blotting. (n=4) Data were based on at least three independent experiments. * $P < 0.05$ vs. control group (0 h or 0 mg/L).

Fig. 2. The expression level of CD36 and PTPRO. (A, B) mRNA and protein levels of PTPRO were detected in PTPRO overexpressed RAW264.7 cells and in PTPRO knockout macrophages. (C, D) The expression levels of CD36 were assayed in PTPRO overexpressed cells and depleted cell after 50mg/L ox-LDL treatment for 24h. (Macrophage, MC; WT, wild type; PTPRO, PTPRO-overexpression; NC, Negative Control plamid transfection) (n=4) Data were based on at least three independent experiments. * $P < 0.05$ vs. control group. # $P < 0.05$ vs. ox-LDL group.

co-culture systems, which indicated that under challenge with 50 mg/L ox-LDL for 24 hours, PTPRO over-expressing RAW264,7 cells significantly inhibited the proliferation of smooth muscle cells, MOVAS. (Fig. 5) These demonstrated that PTPRO promoted the formation of

Fig. 3. Effect of PTPRO on ox-LDL-induced foam cell formation in macrophages. (A) Oil red staining in PTPRO overexpressed RAW264.7 cells and in PTPRO knockout macrophages. (Scale bar: 50 μ m) (B, C) The levels of TG and TC were measured as indicated. (Macrophage, MC; WT, wild type; PTPRO, PTPRO-overexpression; NC, Negative Control plamid transfection) (n=4) Data were based on at least three independent experiments. * $P < 0.05$ vs. control group.

ox-LDL-induced macrophage-derived foam cells and acted as a promoter in atherosclerosis events.

PTPRO improved ox-LDL-induced oxidative stress and macrophage apoptosis

To determine the functional roles of PTPRO in ox-LDL induced cell apoptosis and oxidative stress of macrophages, RAW264.7-PTPRO cells, RAW264.7-NC cells, MC-KO cells and MC-WT cells were exposed to 50 mg/L ox-LDL for 24h. As the results presented, compared to control cells, significantly elevated ROS production and MDA, but reduced SOD were observed in PTPRO-over-expressing cells (Fig. 4A, 4B and 4C). However, in PTPRO-knockout cells, higher level of SOD, but lower level of ROS production and MDA were found (Fig. 4A, 4B and 4C). The flow cytometry analysis demonstrated that cell apoptosis was improved when PTPRO was over-expressed, but decreased after PTPRO depletion (Fig. 4D), which in accordance with the expression of the classical apoptosis protein, caspase-3 (Fig. 6A). These results verified that PTPRO improved ox-LDL-induced oxidative stress and macrophage apoptosis.

PTPRO acted as a regulator in ox-LDL challenging macrophages through TLR4/NF- κ B signaling pathway

As it showed in Fig. 6, under the stimulation of ox-LDL, comparing to control cells, TLR4 and NF- κ B/p65 phosphorylation was significantly enhanced in PTPRO over-expressing cells, and in PTPRO knockout cells, TLR4 and NF- κ B/p65 phosphorylation was more significantly down-regulated (Fig. 6B and 6C). Furthermore, when specific NF- κ B/p65 inhibitor, Bay11-7082 was used, up-regulation of NF- κ B signaling was remarkably inhibited (Fig. 6B and 6C).

Fig. 4. Effect of PTPRO on ox-LDL-induced oxidative stress and cell apoptosis. Cells were challenged with 50 mg/L ox-LDL for 24 h (A, B, C) The oxidative status markers, ROS, MDA and SOD, were detected as indicated. (D) The degree of cell apoptosis was evaluated by flow cytometry. PE for nucleus staining and APC for cyto-membrane staining. (Macrophage, MC; WT, wild type; PTPRO, PTPRO-overexpression; NC, Negative Control plamid transfection) (n=4) Data were based on at least three independent experiments. * $P < 0.05$ vs. control group; ** $P < 0.05$ vs. ox-LDL group.

Discussion

In this study, we focus on the functional role of PTPRO in the development of AS. During the development of AS, persistently existing chronic inflammation finally resulted in the formation of damage within vascular endothelium, along with lipid deposition and the infiltration of inflammatory cells. Macrophages is vitally involved by swallowing over loaded lipid which in turn led to the disorders of metabolism and decreased anti-apoptosis ability of cells [26].

