Statins Attenuate Activation of the NLRP3 Inflammasome by Oxidized-LDL or TNF-α in Vascular Endothelial Cells through a PXR-dependent mechanism

Shaolan Wang, Xinya Xie, Ting Lei, Kang Zhang, Baochang Lai, Zihui Zhang, Youfei Guan, Guangmei Mao, Lei Xiao and Nanping Wang

Cardiovascular Research Center, School of Medicine, Xi’an Jiaotong University, Xi’an 710061, China (SW, XX, TL, KZ, BL, ZZ, LX, NW); The Advanced Institute for Medical Sciences, Dalian Medical University, Dalian, 116044, China (YG, NW); Department of Quantitative Health Sciences, Cleveland Clinic, Cleveland, Ohio, 44195, USA (GM)
Running title page

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b) Corresponding authors:

Dr. Nanping Wang
The Advanced Institute for Medical Sciences,
Dalian Medical University, Dalian, 116044, China
Tel: +86-0411-86110233, Fax: +86-0411-86118981. E-mail: nanpingwang2003@yahoo.com;
or
Dr. Lei Xiao
Cardiovascular Research Center,
Xi’an Jiaotong University, Xi’an, 710061, China,
Tel: +86-29-82655186, Fax: +86-29-82655196. E-mail: xiaolei0122@xjtu.edu.cn

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reverse transcriptase-PCR; TNFα, tumor necrosis factor alpha.
Abstract

Excessive activation of the NLRP3 inflammasome is implicated in cardiovascular diseases. Statins exert an anti-inflammatory effect independent of their cholesterol lowering effect. This study investigated the potential role of statins in the activation of the NLRP3 inflammasome in endothelial cells (ECs). Western blotting and qRT-PCR showed that oxidized-LDL (oxLDL) or tumor necrosis factor alpha (TNFα) activated the NLRP3 inflammasome in ECs. Simvastatin or mevastatin significantly suppressed the effects of oxLDL or TNFα. Promoter reporter assays and siRNA knockdown revealed that statins inhibit oxLDL-mediated NLRP3 inflammasome activation via the pregnane X receptor (PXR). In addition, PXR agonists (rifampicin and SR12813) or overexpression of a constitutively active PXR (VP-PXR) markedly suppressed the NLRP3 inflammasome activation. Conversely, PXR knockdown abrogated the suppressive effect of rifampicin on NLRP3 inflammasome activation. Knockdown of lectin-like oxidized LDL receptor (LOX-1) or overexpression of IκBα attenuated oxLDL- or TNFα-triggered activation of the NLRP3 inflammasome. Chromatin immunoprecipitation assays indicated that mevastatin inhibited NF-κB binding to the promoter regions of the human NLRP3 gene. Collectively, these results demonstrate that the statin activation of PXR inhibits the activation of NLRP3 inflammasome in response to atherogenic stimuli such as oxLDL and TNFα in ECs, providing a new mechanism for the cardiovascular benefit of statins.
Introduction

Endothelial cells (ECs) exert critical functions in maintaining homeostasis in the vascular system. Endothelial inflammation is a major contributor to endothelial dysfunction in cardiovascular disorders such as hypertension, diabetes and atherosclerosis (Davignon and Ganz, 2004). Interleukin-1β (IL-1β) is a key pro-inflammatory mediator involved in endothelial dysfunction and serves as a major atherogenic factor (Fearon and Fearon, 2008). The production of active IL-1β is tightly controlled through the formation and activation of the inflammasome. The NLRP3 inflammasome, the best-characterized family member, is a multiprotein complex consisting of NLRP3, caspase-1 and the adaptor ASC (PYCARD). Activation of the NLRP3 inflammasome promotes the cleavage of caspase-1 and the maturation of IL-1β and IL-18. Excessive activation of the NLRP3 inflammasome and the ensuing IL-1β secretion have recently emerged as central mechanisms in the pathogenesis of metabolic diseases including type II diabetes, obesity, and atherosclerosis (Mitroulis et al., 2010; Strowig et al., 2012). Activation of NLRP3 in ECs, triggered by dyslipidemia, disturbed blood flow, and visfatin, may contribute to the development of atherosclerosis and restenosis (Xia et al., 2014; Xiao et al., 2013).

