Lovastatin restores the function of endothelial progenitor cells damaged by oxLDL

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Aim: The aim of the study was to investigate whether lovastatin restores the survival and function of endothelial progenitor cells (EPCs) damaged by oxLDL.

Methods: EPCs were preincubated with different concentrations of lovastatin (2, 10, and 50 µmol/L) with or without the Akt inhibitor triciribine for 24 h and were then exposed to 50 µg/mL oxLDL for 48 h. The survival of EPCs, as well as the cellular migration, adhesion, and tube formation of these cells, was examined. To explore the mechanisms of lovastatin’s effects on EPCs, the levels of phosphorylated Akt and eNOS and of total eNOS protein and mRNA were assayed.

Results: Incubation of EPCs with oxLDL resulted in significant apoptosis and impaired cellular migration, adhesion and tube structure formation. The detrimental effects of oxLDL on EPC survival and function were attenuated by pretreatment of EPCs with lovastatin. However, when EPCs were pretreated with lovastatin and triciribine at the same time, the beneficial effects of lovastatin were abolished by triciribine. Furthermore, oxLDL caused a significant downregulation of eNOS mRNA and protein expression, as well as a suppression of Akt and eNOS phosphorylation. However, the effects of oxLDL on Akt/eNOS activity and eNOS expression were reversed by lovastatin.

Conclusion: Lovastatin reverses the survival and function of EPCs by regulating the Akt/eNOS signaling pathway and the gene transcription of eNOS.

Keywords: oxidized low density lipoprotein; endothelial progenitor cells; endothelial nitric oxide synthase

Acta Pharmacologica Sinica (2009) 30: 545–552; doi: 10.1038/aps.2009.41

Introduction

Postnatal neovascularization is a crucial compensatory response in chronic ischemic diseases. Recently, increasing numbers of studies have indicated that postnatal neovascularization is involved in the incorporation of endothelial progenitor cells (EPCs) [1, 2]. Therefore, understanding the regulation of EPCs and their mechanisms may provide new insights into therapeutic neovascularization. Our group and others have investigated the detrimental effects of oxLDL, a risk factor for cardiovascular disease, on the number and activity of EPCs [3, 4]. HMG-CoA reductase inhibitors (statins) have been developed as lipid-lowering drugs and have been documented to reduce the morbidity and mortality of coronary artery disease (CAD) [5]. To gain further insight into the mechanisms by which statins improve cardiovascular function, we investigated whether statins inhibited the detrimental effects of oxLDL on EPCs.

Recent studies have demonstrated that vascular endothelial growth factor (VEGF) and statins activate the serine/threonine kinase Akt in EPCs and induce the mobilization of bone marrow-derived EPCs [6, 7]. Akt serves as a multifunctional regulator of cell biology [8]. Phosphorylated Akt becomes available to phosphorylate its downstream substrates, such as endothelial nitric oxide synthase (eNOS), leading to eNOS activation [9]. Furthermore, recent studies have suggested that eNOS serves as a significant regulator of hemangioblast activity [10]. Thus, we hypothesize that the Akt/eNOS signaling pathway is involved in the effects of statins on the biology of EPCs.

Materials and methods

Preparation and oxidation of LDL Written informed consent was obtained from all volunteers in accordance with the institutional Ethics Committee. Blood samples for LDL preparation were taken from healthy volunteers after 12 h of
fasting. LDL was separated by density gradient ultracentrifuga-
tion. The purity of the LDL was determined by agarose gel
electrophoresis and the protein content was determined by
the modified Lowry method. LDL was oxidized by exposure
to CuSO₄. EDTA was then added, and the sample was dia-
lized against PBS to stop the oxidation process. Compared
with LDL, oxidized LDL showed increased electrophoretic
mobility in agarose gels and had higher concentrations of
thiobarbituric acid reactive substances.

