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Nifedipine inhibits ox-LDL-induced lipid accumulation in human blood-derived macrophages

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ABSTRACT

Studies have shown that nifedipine, an anti-hypertensive drug, protects against atherosclerotic progression, but the underlying mechanisms remain elusive. Oxidized low-density lipoprotein (ox-LDL) is critically implicated in macrophage lipid deposition seen in atherosclerosis. In this study, we examined the effects of nifedipine on some ox-LDL-associated changes in human blood-derived macrophages. We isolated monocytes from normal human blood and differentiated them into macrophages. We then treated these human macrophages with ox-LDL and/or nifedipine, and examined lipid accumulation and expression levels of two scavenge receptors CD36 and SR-A as well as a protein kinase PKC-θ. Nifedipine treatment substantially reduced lipid accumulation and the expression of CD36, SR-A, and protein kinase C (PKC)-θ in human macrophages treated with ox-LDL. Silencing of PKC-θ using siRNA also reduced the expression of CD36 and SR-A in these cells. Our results thus suggest that nifedipine may inhibit atherosclerosis by reducing ox-LDL-induced lipid deposition through suppression of the CD36/SR-A-mediated uptake of ox-LDL by macrophages via a PKC-θ-dependent mechanism.

1. Introduction

Atherosclerosis is a chronic inflammatory disease characterized by early and prolonged presence of macrophages within the arterial intima [1]. During atherogenesis, circulating monocytes migrate into the arterial intima where the majority of them differentiate into macrophages [2]. After uncontrolled uptake of ox-LDL via different scavenger receptors, macrophages are transformed into lipid-laden foam cells and release growth factors and cytokines that drive the growth and inflammatory responses of atherosclerotic plaque [3]. The scavenger receptors CD36 and SR-A are the main receptors for ox-LDL on the macrophage membrane [4]. SR-A, a member of the class A scavenger receptor family, has been implicated in atherosclerosis [5]. CD36 belongs to the scavenger receptor B family, and its activation has also been involved in foam cell formation. SR-A and CD36 may account for greater than 90% of the lipid uptake by macrophages treated with oxidized LDL [6].

Nifedipine is a calcium channel blocker and it has been widely used to treat hypertension. Nifedipine can inhibit vascular smooth muscle cell proliferation and reactive oxygen species (ROS) production via an AMP-activated protein kinase signaling pathway [7]. In a clinical study, Effect of Long-acting Nifedipine on Mortality and Cardiovascular Morbidity in Patients with Hypertension (ACTION) and Symptomatic Stable Angina Trial, the cardiovascular morbidity of patients with symptomatic coronary artery disease and hypertension are significantly reduced by the addition of nifedipine-GITS to the basic treatment [8]. Nifedipine also improves endothelial function as well as coronary and brachial artery vasodilation to Ach in patients with coronary artery disease [9]. Although nifedipine has been shown to have anti-hypertensive and anti-atherogenic effects [10], the underlying mechanisms remain largely unknown. In this study, we explored the effects of nifedipine on the lipid accumulation and scavenge receptor expression associated with ox-LDL in human blood-derived macrophages.

2. Materials and methods

2.1. Materials

Nifedipine was purchased from Sigma (USA). Total RNA extraction reagent RNAiso Plus, PrimeScript RT reagent kit, and SYBR-Green PCR kit were purchased from Takara (Japan). Immunoblot reagents were purchased from the Beyotime Institute of

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Biotechnology (China). All other chemicals were of the best grade available from commercial sources.

2.2. Cell culture

Human peripheral blood monocytes (PBM) were isolated from healthy volunteers using Ficoll/Hypaque gradient centrifugation. The pooled monocytes from the volunteers were incubated in DMEM supplemented with 10% autologous serum for 10 days for differentiation into macrophages.

2.3. Effects of nifedipine on lipid accumulation induced by ox-LDL in human macrophages

Human macrophages were treated with control, ox-LDL (1 mg/ml) + nifedipine (10 nmol/l), or ox-LDL (1 mg/ml) for 24 and 48 h. Lipid depostion was visualized after oil red O staining.