Statins comprise a class of cholesterol-lowering drugs widely used in the treatment of hypercholesterolemia and coronary artery diseases (CAD). Acting as HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase inhibitors, statins increase the expression of low-density lipoprotein (LDL) receptor in the liver, resulting in increased cholesterol uptake and lowering circulating cholesterol levels. In addition, statins have immunomodulatory and anti-inflammatory properties (Morimoto et al., 2006; Spyridopoulos et al., 2004). Statins also improve endothelial function (Kesavan et al., 2014). Recent studies have shown that statins
affect the anti-NLRP3 in patients with cardiovascular disease. A randomized clinical study showed that atorvastatin markedly diminished NLRP3 inflammasome levels in CAD patients (Satoh et al., 2014). In monocytic THP-1 cells, atorvastatin inhibited activation of the NLRP3 inflammasome (Satoh et al., 2014). Rosuvastatin has been reported to alleviate diabetic cardiomyopathy by inhibiting NLRP3 inflammasome and mitogen-activated protein kinase (MAPK) pathways in a rat model of type II diabetes (Luo et al., 2014). However, the mechanisms by which statins regulate NLRP3 inflammasome remain largely unknown.

The nuclear pregnane X receptor (PXR; NR1I2) is a member of the nuclear receptor superfamily and functions as the key regulator of the body’s defense against xenobiotics, including pollutants, drugs, dietary compounds, and their metabolites (Kliewer et al., 2002). In addition to its conventional role in xenobiotic metabolism in liver and intestines, we and others also found that PXR is expressed in the vasculature where it regulates vascular detoxification and inflammation (Wang et al., 2013). In particular, PXR suppresses the expression of pro-inflammatory adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and endothelial selectin (E-selectin) in response to TNF-α and lipopolysaccharide (LPS) in ECs. Overexpression of a constitutively active PXR in rat carotid arteries attenuated pro-inflammatory responses (Wang et al., 2013). Notably, PXR appears to orchestrate the regulated expression of the key genes responsible for detoxification and innate immunity programs in ECs (Wang et al., 2014). Statins have been known to activate PXR (Howe et al., 2011). Thus, we sought to investigate the effects of statins in activation of NLRP3 inflammasomes in ECs and the underlying mechanisms.
Materials and Methods

Cells and Reagents

Human umbilical vein endothelial cells (HUVECs) were cultured in medium 199 containing heparin (0.1 mg/ml), acidic fibroblast growth factor (FGF) (10 ng/ml), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 U/ml) and 20% FBS. Bovine aortic endothelial cells (BAECs) were maintained in Dulbecco’s modified Eagle medium (DMEM) with 10% FBS, penicillin (100 U/ml), streptomycin (100 U/ml). Rifampicin was from Cayman (Ann Arbor, MI). Antibodies against NLRP3 and NF-κB p65 were from Abcam (Cambridge, UK). Antibody to caspase-1 was from Cell Signaling Technology (Danvers, MA). Antibodies against PXR, IκBα, IgG, GADPH, Histone and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against LOX-1 was from Sigma-Aldrich (St Louis, MO). Human recombinant TNF-α was from R&D Systems (Minneapolis, MN). Reactive oxygen species (ROS) inhibitors (NAC and DPI) were from Sigma-Aldrich (St Louis, MO). Oxidized LDL (oxLDL) was from Yiyuan biotechnology (Guangzhou, China). Other reagents were from Sigma-Aldrich.

Quantitative Reverse Transcriptase-PCR (qRT-PCR)

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA), then reverse transcribed into cDNA by using iScript cDNA synthesis kit (Bio-rad, Hercules, CA). qRT-PCR was performed by using SYBR Green Supermixes (Bio-rad) and a 7500 Real-time PCR machine (Applied Biosystems, Foster City, CA). Fold changes of gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method. The qRT-PCR primers used were as follows: NLRP3 forward primer: 5’-CTTCTCTGATGAGGCCCAAG-3’, reverse primer: 5’-GCAGCAAAACTGGAAAGGAAG-3’; LOX-1 forward primer:
5’-TTACTCTCCATGGTGCTG-3’, reverse primer: 5’-AGCTTCTTCTGCTTGGC-3’;
MDR1 forward primer: 5’-CTTGTTAGACAGCCTCATA-3’, reverse primer:
5’-TCATACAGTCAGAGTTCAC-3’; GADPH forward primer:
5’-ACCACAGTCATGCCATCAC-3’, reverse primer: 5’-TCCACCACCCCTGTTGCTGTA-3’.
GAPDH was used as an internal control.