Culture and identification of EPCs Mononuclear
cells (MNCs) were isolated by histopaque-density gradient
centrifugation within 4 h of human umbilical cord blood
collection. Written informed consent was obtained from all
mothers in accordance with the institutional Ethics Com-
mittee. CD34⁺ cells were isolated from MNCs with the
CD34 Isolation Kit (MACS, Miltenyi Biotec) according to
the manufacturer’s instructions. CD34⁺ cells were seeded in
fibronectin-coated culture flasks (Invitrogen) and cultured
in endothelial cell growth medium-2 (EGM-2) (Clonetics)
supplemented with 50 ng/mL vascular endothelial growth
factor (VEGF, Pepro Tech), and 20 ng/mL interleukin-3 (IL-3,
kindly provided by Kirin Pharmaceuticals). After 7−10 d of
culture, non-adherent cells were removed.

To examine the expression of surface markers on EPCs,
cells were incubated with monoclonal mouse anti-human
antibodies against VE-cadherin or vWF (BD PharMingen)
for 30 min at 4 °C. Cells were further incubated with fluores-
ccein isothiocyanate (FITC)-conjugated rat anti-mouse IgG.
For additional analysis, cells were incubated for 30 min at
4 °C with phycoerythrin (PE)-conjugated mouse anti-hu-
man CD133 (Miltenyi Biotec) or FITC-conjugated mouse
anti-human CD34, CD31, CD106, and CD14 antibodies
(BD PharMingen). Isotype-matched antibodies served as a
control. The cells were then fixed in 1% paraformaldehyde
and quantitatively analyzed with a flow cytometer (Becton
Dickinson).

EPCs were incubated with 10 µg/mL Dil Ac-LDL
(Molecular Probes) at 37 °C for 4 h and fixed with 2% paraformaldehyde for 10 min. Cells were washed and then
incubated with 10 µg/mL FITC-UEA-1 (Sigma) at 4 °C for
30 min. The incorporation of Ac-LDL and the binding of
UEA-1 were detected with a fluorescent microscope (Leica
Microsystems, GmbH).

Treatment of EPCs Except for the control cells, EPCs
were stimulated with 50 µg/mL oxLDL for 48 h. Samples of
the EPCs were preincubated with different concentrations of
lovastatin (2, 10, and 50 µmol/L) for 24 h and then exposed
to oxLDL for 48 h. To determine the role of Akt in the pro-
cess, EPCs were co-incubated with 50 µmol/L lovastatin and
1 µmol/L triciribine for 24 h and then exposed to oxLDL for
48 h.

Apoptosis assay For in situ detection of apoptotic cells,
EPCs were fixed with 2% paraformaldehyde. Then, a terminal
deoxyribonucleotidyl transferase-mediated dUTP nick end
labeling (TUNEL) assay was applied according to the manu-
ufacturer’s instructions (Roche Molecular Biochemicals); The
EPCs were counterstained with DAPI (Sigma). TUNEL
positive cells were examined in a blinded fashion and the
percentage of TUNEL positive cells was evaluated.

Adhesion assay After detachment and centrifugation,
EPCs were resuspended in adhesion buffer (0.5% BSA in
EBM-2). Identical numbers of cells were replated onto
fibronectin-coated culture plates, incubated for 30 min at
37 °C, and then carefully washed three times with adhesion
buffer to remove non-adherent cells. The adherent cells were
stained with Dil Ac-LDL and counted under a fluorescence
microscope at a 200×magnification by independent blinded
investigators. Six independent fields were assessed for each
well, and the average number of adherent cells/200×field
was determined.