2.4. RNA isolation and quantitative real-time PCR analyses

Total RNA was extracted using RNAiso Plus reagent in accordance with manufacturer's instructions. PCR primers were synthesized by Shanghai Sangon (Shanghai, China). The forward and reverse primer sequences for each gene are listed below: CD36: 5′-GTC TTC CCA ATA AGC ATG TCT CC-3′ and 5′-GTC TTC CCA ATA AGC ATG TCT CC-3′; SR-A: 5′-TGA ACG AGA GGA TGC TGA CTG-3′ and 5′-TGT CAT TGA ACG TGC GTC AAA-3′; PKC-θ: 5′-ATC CTG GAG AGA GCT CTC CAA T-3′ and 5′-CGC CTG AGC TCT CTA TCA TCG T-3′; and GAPDH: 5′-AGG CCG GTG CTG AGT ATG TC-3′ and 5′-TGC CTG CTT CAC CAC CTT CT-3′. Real-time PCR was performed with SYBR® *Premix Ex Taq*™ II, on a Bio-Rad iCycler with an iQ3.1 real-time PCR system. The ΔΔ Ct method was used to determine relative abundance of mRNA, and GAPDH was used as an internal invariant control.

2.5. Western blot analysis

Cells were harvested and protein extracts prepared in accordance with the manufacturer's instructions. Immunoblot analysis [12% SDS-PAGE; 30 μg protein per lane] was then performed using rabbit anti-CD-36, anti-SR-A, anti- PKC-θ or anti-GAPDH (Abcam, USA)-specific antibodies. Proteins were visualized using enhanced chemiluminescence.

2.6. Selection of PKC-θ siRNAs in human macrophages

The PKC-θ-siRNA specific for human PKC-θ and nonsilencing (control) siRNAs were synthesized by Shanghai Genechem (Shanghai, China). Human macrophages (1×10^6 cells/well) were transfected using Lipofectamine 2000 (Invitrogen). Following 48 h transfection, a siRNA fragment of PKC-θ suppressed expression of PKC-θ gene by 70% according to RT-PCR analyses. This siRNA (PKC-θ-siRNA) was used for the experiments and its sequence is 5-ATC TGC ATG CTG ATG ACT T-3.

2.7. PKC-θ siRNA transfection and Western blot analyses

Human macrophages were grown in culture flasks at a density of 1×10^7/ml for 12 h, washed with PBS, and then treated with PKC-θ-siRNA or control siRNA in the serum-free DMEM containing ox-LDL at 10 μg/ml and in absence or presence of nifedipine (10 nmol/L). The cells were harvested 96 h later for protein extraction and immunoblotting [12% SDS-PAGE; 60 μg protein per lane] using rabbit anti-CD36, anti-SR-A, anti-PKC-θ and anti-GAPDH (Abcam, USA)-specific antibodies.

2.8. Statistical analyses

Data are expressed as mean ± standard error of the mean (SEM). Results were analyzed using One-way ANOVA with SPSS 13.0 software. $P < 0.05$ was considered statistically significant. All experiments were performed at least in triplicate.

3. Results

3.1. Nifedipine inhibits ox-LDL-induced lipid accumulation in human macrophages

Macrophages accumulate lipids in the cytoplasm after treatment with ox-LDL. To determine if nifedipine modulates this phenomenon, we treated human peripheral blood monocyte (PBM)-derived macrophages with ox-LDL in the absence or presence of nifedipine. A substantial reduction in Oil red O-stained lipids was observed in macrophages treated with ox-LDL (1 mg/ml) and nifedipine (10 nmol/l) relative to ox-LDL (1 mg/ml) alone after 24 h or 48 h of treatment (Fig. 1). This observation indicates that nifedipine prevents ox-LDL-induced lipid accumulation in human macrophages.

3.2. Nifedipine suppresses ox-LDL-induced increases in expression levels of CD36, SR-A, and PKC-θ in human macrophages

To determine the potential mechanisms involved in the above observation, we first examined CD36 and SR-A, two cell surface receptors critically implicated in the uptake of ox-LDL, and PKC-θ, a protein kinase involved in lipid-associated signal transduction. We found that the mRNA expression levels of CD36, SR-A, and PKC-θ increased 260%, 452%, and 410%, respectively, in human macrophages treated with ox-LDL at 5 μg/ml (Fig. 2A–C). At 10 μg/ml, the increases were 320%, 630%, and 550%, respectively (Fig. 2A–C). Similar alterations were seen for the protein levels of CD36, SR-A and PKC-θ (230%, 320%, and 280% increases at 5 μg/ml of ox-LDL; 290%, 415% and 390% increases at 10 μg/ml, respectively). (Fig. 2D–G)