**Western blotting**

Total protein was extracted from cells with lysis buffer (10 mM Tris·HCl at pH 7.4, 150 mM NaCl, 0.1% Triton X-100) supplemented with a protease inhibitor cocktail (Roche Diagnostics). Cytoplasmic proteins were extracted with hypotonic lysis buffer (10 mM Tris·HCl, pH 7.5, 1.5 mM MgCl₂, 10 mM KCl, 0.5% Nonidet P-40). Nuclear proteins were extracted with high-salt buffer (20 mM Tris·HCl, 1.5 mM MgCl₂, 420 mM NaCl, 10% glycerol, 0.2 mM EGTA). BCA protein assay reagents (Pierce, Rockford, USA) were used to assess protein concentration. Protein samples were separated on SDS-PAGE and transferred onto PVDF membrane. Immunoblotting was performed with primary antibodies and appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies, visualized using the ECL chemoluminescence system.

**Adenoviral vectors and infection**

Recombinant adenoviruses encoding VP-PXR (Ad-VP-PXR), Ad-tTA (an adenovirus expressing tTA, a tetracycline-responsive transactivator), Ad-LacZ (β-galactosidase), and Ad-IκBα were as previously described (Wang et al., 2013). HUVECs were co-infected with Ad-tTA and Ad-VP-PXR in the presence or absence of tetracycline (0.1 μg/ml).
Small interfering RNA (siRNA) and Transfection

HUVECs were transfected with PXR siRNA (sense: 5’-CAGGAGCAAUUCGCCAUUATT-3’; antisense: 5’-UAAUGCGAAAUUGCUCUGTT-3’), LOX-1 siRNA (sense: 5’-GGUCUUCAGUUUCUUACUTT-3’; antisense: 5’-AGUAAGAAACUGAAGACCTT-3’) or scrambled siRNA (sense: 5’-CGGGUUGCCCAAAGACGACAA-3’; antisense: 5’-UUGUCGUCUUUGGGCAAACCG-3’) with Lipofectamine 2000 (Invitrogen). Experiments were performed with these cells at 24 h after transfection.

Chromatin Immunoprecipitation (ChIP) Assay

HUVECs were treated with 1% formaldehyde for DNA-protein crosslinking and then chromatin was sheared by sonication (3 times × 15 seconds). Immunoprecipitations were performed with the use of a rabbit polyclonal antibody against NF-κB/p65 or control IgG, and protein A/G Sepharose beads. After washing, the beads were eluted in elution buffer (1% SDS, 100 mM NaHCO₃). DNA samples were extracted and purified after proteinase K digestion. The bound DNAs were amplified using qPCR with primers flanking the NF-κB binding sites in the human NLRP3 promoter regions (Table 1). Relative DNA binding was expressed as fold enrichment compared to immunoprecipitates using control IgG (Fan et al., 2008).

Plasmids and Transfection

The genomic fragment harboring -2977 to +151 bp in relation to transcription start site (Anderson et al., 2008) of human NLRP3 gene was cloned using PCR from the genomic DNA. The primer sequences were 5’-CGGGCTAGCGGTACGCTACGTTCTA-3’ (forward) and 5’-CGGCTCGAGGCGAAGAAATTCTTAG-3’ (reverse). The amplified product was
subcloned into pGL3-basic plasmid containing the firefly luciferase reporter gene (Promega) with the use of Nhe I and Xho I restriction enzymes to generate NLRP3-luc. The PXRE-luciferase reporter plasmid (PXRE-Luc), pCMX-PXR and 5×NF-κB-Luc have been described previously (Wang et al., 2013). Plasmids were transfected into BAECs using Lipofectamine 2000. In order to normalize transfection efficiency, a plasmid expressing β-galactosidase (pRSV-gal) was co-transfected. Results were expressed as fold induction compared to basal promoter activity.

Statistical analysis

Data are presented as mean ± SD from at least three independent experiments. Data analysis was performed using Microsoft Excel (Microsoft, Redmond, WA) and GraphPad Prism (versions 6, GraphPad Software, La Jolla, CA). Student t test (paired groups) or one-way ANOVA followed by Newman–Keuls post hoc test (multi-group comparisons) were used to analyze the statistical significance. P <0.05 were considered significant.
Results

Effects of statins on oxLDL- or TNFα-induced NLRP3 inflammasome activation in ECs.