Migration assay The migration of EPCs was per-
fomed in a Transwell Chamber (Corning Costar). In brief,
EPCs were gently detached, harvested and resuspended in
chemotaxis buffer (EBM-2, 0.5% BSA). Chemotaxis buf-
fer (100 µL) containing 1×10⁵ cells was added to the upper
compartment and 600 µL of chemotaxis buffer with 100
ng/mL VEGF was added to the lower compartment. After
incubation at 37 °C for 4 h, the filters were removed and the
cells in the lower compartment were counted using flow
cytometry with appropriate gating for 20 s at a high flow
rate. Chemotaxis buffer (600 µL) with 1×10⁵ cells was also
counted in the same way as the standard. The migratory rate
was determined by calculating the percentage of the input
cells that migrated into the lower chamber divided by the
number of input cells originally added to the upper compart-
ment (1×10⁵). All groups of experiments were studied in
triple.

Tube formation assay ECM gel (Sigma) was thawed at
4 °C overnight and placed in a 96-well culture plate at 37 °C
for 1 h to allow for solidification. EPCs were harvested and
replated (10 000 cells/well) on top of the solidified ECM gel
in EBM-2 medium supplemented with 0.5% BSA and VEGF
(100 ng/mL). Cells were incubated at 37 °C for 24 h. Tube
formation was defined as a structure exhibiting a length four
times its width. The networks of tubes were photographed
from six randomly chosen fields under a microscope. The
total length of the tube structures in each photograph was
Semiquantitative reverse transcription-PCR
Total RNA was extracted from EPCs using the RNA extraction kit (Qiagen). To generate cDNA, RNA (250 ng) was reverse transcribed with reverse transcriptase. For PCR amplification, two specific primers were used to identify and amplify eNOS (sense primer: 5′-ATGAAGCAGCCTGGAGATGAG-3′; antisense primer: 5′-TCGGAGCCATACAGGATTG-3′). The PCR product was 299 bp. For PCR, 30 cycles were used at 94°C for 40 s, 50°C for 1 min and 72°C for 1 min. The reverse transcription PCR-amplified samples were visualized on 1% agarose gels using ethidium bromide. Each mRNA band was normalized with internal reference β-actin mRNA. Bands of interest were analyzed by scan analysis software and normalized to β-actin mRNA bands.

Western blot analysis
EPC lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked by incubation in Tris-buffered saline [10 mmol/L Tris (pH 7.5), 100 mmol/L NaCl] containing 0.1% (v/v) Tween 20 and 5% (v/v) non-fat dry milk for 2 h, followed by incubation at room temperature with rabbit polyclonal antiphospho-Akt-Ser 473 and eNOS-Ser1177 antibodies (Cell Signaling Technology) or mouse monoclonal anti-eNOS antibodies (BD Transduction Laboratories) for 2 h. The membranes were washed extensively in Tris-buffered saline containing 0.1% (v/v) Tween 20 before incubation for 1 h with a horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (Amersham Life Sciences). The membranes were then washed and developed using enhanced chemiluminescence (ECL) substrate (Amersham Life Sciences). The membranes were reprobed with an anti-actin antibody (Santa Cruz Biotechnology) or an anti-Akt antibody (Cell Signaling Technology).

Statistical analysis
Data were expressed as means±SD. Differences between group means were determined by a one-way ANOVA, followed by a Newman-Keuls test. P<0.05 was considered statistically significant.

Results
The identification of EPCs
After the CD34+ cells were sorted twice by immunomagnetic sorting with a CD34 antibody, the purity of enriched CD34+ cells was higher than 90% (Figure 1A). When CD34+ cells were cultured on fibronectin-coated dishes, many cell clusters appeared and numerous spindle-shape cells sprouted from the edge of the cell clusters after cultivation for 4–7 d. Flow cytometric analysis revealed that the ratio of CD31 expression was 67%±7%, the ratio of VE-cadherin expression was 70%±3%, and the ratio of vWF expression was 47%±5%. The expression levels of CD14, CD106, and CD45 were very low. The stem cell marker CD133 was still expressed by the EPCs (Figure 1B). To confirm that the cells isolated were EPCs, cells were double stained with CD31 and VE-cadherin or CD31 and CD45. Of the total cellular pool, 65%±8% of cells stained positively for CD31 and VE-cadherin; however, few cells were stained with both CD31 and CD45 (Figure 1C). EPCs took up Dil Ac-LDL and bound FITC-UEA-1 (Figure 1D), indicating that they expressed the scavenger receptor of Ac-LDL and the ligand of UEA-1.

