Eplerenone inhibits oxidized low-density lipoprotein-induced proliferation and migration of vascular smooth muscle cells by downregulating GPER expression

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Abstract

Background. Excessive proliferation and migration of vascular smooth muscle cells (VSMCs) are associated with the pathogenesis of atherosclerosis (AS). Eplerenone (EPL), a novel selective aldosterone receptor blocker, plays a substantial role in the treatment of cardiovascular disease. The G protein-coupled estrogen receptor (GPER) is a target of EPL as the STITCH website predicated.

Objectives. We aimed to investigate the roles of EPL in AS and identify its potential mechanisms of action.

Materials and methods. Oxidized low-density lipoprotein (ox-LDL) was employed to stimulate VSMCs to establish a cellular model of AS. The ability of cell proliferation was examined using a Cell Counting Kit-8, and the expression of proliferation-related proteins was tested using immunofluorescence staining and western blot analysis. Subsequently, cell migration and the expression of migration-associated proteins were evaluated with a wound healing assay, transwell assay and western blot analysis. Then, GPER expression was determined using western blot analysis in the absence or presence of EPL. To explore the regulatory mechanisms of EPL in ox-LDL-stimulated VSMCs, GPER was overexpressed, followed by measurement of cell proliferation and migration.

Results. The Ox-LDL stimulation notably upregulated GPER expression, whereas EPL treatment downregulated GPER expression in a dose-dependent manner. Additionally, EPL markedly inhibited proliferation and migration of VSMCs, and the highest dose of EPL resulted in the most marked effect. By contrast, GPER overexpression reversed the inhibitory effects of EPL on proliferation and migration of VSMCs.

Conclusions. Eplerenone suppressed ox-LDL-induced proliferation and migration of VSMCs partly through downregulation of GPER, providing a new mechanism of support for EPL use in the clinical treatment of AS.

Key words: atherosclerosis, proliferation, migration, vascular smooth muscle cells, eplerenone
**Background**

Atherosclerosis (AS), a chronic degenerative disease of the arterial wall, is the leading cause of peripheral vascular disease and cardiac-cerebral vascular disease.\(^1\) It is well known to cause high morbidity and mortality in aged individuals worldwide.\(^2,3\) Vascular smooth muscle cells (VSMCs) are involved in the reconstruction of arterial wall by maintaining blood flow in affected vessels due to atherosclerotic alteration.\(^4\) A growing body of literature has shown that abnormal proliferation and migration of VSMCs are closely associated with AS progression.\(^5,6\)

Oxidized low-density lipoprotein (ox-LDL), a well-established risk factor for AS, can induce proliferation and migration of VSMCs.\(^7\) Therefore, ox-LDL was employed in the present study to stimulate VSMCs to establish an AS cell model, providing a similar environment to explore the regulatory mechanisms involved in AS.

Eplerenone (EPL), a selective aldosterone receptor antagonist, is approved by Federal Drug Administration (FDA) for the treatment of left-sided heart failure and systemic hypertension.\(^8\) Compelling evidence has indicated that EPL is effective in remediating cardiovascular diseases secondary to hypertension.\(^9\) One report supports the notion that EPL inhibits Tregs by inactivation of Kv1.3 channel to reverse cardiac fibrosis.\(^10\) Additionally, EPL can suppress proliferation of contralateral renal cells in rats with unilateral ureteral obstruction.\(^11\) What is more, EPL has been affirmed to reduce renal fibrosis.\(^10\) Furthermore, blocking the function of GPER can alleviate the bisphenol A-induced proliferation of VSMCs.\(^12\) However, the role of EPL in AS and whether it functions through targeting GPER remain to be elucidated.

**Objectives**

In the present study, we probed the impacts of EPL on proliferation and migration of VSMCs stimulated by ox-LDL, as well as its underlying molecular mechanism. This study is of great significance since it provides experimental support for EPL therapy in the clinical treatment of AS.

**Materials and methods**

**Cell culture and treatment**

Human VSMCs were purchased from China Center for Type Culture Collection (Wuhan, China) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, USA) at 37°C in fully humidified air of 95% air and 5% CO\(_2\). The VSMCs were seeded in six-well plates at the density of 1 x 10\(^6\) cells/well. To construct the AS model in vitro, 100 μg/mL of ox-LDL (Yiyuan Biotech, Guangzhou, China) were utilized to treat cells for 48 h. After that, the cells were treated with 0.3 μM, 1 μM and 3 μM EPL (Pfizer, New York, USA) for 24 h to evaluate the effects of EPL on ox-LDL-induced VSMCs.