OxLDL and TNFα are critical atherogenic mediators known to induce the expression of pro-inflammatory genes in ECs (Yokode, 2001; Zhang et al., 2009). To test the effects of oxLDL and TNF-α on the NLRP3 inflammasome in ECs, we treated HUVEC with various concentrations of oxLDL (0 ~ 200 μg/ml) or TNFα (0 ~ 10 ng/ml) for 12 h. Western blotting showed that both oxLDL and TNFα dose-dependently increased the protein levels of NLRP3 and cleaved caspase-1 (Fig. 1A). A similar dose-dependent action was observed at the NLRP3 mRNA level as measured by using qRT-PCR (Fig. 1B). Next, we examined the effects of statins on NLRP3 inflammasome activation. We treated HUVEC with simvastatin (0.5 μM) or mevastatin (5 μM) for 12 h before exposure to oxLDL or TNFα. As shown in Figure 1C, simvastatin or mevastatin significantly inhibited the oxLDL- or TNFα-induced increases in protein levels of NLRP3 and cleaved caspase-1. The mRNA level of NLRP3 was also decreased by the statins (Fig. 1D). Taken together, these results indicated that statins attenuate the activation of NLRP3 inflammasome induced by oxLDL or TNFα in ECs.

Role of PXR in the suppressive effect of statins on oxLDL- or TNFα-activated NLRP3 inflammasome in ECs.

Statins have been reported to activate PXR in liver cells (Hoffart et al., 2012; Howe et al., 2011; Plee-Gautier et al., 2012). Thus, we examined the effect of statins on PXR in ECs. We co-transfected pCMX-PXR and PXRE-Luc into BAECs, and then treated with atorvastatin, mevastatin or simvastatin at various concentrations for 24 h. PXR reporter activity was significantly increased by all three statins (Fig. 2A). In HUVECs, simvastatin and mevastatin
also increased the mRNA level of MDR1, a PXR target gene (Fig. 2B). These results indicated that statins can activate PXR in ECs.

To determine whether PXR mediates the inhibitory effect of statins on inflammasome activation, we used siRNA to silence the expression of endogenous PXR in ECs. The suppressive effects of the statins on the oxLDL-activated NLRP3 inflammasome were significantly reduced in ECs transfected with PXR siRNA (Fig. 2C and D). Knockdown of PXR protein and mRNA levels were validated with Western blotting and qRT-PCR (Fig. 2E and F). Together, these results demonstrated that statins attenuated oxLDL or TNFα-activated NLRP3 inflammasome activation via a PXR-dependent mechanism.

Effects of PXR agonists on oxLDL- or TNFα-induced NLRP3 inflammasome.

To examine whether other specific PXR agonists have a similar inhibitory effect on NLRP3 inflammasome activation, we pretreated ECs with rifampicin (10 µM) or SR12813 (1 µM) before exposure to oxLDL or TNFα. As shown in Fig. 3A, rifampicin and SR12813 both inhibited the oxLDL- or TNFα-stimulated increases in NLRP3 and cleaved caspase-1. The mRNA level of NLRP3 was also decreased (Fig. 3B). To rule out potential off-target effect of individual agonists, we infected HUVECs with Ad-VP-PXR. We found that overexpression of PXR attenuated the NLRP3 inflammasome activation in response to oxLDL or TNFα (Fig. 3C). Furthermore, PXR knockdown abrogated the suppressive effect of rifampicin on oxLDL-induced NLRP3 inflammasome activation (Fig. 3D and E). Taken together, these results suggested that PXR agonists inhibit oxLDL- or TNFα-activated NLRP3 inflammasome in a PXR-dependent manner in ECs.
Role of the LOX-1/NF-κB pathway in activation of the NLRP3 inflammasome by oxLDL or TNFα.

Lectin-like oxLDL receptor (LOX-1) is the major receptor for oxLDL in ECs and is thought to be a marker of endothelial dysfunction in many cardiovascular diseases (Pirillo et al., 2013; Ulrich-Merzenich and Zeitler, 2013). LOX-1 expression was increased following oxLDL or TNFα exposure (supplemental Fig. 1). Knockdown of LOX-1 in ECs attenuated the activation of NLRP3 inflammasome induced by oxLDL (Fig. 4A and B). These results indicated an important role of LOX-1 in ox-LDL activation of the NLRP3 inflammasome in ECs. It has been previously shown that oxLDL binding to endothelial LOX-1 can activate the NF-κB signal pathways (Hu et al., 2008). We investigated whether NF-κB was also involved in oxLDL or TNFα-induced NLRP3 inflammasome activation. HUVECs were infected with Ad-IκBα to block NF-κB activation before exposure to oxLDL or TNFα. As shown in Figures 4C and D, Ad-IκBα abolished both oxLDL- and TNFα-induced NLRP3 inflammasome activation. These results indicated that the LOX-1/NF-κB pathway contributes to the activation of the NLRP3 inflammasome by oxLDL or TNFα.

Role of the LOX-1/NF-κB pathway in the effects of statins on the NLRP3 inflammasome.