The effect of lovastatin on EPC apoptosis
EPC apoptosis was examined by in situ TUNEL assay, in which TUNEL-positive cells were labeled with FITC and total adherent cells were dyed blue (Figure 2A). The percentage of apoptotic cells significantly increased when EPCs were exposed to 50 µg/mL oxLDL (oxLDL group: 21.12%±1.32% versus the control group, 8.99%±1.09%; P<0.05). The percentage of cells that became apoptotic in response to oxLDL was dose-dependently reduced when EPCs were pretreated with lovastatin (2, 10, and 50 µmol/L lovastatin group: 17.92%±1.54%, 13.63%±1.87%, 9.07%±0.63% versus the oxLDL group; P<0.05). However, the percentage of apoptotic cells was increased when EPCs were pretreated with lovastatin and triciribine at the same time (50 µmol/L lovastatin+triciribine group: 19.12%±2.64% vs control; P<0.05) (Figure 2B).

The effect of lovastatin on EPC adhesion
Adhesion of EPCs to extracellular matrix proteins is important for the formation of new blood vessels. EPCs treated with oxLDL showed a significant reduction in adhesion to fibronectin (oxLDL group: 27±2 vs control group: 37±5; P<0.05). When EPCs were pretreated with 2, 10, and 50 µmol/L lovastatin, adherent cells were partially or completely restored to the level of the control (2, 10, and 50 µmol/L lovastatin group: 32±2, 38±2, and 39±2 vs oxLDL group; P<0.05). However, when EPCs were pretreated withLovastatin and triciribine at the same time, the beneficial effect of lovastatin on EPC adhesion was abolished (50 µmol/L lovastatin+triciribine group: 27±2 vs 50 µmol/L lovastatin group; P<0.05) (Figure 3A and B).

The effect of lovastatin on EPC migration
We analyzed the migratory activity of EPCs in response to VEGF. Figure 4 shows a significant reduction in EPC migration after treatment with oxLDL (oxLDL group: 5.0%±0.6% vs the control group: 9.6%±0.9%; P<0.05). Pretreatment of
EPCs with lovastatin markedly inhibited the reduction of EPC migration in response to oxLDL (2, 10, and 50 μmol/L lovastatin groups: 6.8%±0.7%, 9.5%±0.7%, and 10.0%±0.6% vs oxLDL; P<0.05). However, when triciribine was added,
the beneficial effect of lovastatin was abolished (50 μmol/L lovastatin+triciribine group: 4.9±0.5 vs 50 μmol/L lovastatin group; *P*<0.05) (Figure 4).

**The effect of lovastatin on EPC tube formation**  EPCs incubated on matrigel for 24 h formed an extensive and enclosed tube network. OxLDL impaired the ability of EPCs to form this tube network (Figure 5A). The total length of the tube network in each photograph was decreased (oxLDL group: 2.1±0.4 mm vs control group: 4.6±0.6 mm; *P*<0.05). Furthermore, the tube network was severely disrupted, resulting in an incomplete and sparse tube network. Lovastatin restored the ability of EPCs to form a tube network (2, 10, and 50 μmol/L lovastatin groups: 3.2±0.2, 4.5±0.6, and 4.8±0.4 mm vs oxLDL group; *P*<0.05). Triciribine abolished the effect of lovastatin (50 μmol/L lovastatin+triciribine group: 2.1±0.4 mm vs 50 μmol/L lovastatin group; *P*<0.05) (Figure 4B).