We then examined whether nifedipine has any effects on the expression of CD36, SR-A, and/or PKC-θ in human macrophages treated with ox-LDL at 10 μg/ml. Indeed, nifedipine treatment at 10 nmol/L significantly reduced the mRNA levels of CD36, SR-A, and PKC-θ by 57.0%, 65.3%, and 62.8%, respectively, in these cells (Fig. 3A–C). Similar changes were observed when nifedipine was used at 100 nmol/L, suggesting that there was no dose-dependent effect in the range of the doses. Nifedipine treatment also significantly reduced the protein expression levels of CD36, SR-A, and PKC-θ by 53%, 58%, and 57.3%, respectively, in human macrophages treated with ox-LDL at 10 μg/ml (Fig. 3D–G). These findings, together with key roles of CD-36 and SR-A in ox-LDL uptake, suggest that nifedipine may prevent ox-LDL-induced lipid accumulation by suppressing cellular uptake of ox-LDL particles.

3.3. PKC-θ modulates ox-LDL-induced expression of CD-36 and SR-A in human macrophages

PKC-θ senses cellular lipids to alter signal transduction, which has the potential to alter gene expression. Therefore, we examined whether there is any crosstalk among PKC-θ, CD36 and SR-A in human macrophages. The siRNA-mediated silencing of PKC-θ, like nifedipine at 10 nmol/l, reduced the protein levels of CD36 and SR-A (by 73%, 79.5% for PKC-θ siRNA and 52%, 59% for nifedipine, respectively) (Fig. 4A–D). This result implies that PKC-θ may be involved in the regulation of SR-A and CD-36 expression induced by ox-LDL in human macrophages.

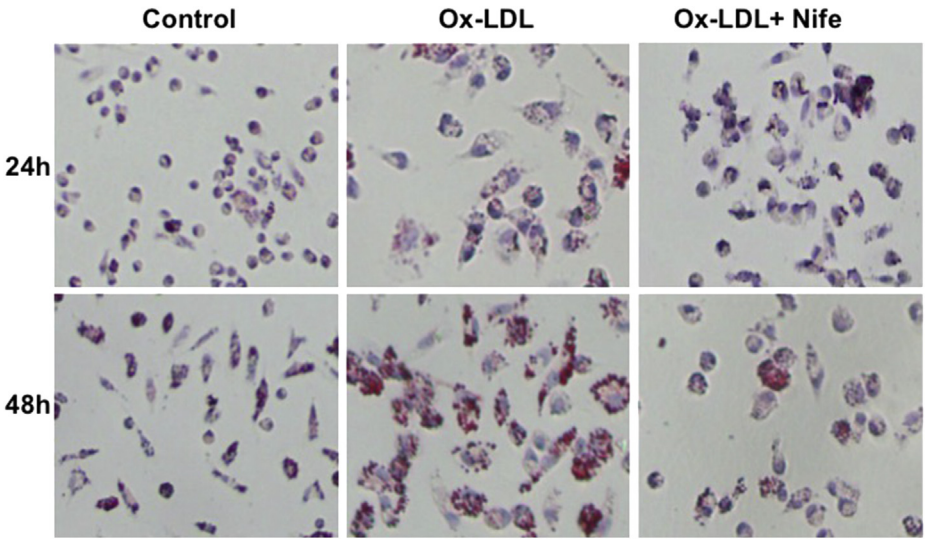

Fig. 1. Nifedipine inhibits ox-LDL-induced lipid accumulation in human macrophages. Human macrophages were treated with ox-LDL (1 mg/ml) and nifedipine (10 nmol/l) or ox-LDL alone for 24 h or 48 h and then subjected to Oil red O staining.

4. Discussion

Previous studies have shown that nifedipine inhibits the expression of macrophage inflammatory cytokine MCP-1 via suppressing phosphorylation and activation of PPAR, and protects against atherosclerotic progression in apoE−/− mice [10]. Ox-LDL plays a critical role in the pathogenesis of atherosclerosis [11,12]. Here we demonstrated that nifedipine prevents ox-LDL-induced lipid accumulation in human blood-derived macrophages. We further showed that nifedipine inhibits ox-LDL-associated expression of CD36, SR-A, and PKC-θ in these cells. Considering the critical roles of CD36 and SR-A in macrophage uptake of ox-LDL particles, our data suggest that nifedipine may protect against atherosclerosis by inhibiting ox-LDL-induced lipid accumulation in macrophages through down-regulating ox-LDL uptake.