To investigate whether statins attenuate NLRP3 inflammasome activation by inhibiting the LOX-1/NF-κB pathway, we pretreated ECs with statins for 12 h before stimulation with ox-LDL. As shown in Figures 5A and B, oxLDL increased LOX-1 expression; this response was significantly diminished by simvastatin or mevastatin. Similarly, PXR agonists (rifampicin and SR12813) or overexpression of PXR also decreased protein and mRNA levels of LOX-1 (Fig.
Furthermore, PXR knockdown abolished the suppressive effects of statins and rifampicin on LOX-1 expression (Fig. 5E-H). Taken together, these results indicated an inhibitory effect of PXR on LOX-1 expression.

LOX-1 activation results in the excessive generation of reactive oxygen species (ROS), which are known to trigger the activation of the NLRP3 inflammasome (Schroder and Tschopp, 2010). We found that simvastatin or mevastatin significantly reduced ROS generation in response to TNFα (supplemental Fig. 2). Furthermore, the PXR agonists rifampicin and SR12813 also decreased NF-κB activation by oxLDL or TNFα (supplemental Fig. 3). Thus, these results suggested that PXR inhibition of ROS may contribute to the inhibitory effects of statins on activation of the LOX-1/NF-κB pathway and the NLRP3 inflammasome.

Effects of statins on PXR inhibition of NF-κB binding to the NLRP3 promoter.

Since NF-κB plays a central role in the transcriptional regulation of the NLRP3 gene, and PXR suppressed activation of NF-κB target genes (Gu et al., 2006; Qiao et al., 2012; Xie and Tian, 2006), we examined the effects of statin-activated PXR on NF-κB binding activity to the human NLRP3 promoter. Bioinformatics analysis using the transcription element search system (TESS, http://www.cbil.upenn.edu/cgi-bin/tess) revealed 3 canonical NF-κB-responsive elements in the 5′-flanking region of the human NLRP3 gene (Fig. 6A). ChIP assays demonstrated that both oxLDL and TNFα increased NF-κB binding at these motifs, and that these responses were inhibited by mevasatin (Fig. 6B) or SR12813 (Fig. 6C). Furthermore, we performed the NLRP3 promoter reporter assay in BAECs. As shown in Figure 6D, TNFα increased the NLRP3-Luciferase reporter activity, which was inhibited by SR12813. These results indicated...
that the statin-activated PXR inhibits NF-κB binding to the NLRP3 promoter in ECs.
Discussion

In this study, we demonstrated for the first time that statins can inhibit ox-LDL or TNF-α-induced NLRP3 inflammasome activation in ECs, and that this inhibitory effect is mediated by activation of the xenobiotic nuclear receptor PXR.

A variety of intracellular and extracellular stimuli have been known to trigger the activation of NLRP3 inflammasomes. Such “danger” signals include microbial components, ATP, glucose, cholesterol crystal, hyaluronan, and ROS (Jin and Flavell, 2010; Sutterwala et al., 2014). As a part of innate immunity system, the NLRP3 inflammasome is essential to the maintenance of homeostasis against pathogens and xenobiotics. However, excessive activation of inflammasomes may lead to tissue damage and is implicated in pro-inflammatory diseases (Kim and Jo, 2013). Cholesterol crystals and oxLDL activate NLRP3 inflammasome in macrophages and play pathological roles in atherosclerosis (Duewell et al., 2010; Sheedy et al., 2013). A randomized clinical study has shown that mRNA and protein levels of NLRP3 in peripheral blood mononuclear cells are higher in the CAD patients and are positively correlated with the plasma levels of IL-1β and IL-18. Interestingly, NLRP3 and IL-1β/IL-18 levels were significantly reduced after treatment with atorvastatin for 8 month (Satoh et al., 2014). In the current study, we found that oxLDL and TNFα triggered NLRP3 inflammasome activation in ECs and, most importantly, that statins inhibited oxLDL- or TNF-triggered NLRP3 inflammasome activation (Fig 1).

One of the important findings in this study is that PXR plays a critical role in the suppression of TNFα- and oxLDL-induced NLRP3 inflammasome activation in human ECs. This notion is
supported by the following results: 1) Statins (simvastatin, mevastatin and atrovastatin) activated the PXR reporter and endogenous target gene MDR1 in ECs (Fig. 2A-B); 2) Gene silencing of PXR abrogated the suppressive effect of statin on NLRP3 inflammasome (Fig. 2C-D), whereas PXR agonists or overexpression of PXR elicited similar effects (Fig. 3A-C). It is worth noting that the pharmacologic actions of PXR are highly divergent across species (Zhou et al., 2009). The species divergence may be explained by the low homology in the ligand-binding domain (LBD), where human and mouse genes share less than 80% in amino acid sequences (Zhou et al., 2009). Thus, we used rifampicin and SR12813, two potent agonists for human PXR with little agonism toward rodent PXR (Jones et al., 2000).