**Cell transfection**

Cells were plated into six-well plates (1 x 10\(^6\) cells per well) and transfection was performed once cells in the logarithmic growth phase reached 80% confluence. The overexpression plasmids of GPER (Oe-GPER-1 and Oe-GPER-2) and empty vector (Oe-NC) were designed and synthesized by Ribobio Co., Ltd. (Guangzhou, China). Transfection experiments were carried out with Lipofectamine 3000 (Invitrogen, Carlsbad, USA) following manufacturer’s recommendations. At 24 h after transfection, VSMCs were collected and the successful transfection was determined using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.

**Cell viability assay**

A Cell Counting Kit-8 (CCK-8; Shanghai Yi Sheng Biotechnology Co. Ltd., Shanghai, China) was adopted for detecting cell viability after appropriate treatments according to standard techniques. In brief, VSMCs (about 5 x 10\(^5\) cells/per well) were seeded in a 96-well plate. After exposure to EPL or ox-LDL, a volume of 10 μL of CCK-8 solution was added to each well. Following further incubation at 37°C for 1 h, the absorbance was determined at the wavelength of 450 nm using a microplate reader (Molecular Devices, Sunnyvale, USA).

**Immunofluorescence staining**

The VSMCs were plated on coverslips in 24-well plates following transfection and cultured until 80% confluence was reached. After different treatments, cells were fixed with 4% paraformaldehyde for 15 min, followed by permeabilization with 0.05% Triton X-100 for 20 min at room temperature. After blocking in 5% bovine serum albumin for 30 min, cells were cultivated with a primary antibody against Ki67 (Cell Signaling Technology, Inc., Boston, USA) overnight at 4°C. Following three-time washing with phosphate-buffered saline (PBS), cells were incubated DyLight™ 488-conjugated secondary antibody (Thermo Fisher Scientific, Inc., Waltham, USA) for 1 h at room temperature. Subsequently, the nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Merck KGaA, St. Louis, USA) for 5 min and then washed 3 times with PBS in the dark. Immunofluorescence was detected under a fluorescence microscope (Olympus Corp., Tokyo, Japan).
Wound healing assay

The VSMCs (2 × 10^5 cells/well) were placed in six-well plates and cultured overnight at 37°C. Thereafter, cells were incubated with serum-free medium for 12 h prior to the experiment. Afterwards, a wound was gently created by a 10 μL sterile pipette tip and the cell debris was washed twice with PBS. Wound closure was monitored by comparing digital photographs of the same region of interest taken at 0 h and 24 h time points using a fluorescence microscope (Olympus Corp.). Quantitative analysis of the wound healing area was performed using ImageJ software (National Institutes of Health (NIH), Bethesda, USA).

Transwell migration assay

For the transwell migration assay, serum-free media containing 5 × 10^4 VSMCs were placed into the upper chamber of a 24-well transwell filter with 8 µm pore size. The lower chamber was filled with media supplemented with 10% FBS. Migratory cells would transgress through the porous filters at 37°C within 24 h. Then, VSMCs were fixed with 4% paraformaldehyde for 20 min. Cells that migrated through the pores of the filters were stained with 1% crystal violet for 30 min. The images were photographed under a fluorescence microscope (Olympus Corp.) and the number of migrated cells was calculated using ImageJ software.

RT-qPCR analysis

Total RNA was isolated from VSMCs using TRIzol reagent (Invitrogen) according to standard techniques. The complementary DNA (cDNA) was synthesized using a PrimeScript RT Reagent Kit (TaKaRa, Tokyo, Japan). The PCR then was performed with 2 μg cDNA as the template using Power SYBR Master Mix (Applied Biosystems, Foster City, USA) on the ABI 7500 PCR system (Applied Biosystems). The following thermocycling conditions were used: initial denaturation at 95°C for 7 min; 40 cycles of 95°C for 15 s and 60°C for 30 s; and a final extension at 72°C for 30 s. Sequences of the gene-specific primers were synthesized by Ribobio Co., Ltd. (Guangzhou, China). Relative expression was calculated using the 2^ΔΔCt method. Gene expression was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Western blot analysis

Whole proteins were extracted using a protein lysis buffer in the presence of a protease inhibitor cocktail (Beyotime, Shanghai, China), which were further quantified using a bicinchoninic acid protein assay kit (Beyotime). Then, equal amount of total protein (50 μg) was subjected to 10% SDS-PAGE electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, USA). The membranes were subsequently blocked with 5% non-fat milk for 1 h at room temperature and incubated with primary antibodies (Santa Cruz Biotechnology, Dallas, USA) overnight at 4°C. Following this, the membranes were further probed with horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology) for 1.5 h at room temperature. The immunoreactive protein bands on the membranes were visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, USA). ImageJ software was used for quantitative analysis of the gray values of protein bands. The relative expression was normalized to the internal control GAPDH.