PKC-θ is critically involved in the T cell receptor (TCR) signaling selectively required for the activation of T cell in vivo [13]. PKC-θ

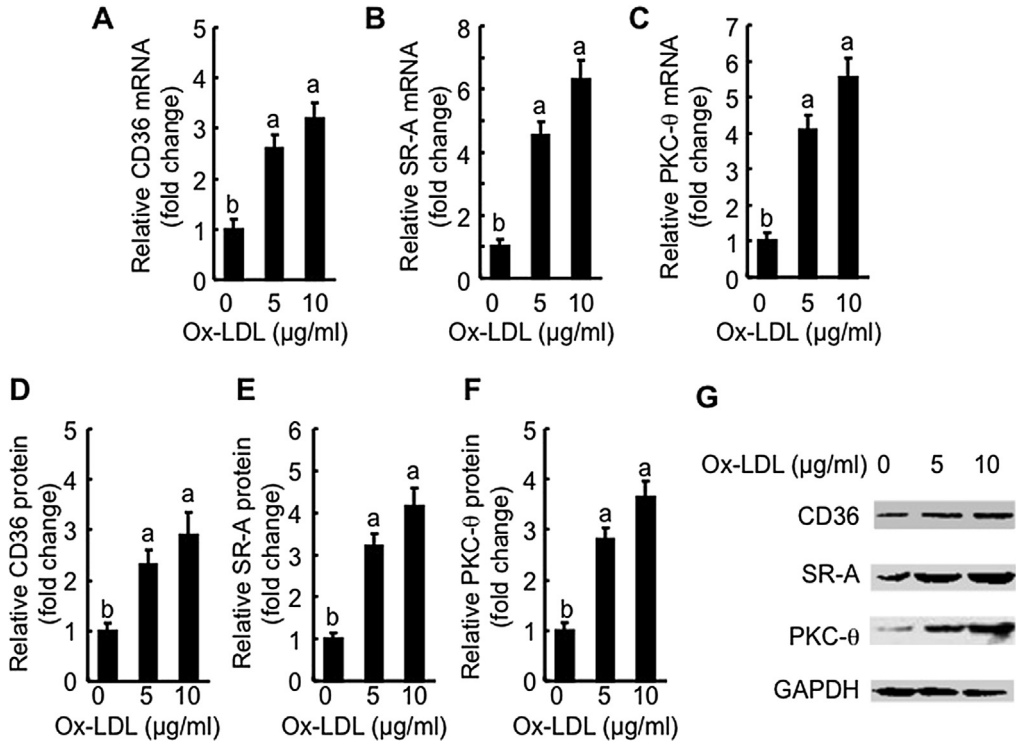

Fig. 2. Ox-LDL induces expression of CD36, SR-A and PKC-θ in human macrophages. (A–C) The mRNA levels CD36, SR-A and PKC-θ in human macrophages treated with ox-LDL for 24 h (D–G) The protein levels of CD36, SR-A, and PKC-θ in human macrophages treated with ox-LDL for 48 h. Values associated with different small letters are statistically different.