Atherogenic effects of LOX-1 have been well established and are involved in endothelial dysfunction, foam cell formation, platelet activation, and plaque instability (Pirillo et al., 2013). Expression of LOX-1 can be induced in response to many atherogenic stimuli including TNFα, angiotensin II, endothelin-1, free radicals, and oxLDL (Pirillo et al., 2013). LOX-1 activation leads to ROS generation, mitochondrial DNA damage and the induction of NLRP3 expression in THP-1 cells (Ding et al., 2014). We found that LOX-1 was essential for oxLDL-triggered NLRP3 inflammasome activation in ECs (Fig. 4A-B). Activation of LOX-1 by oxLDL triggers the NFκB signaling pathway (Matsunaga et al., 2003; Mattaliano et al., 2009). Here, we demonstrated that the PXR activation either by statins or rifampicin treatment, or by PXR overexpression, inhibited LOX-1 expression (Fig 5A-D). Conversely, knockdown of PXR diminished the inhibitory effects of statins and rifampicin on LOX-1 (Fig 5E-H), indicating that statins inhibit LOX-1 expression in a PXR-dependent manner. These results suggest that inhibition of LOX-1/NFκB is a key mechanism by which statin/PXR signaling inhibits oxLDL-
or TNFα- induced NLRP3 inflammasome activation in ECs (Fig. 4C-D). This finding is consistent with the previously described reciprocal inhibition between PXR and NFκB pathways (Zhou et al., 2006). In light of the well-established roles of NFκB in atherogenesis (Baker et al., 2011) and pleotropic effects of statins on endothelial pro-inflammatory response (Greenwood and Mason, 2007), the PXR/NFκB/NLRP3-inflammasome signaling pathway may represent a novel mechanism underlying the athero-protective actions of statins.

Intriguingly, our previous study showed that PXR has recurrent cis-elements in the regulatory regions of NLRP3 genes that, when activated, can transcriptionally induce the expression of NLRP3 (Wang et al., 2014). Such a mechanism may play an important role in the maintenance of vascular homeostasis by coupling endothelial detoxification with immune surveillance program against the xenobiotics and concurrent tissue injury. On the other hand, atherogenic stimuli provoke excessive expression of NLRP3 and a much more robust activation of the NLRP3 inflammasome via the AP-1 (Yang et al., 2014) and NFκB pathways. The results in the present study also revealed functional NFκB motifs in the NLRP3 promoter region. Statin or PXR agonist SR12813 diminished NFκB/p65 biding to these motifs, supporting that statin-activated PXR can counteract oxLDL-induced inflammasome activation by blocking the LOX-1/NFκB pathway. It is also conceivable that the statin-activated PXR may antagonize the NFκB-mediated transcriptional complexes at the promoter level by “trans-repression”, a mechanism involving the competition and relocation of transcriptional co-factors that is responsible for the anti-inflammatory actions of nuclear receptors (Glass, 2006; Gu et al., 2006). Furthermore, our recent study demonstrated that laminar shear stress is an athero-protective flow pattern that enhances endothelial PXR activity. The shear stress significantly suppresses the
pro-inflammatory response but promotes endothelial survival in response to xenobiotic injuries, reinforcing the importance of context- and tissue-specific effects of PXR in ECs (Wang et al., 2013).

In conclusion, our results demonstrate that statins inhibit NLRP3 inflammasome via PXR activation in ECs. Since the conversion of endothelium to a pro-inflammatory state is an early step in atherogenesis, the suppressive role of statins on endothelial inflammasome activation may provide new molecular insights into their pharmacological actions in the primary prevention of cardiovascular morbidity and mortality.
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Authorship Contributions

Participated in research design: T. Lei, L. Xiao, N. Wang.

Conducted experiments: S. Wang, X. Xie, K. Zhang, Z. Zhang, L. Xiao.

Contributed new reagents or analytic tools: B. Lai.