So here in this study, in order to build the AS cell model, we used moderate density of ox-LDL (50 mg/L) to challenge RAW264.7 for 24 h. As we observed, under the treatment of ox-LDL, the expression of PTPRO was promoted in a dose-dependent and time-dependent manner, which in accordance with the cell apoptosis presenting in the similar way. This indicated that PTPRO may be a participator in regulating cell apoptosis and the development

Fig. 5. The proliferation of MOVAS cells when co-cultured with RAW264.7. (A, B) The cell proliferative rates were evaluated with EdU assay. Cell nucleus was stained with DAPI. (PTPRO-overexpression; NC, Negative Control plasmid transfection) (n=4) Data were based on at least three independent experiments. * $P < 0.05$ vs. not co-cultured group; # $P < 0.05$ vs. co-cultured with RAW264.7-NC group.

of AS. In the pathological process of AS progression, the formation of foam cells is a hallmark event. So we further investigated the effect of PTPRO in the formation of ox-LDL-induced macrophage-derived foam cells. As we found, in PTPRO over-expressing cells, the level of oil-red staining, TC and TG were significantly increased; nonetheless, adverse results were showed in PTPRO knock-out cells. And under the exposure of ox-LDL, the expression of the macrophage scavenger receptor, CD36 was accordingly regulated, higher in PTPRO over-expressing cells and lower when PTPRO was depleted. These suggested that PTPRO played vital roles in the formation of macrophage-derived foam cells. Generally, in ox-LDL induced AS, increased cell apoptosis and oxidative stress of macrophages was the main mechanism [29], so we detected that whether PTPRO functionally affected the cell apoptosis and oxidative stress of macrophages. As the results showed that over-expression of PTPRO resulted in improved cell apoptosis and oxidative stress and depletion of PTPRO reversed these effects.

In addition, we detected the expression of TLR4 in ox-LDL challenging macrophages. As we found, the level of TLR4 was significantly increased or reduced when PTPRO was over-expressing or depleted, which indicated that in ox-LDL-induced macrophage-derived foam cells, TLR4 was a downstream point of PTPRO. TLR4 is an important regulator of NF- κ B pathway, which was also of great significance in the development of AS [25]. NF- κ B is a vital transcript factor involved in inflammation, adaptive immune and innate immune. Activated NF- κ B exacerbates inflammatory response and intensifies tissue injury through increasing pro-inflammatory and reducing anti-inflammatory cytokines. NF- κ B can also be regulated under ox-LDL stimulation. As formerly reported, ox-LDL might upregulate IL-6 via increasing NF- κ B in an IGF2-dependent way in THP-1 macrophages [24], ox-LDL stimulation can activate the TLR4/MyD88/NF- κ B inflammatory signaling pathway in vascular smooth muscle cell and finally promotes the formation of foam cell [25], and in peripheral blood mononuclear

Fig. 6. Effect of PTPRO on the expression of caspase 3 and TLR4/NF- κ B signaling pathway. Investigations were performed at 24h. (A, B) The expression levels of caspase 3, TLR4 and GAPDH were investigated by western blot. (C) The expression levels of p-p65 and GAPDH were detected by western blot. Cells were treated with NF- κ B inhibitor, Bay11-7082 (1 μ M) for 24 hours. (Macrophage, MC; WT, wild type; PTPRO, PTPRO-overexpression; NC, Negative Control plamid transfection) (n=4) Data were based on at least three independent experiments. * P < 0.05 vs. control group; & P < 0.05 vs. ox-LDL treated PTPRO overexpressed group; # P < 0.05 vs. ox-LDL group.

cells extracted from unstable angina patients, ox-LDL may induced the activation of NF- κ B [8]. PTPRO has been shown to influence the activation of transcription factors NF- κ B, which mediates classical inflammatory signaling pathways in various diseases. In order to investigate the association between PTPRO/TLR4 and NF- κ B, we detected the expression of NF- κ B and activated NF- κ B, phosphorylated I κ B. As the results indicated, we demonstrated a novel PTPRO/TLR4/NF- κ B signaling pathway in ox-LDL challenging macrophages and macrophage mediated AS. High level of activated NF- κ B was observed in PTPRO over-expressing RAW264.7 treated with ox-LDL, which elucidated the positive relation between PTPRO and TLR4 was involved by NF- κ B signaling pathway. However, rarely evidence was validated to clarify how PTPRO realized its functional regulation of TLR4. Further research on the exact mechanism of AS and the regulatory effects of PTPRO in AS is urgently warranted for exploration.

In conclusion, we firstly reported the functional roles of PTPRO in the AS, and further demonstrated a PTPRO/TLR4/NF- κ B signaling pathway in macrophages associated AS. In

combination with previous findings, our findings further suggested that PTPRO and TLR4 may be promising therapeutic targets for AS.

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Disclosure Statement

Authors involved have contributed adequately to this work and have no conflicts of interest to disclose.

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