Statistical analyses

All data are represented as mean values ± standard deviation (SD) from at least 3 independent experiments. Statistical analysis was conducted with GraphPad Prism v. 6 (GraphPad Software, Inc., San Diego, USA). Student’s t-test was utilized to analyze data between 2 groups. Comparisons involving multiple samples were conducted using one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test. The acceptable value of significance was p < 0.05.

Results

EPL dose-dependently inhibited cell proliferation in ox-LDL-stimulated VSMCs

Firstly, a CCK-8 assay was used to assess cell viability after VSMCs were treated with increasing EPL. As displayed in Fig. 1A, there was no significant effect on cell viability in response to EPL treatment (0.3 μM, 1 μM and 3 μM) relative to the control group. By contrast, ox-LDL challenge significantly enhanced cell viability, which was reduced with EPL co-treatment in a dose-dependent manner (Fig. 1B). Additionally, notably elevated Ki67 expression was observed in ox-LDL-stimulated VSMCs relative to the untreated group, whereas EPL intervention significantly decreased Ki67 expression, especially under co-treatment with 3 μM EPL (Fig. 1C). Consistently, EPL markedly downregulated the expression of minichromosome maintenance-2 (MCM-2) and proliferating cell nuclear antigen (PCNA), which are key proliferation-related proteins, in ox-LDL-exposed VSMCs (Fig. 1D). These results implicate that EPL attenuates proliferation of VSMCs stimulated by ox-LDL.

EPL treatment suppressed migration of VSMCs induced by ox-LDL

The influence of EPL on cell migration in ox-LDL-induced VSMCs was explored in the following experiments. Results presented in Fig. 2A,B show that ox-LDL challenge...
remarkably promoted the migration of VSMCs relative to the control group. Conversely, EPL dose-dependently declined the ox-LDL-promoted ability of cell migration. As expected, results of transwell migration assay presented the same trends with those of the scratch wound healing assay (Fig. 2C,D). Simultaneously, EPL dramatically downregulated the expression of migration-associated proteins including MMP2 and MMP9 in a dose-dependent manner (Fig. 2E). Overall, these data suggest that EPL treatment represses migration of VSMCs boosted by ox-LDL.

**EPL downregulated the expression of GPER in VSMCs exposed to ox-LDL**

To uncover the potential mechanisms of EPL in VSMCs under ox-LDL exposure, the STITCH website (http://stitch.embl.de) was applied to search the potential proteins interacting with EPL. The GPER was assumed to combine with EPL (Fig. 3A). It is observable in Fig. 3B that GPER expression was gradually enhanced with the increased concentrations of ox-LDL. Moreover, under exposure to 100 μg/mL of ox-LDL at time points from 12 h to 72 h, the level of GPER in VSMCs was markedly upregulated compared with the control group, and the highest level of GPER was noted at 48 h (Fig. 3C). Afterwards, the effect of EPL on GPER expression was assessed using western blot analysis. Figure 3 D displays that EPL dose-dependently lowered the level of GPER induced by ox-LDL. To sum up, these observations reveal that EPL can inhibit GPER expression in VSMCs exposed to ox-LDL.

**GPER overexpression reversed the inhibitory impacts of EPL exposure on proliferation and migration of VSMCs treated with ox-LDL**

Subsequently, to study the exact regulatory mechanisms of EPL on GPER, GPER was overexpressed by transfection with overexpression plasmids. Successful transfection was presented in Fig. 4A, and VSMCs transfected with Oe-GPER-1 were selected for the following experiments due to Oe-GPER-1 producing higher expression of GPER.
Fig. 2. EPL treatment suppressed migration of VSMCs induced by ox-LDL

A and B. Cell migration was determined using scratch wound healing assay (magnification x100); C and D. Cell migration was assessed using transwell migration assay (magnification x200); E. The expression of MMP2 and MMP9 was examined using western blot analysis. The experiments were generated from 3 independent repeats (n = 3). ***p < 0.001 compared to control; #p < 0.05 and ###p < 0.001 compared to ox-LDL. EPL – eplerenone; ox-LDL – oxidized low-density lipoprotein; VSMCs – vascular smooth muscle cells; MMP – matrix metalloproteinase.