Wrote or contributed to the writing of the manuscript: S. Wang, L. Xiao, N. Wang, Y. Guan.
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Footnotes

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Legends for Figure

Figure 1. Effects of statins on oxLDL- or TNFα-induced NLRP3 inflammasome activation in ECs. (A) HUVECs were treated with oxLDL (0-200 μg/ml) or TNFα (0-10 ng/ml) for 12 h. Cell lysates were analyzed for NLRP3 and caspase-1 proteins by Western blotting. (B) Total RNA was extracted and subjected to qRT-PCR for the assessment of NLRP3 mRNA. (C) HUVECs were pretreated with simvastatin (SIM, 0.5 μM) or mevastatin (Mev, 5 μM) for 12 h before stimulation with oxLDL (100 μg/ml) or TNFα (10 ng/ml) for 12 h. Cell lysates were analyzed for NLRP3 and caspase-1 protein levels by Western blotting. (D) Total RNA was extracted and subjected to qRT-PCR for the assessment of NLRP3 mRNA. Data represent mean ± SD, n=3-5, *P<0.05, **P<0.01 vs. control, ##P<0.01 vs. oxLDL.

Figure 2. Effect of PXR knockdown on the effects of statins on oxLDL- or TNFα-activated NLRP3 inflammasome in ECs. (A) BAECs were transfected with PXRE-luc and pCMX-PXR and, 24 h later, exposed to atovastatin (ATO, 1-10 μM), Mevastatin (Mev, 1-10 μM) or simvastatin (SIM, 0.5-5 μM) for 24 h. Luciferase activities were measured and normalized to β-galactosidase activity. SR12813 (1 μM) was used as a positive control. (B) HUVECs were incubated with SIM (0.5 μM) or Mev (5 μM) for 12 h. The mRNA level of MDR1 was measured by using qRT-PCR. (C) HUVECs were transfected with PXR siRNA or scrambled (Scr) siRNA for 24 h and then pretreated with Mev for 12 h before exposure to oxLDL (100 μg/ml, 12 h). (D) qRT-PCR for the assessment of NLRP3. (E) PXR protein was detected by Western blotting. (F) The relative mRNA level of PXR was assessed by qRT-PCR. Data represent mean ± SD, n=3, *P<0.05, **P<0.01 vs. control, ##P<0.01 vs. oxLDL.
Figure 3. Effects of PXR agonists on oxLDL- and TNFα-induced NLRP3 inflammasome activation. (A) HUVECs were pretreated with rifampicin (Rif, 10 μM) or SR12813 (SR, 1 μM) for 12 h, and then incubated with oxLDL (100 μg/ml) or TNFα (10 ng/ml) for 12 h. Protein levels of NLRP3 and caspase-1 were assessed by Western blotting. (B) The NLRP3 mRNA level was assessed using qRT-PCR. (C) HUVECs were infected with Ad-VP-PXR for 24 h, and then incubated with oxLDL or TNFα for 12 h. NLRP3 and caspase-1 protein levels were assessed by Western blotting. (D) HUVECs were transfected with PXR or scrambled siRNA for 24 h, and then pretreated with Rif for 12 h before exposure to ox-LDL. The protein levels of NLRP3 and caspase-1 were measured by Western blotting. (E) NLRP3 mRNA level was assessed by qRT-PCR. Data are expressed as mean ± SD, n=3-4, *P<0.05, **P<0.01 vs. control, #P<0.05, ##P<0.01 vs. oxLDL or TNFα.

Figure 4. Effects of LOX-1 and NF-κB knockdown on activation of NLRP3 inflammasome by oxLDL or TNFα. (A) HUVECs were transfected with LOX-1 or scrambled siRNAs for 24 h, and then incubated with oxLDL for 12 h. Cell lysates were analyzed for NLRP3 and caspase-1 protein levels by Western blotting. (B) Total RNA was extracted and subjected to qRT-PCR for assessment of NLRP3 mRNA. (C) HUVECs were infected with Ad-Lac or Ad-IκBα for 24 h and then simulated with oxLDL or TNFα for 12 h. Protein levels of NLRP3 and caspase-1 were assessed by Western blotting. (D) NLRP3 mRNA level was assessed by qRT-PCR. Data represent mean ± SD, n=3-4, *P<0.05 vs. control.