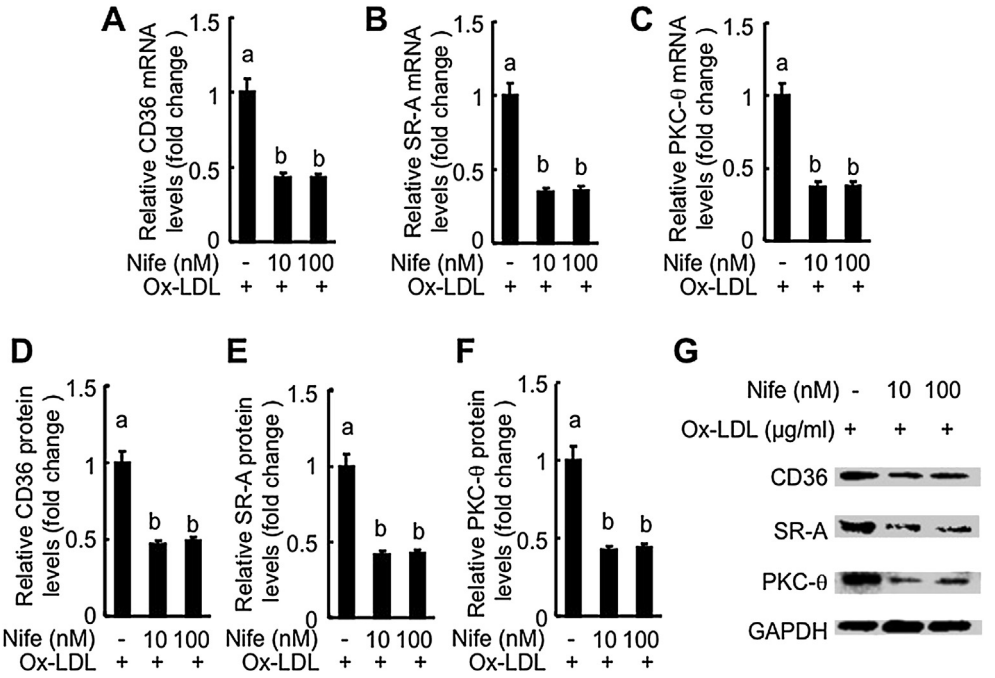

Fig. 3. Nifedipine suppresses ox-LDL-associated expression of CD36, SR-A and PKC-θ in human macrophages. (A–C) The mRNA levels CD36, SR-A and PKC-θ in human macrophages treated with ox-LDL at 10 μg/ml for 24 h in the absence or presence of nifedipine. (D–G) The protein levels CD36, SR-A and PKC-θ in human macrophages treated with ox-LDL at 10 μg/ml for 48 h in the absence or presence of nifedipine. *P < 0.05 vs control. Values associated with different small letters are statistically different.

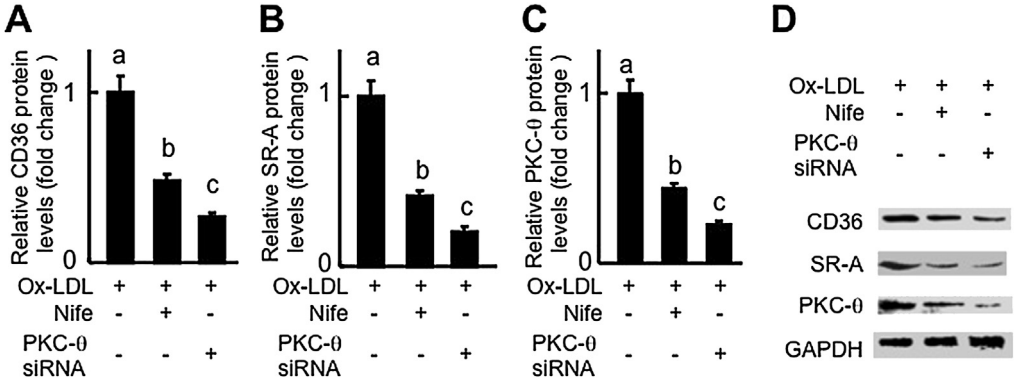

Fig. 4. Silencing of PKC-θ with siRNA suppresses ox-LDL-associated expression of CD36 and SR-A. Human macrophages were treated with ox-LDL at 10 μg/ml for 96 h in the absence or presence of PKC-θ siRNA or nifedipine at 10 nmol/L. The protein expression levels of CD36, SR-A and PKC-θ were measured by Western blots and densitometry. Values associated with different small letters are statistically different.

modulates the activation of various transcription factors such as nuclear factor of activated T cells (NFAT), AP-1 and NF-kB [14,15]. Mature PKC-$\theta^{-/-}$ T cells cannot activate NF-kB and AP1 upon TCR stimulation and fail to proliferate and produce interleukin 2 (IL-2) [16]. Mice deficient in other isoforms of PKC do not have the same effects in T cell activation, thereby indicating the importance of PKC-θ in T cell activation [17]. Here we provided some evidence in support of a role of PKC-θ in regulating CD36 and SR-A expression associated with ox-LDL in human macrophages. Detailed studies are required to study how nifedipine alters PKC-θ expression and activation in the future.

In conclusion, our results suggest that nifedipine inhibits ox-LDL-induced lipid deposition in human macrophages, at least in part through inhibition of CD36 and SR-A expression via a PKC-θ-dependent pathway.

Conflict of interest

None declared.

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Transparency document

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