Fig. 3. EPL decreased GPER expression in ox-LDL-treated VSMCs

A. The EPL-protein interaction network was predicted using the STITCH website; B. Western blot analysis of the expression of GPER when VSMCs were exposed to 25 mg/L, 50 mg/L and 100 mg/L of ox-LDL, respectively. C. The level of GPER was examined using western blot analysis in VSMCs stimulated by 100 mg/L of ox-LDL for 12 h, 24 h, 48 h, and 72 h, respectively. The experiments were generated from 3 independent repeats (n = 3); D. GPER expression was measured using western blot analysis when VSMCs were treated with a series of concentrations of EPL. All experiments were repeated 3 times independently (n = 3). ****p < 0.001 compared to control; *p < 0.01 and **p < 0.001 compared to ox-LDL; EPL – eplerenone; ox-LDL – oxidized low-density lipoprotein; VSMCs – vascular smooth muscle cells; GPER – G protein-coupled estrogen receptor.
As shown in Fig. 4B, GPER overexpression significantly elevated cell viability relative to the Oe-NC group. Meanwhile, as compared to VSMCs transfected with Oe-NC, the decrease of Ki67, MCM-2 and PCNA was notably reversed after GPER overexpression (Fig. 4C, D). Results presented in Fig. 5A–D revealed that the ability of cell migration was remarkably enhanced in the GPER overexpression group compared with the empty vector group. Moreover, GPER-upregulation conspicuously increased the levels of MMP2 and MMP9. Through the above findings, we prove that GPER overexpression abolished the suppressive effects of EPL exposure on proliferation and migration of VSMCs treated by ox-LDL.

**Discussion**

It is well known that AS is a chronic degenerative disease and has become a major cause of cardiovascular morbidity and mortality.\(^{15}\) Aging, obesity, diabetes mellitus, chronic inflammation, and elevated plasma ox-LDL are risk factors for the disease process.\(^{16,17}\) Moreover, postoperative care, rehabilitation training and health education after AS bypass grafting is important in improving the quality of life of patients and preventing the occurrence of poor prognostic event. Existing studies have demonstrated that EPL possesses protective functions against cardiovascular disease.\(^{18}\) The present study was the first to explore the roles of EPL in the functions of VSMCs as applied to AS.

The VSMCs are the major cell type observed in blood vessel walls that play considerable roles in the regulation of multiple physiological and pathological situations.\(^{19}\) Aberrant proliferation and migration of VSMCs are the key events in the progression of AS and restenosis after percutaneous coronary intervention.\(^{20}\) An increasing number of studies have reported that ox-LDL exerts a promotion effect in the development of AS by stimulating the proliferation of VSMCs within the vessel wall.\(^{21,22}\) Therefore, agents blocking or inhibiting proliferation and migration of VSMCs induced by ox-LDL may contribute to the identification of therapeutic strategies. Eplerenone is a newly found novel selective aldosterone receptor antagonist.\(^{8,9}\) Additionally, it has been reported to alleviate hepatocellular carcinoma growth and angiogenesis in mice.\(^{23}\) Adriamycin nephropathy rats treated with EPL exhibit obvious
Attenuation of mesangial cell proliferation and matrix expansion. Moreover, EPL can inhibit neointimal formation after coronary stent implantation in swine by decreasing collagen accumulation. The present study suggested that EPL treatment significantly hampered proliferation and migration of VSMCs treated with ox-LDL, suggesting a promising therapeutic agent in the treatment of AS.

We further explored the molecular mechanisms underlying the inhibitory roles of EPL on ox-LDL-stimulated VSMCs. The STITCH website (http://stitch.embl.de) was used to detect the potential proteins that could partake in the regulation of EPL. Thereafter, GPER, a G protein-coupled receptor coupled with Gs proteins, was discovered as one binding point of EPL. According to published studies, GPER promotes cell proliferation and migration of triple-negative breast cancer, renal cell carcinoma and ovarian cancer. Furthermore, activation of the GPER can increase neurogenesis and alleviate neuroinflammation in the hippocampus of male spontaneously hypertensive rats. Also, blocking of GPER function mitigates the bisphenol A-stimulated proliferation of VSMCs.

Results of the current work indicated that GPER expression was markedly upregulated in ox-LDL-induced VSMCs and the elevation was abolished under treatment with EPL. Importantly, GPER overexpression dramatically reversed the inhibitory effects of EPL intervention on proliferation and migration of VSMCs stimulated by ox-LDL, which was accompanied by the expression changes of proliferation- and migration-associated proteins.

Conclusions

To the best of our knowledge, the present study was the first to investigate the pivotal roles of EPL on VSMCs function. These findings demonstrate that EPL restricts proliferation and migration of VSMCs stimulated by ox-LDL partly by downregulating GPER expression, providing experimental and mechanistic support for EPL use in the clinical treatment of AS. However, a lack of the study in vivo is a limitation of the present research, and therefore, a comprehensive analysis is required in the future.
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