Figure 5. Effects of statins on NLRP3 inflammasome with LOX-1/NF-κB knockdown. (A) HUVECs were treated with SIM (0.5 μM) and Mev (5 μM) for 12 h before exposure to oxLDL...
for 12 h. Protein level of LOX-1 was assessed by Western blotting. (B) LOX-1 mRNA level was assessed by qRT-PCR. (C) HUVECs were infected with VP-PXR (24 h), Rif or SR (12 h) before exposure to ox-LDL. LOX-1 protein was assessed by Western blotting. (D) LOX-1 mRNA was assessed by qRT-PCR. (E) HUVECs were transfected with PXR or Scr siRNA for 24 h, and then pretreated with SIM or Mev for 12 h before exposure to oxLDL for 12 h. LOX-1 protein was assessed by Western blotting. (F) LOX-1 mRNA was assessed by qRT-PCR. (G) HUVECs were transfected with PXR or Scr siRNA for 24 h, and then pretreated with Rif for 12 h before exposure to oxLDL. LOX-1 protein levels were assessed. (H) LOX-1 mRNA level was measured by qRT-PCR. Data represent mean ± SD, n=3-4, *P<0.05, **P<0.01 vs. control, #P<0.05, ##P<0.01 vs. oxLDL.

**Figure 6. Effects of statins and PXR on NF-κB binding to the NLRP3 promoter.**

(A) The diagram depicts putative NF-κB motifs in the 5′-flanking region of the human NLRP3 gene. HUVECs were treated with ox-LDL (B) or TNF-α (C) in the presence or absence of Mev or SR. ChIP assays were performed with NF-κB/p65 antibody or IgG as a negative control. Immunoprecipitated DNA was eluted and amplified by PCR using specific primers flanking the NF-κB sites. PCR results were expressed as fold change compared to IgG control. (D) BAECs were co-transfected with NLRP3-luc plasmid, then exposed to SR for 12 h before incubating with TNFα. Luciferase activity was measured and normalized to β-gal activity. Data represent mean ± SD, n=4, **P<0.01 vs. control. ##P<0.01 vs. TNFα.
### Table 1. Primers for Chromatin Immunoprecipitation (ChIP) Assay

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward (5'-3’)</th>
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<tr>
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<td>TGAAAAGGACGACCCCAG</td>
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<td>-166 to -157</td>
<td>ACTCGCATGGCATGCTTTA</td>
<td>TCTGGTTCCATACTGGGT</td>
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<tr>
<td>-110 to -101</td>
<td>ACCGAGACACGGTTTGAC</td>
<td>GCTGGACTTACCCAGATGCC</td>
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Figure 1

A) Ox-LDL (µg/ml) and TNF-α (ng/ml) levels.

B) Graph showing relative mRNA levels of NLRP3, Pro-caspase-1, and Cleaved p20.

C) Ox-LDL and TNF-α treatment conditions with SIM and Mev.

D) Graph showing relative mRNA levels of NLRP3, Pro-caspase-1, and Cleaved p20 with various treatments.
Figure 2

A

Relative Luciferase Activity

Control 1 5 10 1 5 10 0.5 1 5 SR

ATO Mev SIM

* **

B

MDR1

Control SIM Mev

* *

C

Scr PXR

siRNA ox-LDL Mev NLRP3

Pro-caspase-1 Cleaved p20

β-actin

D

NLRP3

Control ox-LDL Mev+ox-LDL

* *

E

PXR

Scr PXR

siRNA

β-actin

F

Relative mRNA level

Scr PXR

siRNA

0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0

0 1 2 3 4 5 6 7 8 9 10
Figure 4

A

<table>
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<td>Cleaved p20</td>
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<td>LOX-1</td>
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<td>β-actin</td>
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B

![Graph showing relative mRNA level for Scr siRNA and LOX-1 siRNA]

C

<table>
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<tr>
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<th>Ad-ικBα</th>
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<td>TNF-α</td>
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</table>

D

![Graph showing relative mRNA level for Ad-LacZ and Ad-ικBα]

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Figure 5

A

B

C

D

E

F

G

H

ox-LDL

LOX-1

β-actin

SIM Mev

ox-LDL

LOX-1

β-actin

Mock VP-PXR Rif SR

ox-LDL

LOX-1

β-actin

Scr siRNA PXR siRNA

ox-LDL

LOX-1

β-actin

Scr siRNA PXR siRNA

ox-LDL

LOX-1

β-actin

Scr siRNA PXR siRNA

ox-LDL

LOX-1

β-actin

Scr siRNA PXR siRNA

Relative mRNA level

Relative mRNA level

Relative OLR1 mRNA level

Scr siRNA PXR siRNA

Scr siRNA PXR siRNA

Scr siRNA PXR siRNA
Figure 6

A

CHIP Assay (fold induction)

B

Control
ox-LDL
Mev+ox-LDL

IgG
p65

C

Control
TNF-α
SR+TNF-α

Relative Luciferase Activity

D

Control
TNF-α
SR+TNF-α

IgG
p65

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