**Regulation of eNOS mRNA expression by lovastatin**  We set the eNOS mRNA expression of the control equal to 100%. Figure 6 depicts a significant downregulation of eNOS mRNA expression when EPCs were incubated with oxLDL (oxLDL group: 41%±5% vs control: 100%; *P*<0.05). Lovastatin dose-dependently upregulated eNOS mRNA expression (2, 10, and 50 μmol/L lovastatin groups: 57%±5%, 91%±6%, and 106%±9% vs oxLDL group; *P*<0.05)

**Regulation of Akt and eNOS phosphorylation by lovastatin**  To test our hypothesis that lovastatin influenced EPC activity through the Akt/eNOS pathway, immunoblots...
were performed. OxLDL induced a downregulation of Akt phosphorylation (oxLDL group: 36%±3% vs control: 100%; \( P < 0.05 \)). However, lovastatin restored the phosphorylation of Akt (2, 10, and 50 µmol/L lovastatin group: 59%±7%, 91%±4%, and 98%±6% vs oxLDL group; \( P < 0.05 \)) (Figure 7).

To determine whether lovastatin influenced eNOS protein expression, eNOS protein was also assayed by immunoblots. Incubation of EPCs with oxLDL reduced eNOS protein expression (oxLDL group: 42%±7% vs control: 100%; \( P < 0.05 \)). Lovastatin (10 or 50 µmol/L) inhibited the down-regulation of eNOS protein (10 and 50 µmol/L lovastatin groups: 93%±4% and 105%±6% vs oxLDL group; \( P < 0.05 \)) (Figure 8A, 8B).

To further test our hypothesis that lovastatin influenced EPC activity through the Akt/eNOS pathway, the expression of phosphorylated eNOS at the Ser1177 phosphorylation site was tested. Because the level of eNOS protein was regulated by oxLDL and lovastatin, different amounts of total protein were applied to make the eNOS protein levels similar among groups. OxLDL also reduced eNOS phosphorylation (oxLDL group: 51%±8% vs control: 100%; \( P < 0.05 \)). Lovastatin (10 or 50 µmol/L) inhibited the downregulation of eNOS phosphorylation (10 and 50 µmol/L lovastatin groups: 93%±5% and 101%±4% vs oxLDL group; \( P < 0.05 \)) (Figure 8C, 8D).
Discussion

Recent experimental and clinical studies have indicated that statins, widely used cholesterol-lowering drugs, possess cholesterol-independent effects\(^{[11]}\). Thus, statins have multiple protective effects on EPCs. These findings explain why the number and function of circulating EPCs was augmented by statin therapy in patients with stable CAD\(^{[15]}\). Circulating EPCs serve as a biological marker for vascular function and participate in neovascularization following ischemic injury\(^{[1,14]}\). Therefore, augmentation of circulating EPC numbers and function by statins might be a novel strategy to improve neovascularization after tissue ischemia.

Another contribution of the study was to explore the mechanisms by which lovastatin exerted protective effects on EPCs. Activation of Akt plays a central role in the biology of EPCs, promoting the survival and mobilization of EPCs\(^{[7,15]}\). The present results suggest that Akt is also involved in the effects of lovastatin on EPCs. Phosphorylation of Akt is available to phosphorylate eNOS at Ser 1177, leading to eNOS activation in mature endothelial cells\(^{[9]}\). eNOS is also activated by phosphorylated Akt in EPCs. The activation of eNOS catalyzes nitric oxide (NO) generation, which plays a key role in maintaining cardiovascular homeostasis. NO is a significant regulator of EPC activity. Mice deficient in eNOS display impaired ischemia-induced angiogenesis and have reduced EPC mobilization\(^{[16]}\). Estrogen also increases EPC number by inducing NO generation\(^{[17]}\).

Previous studies have shown that many downstream targets of Akt are involved in cell survival pathways, including GSK-3, BAD, NF-κB, and the FKHR family of forkhead transcriptional factors\(^{[18]}\). Indeed, the forkhead transcription factor AFX (FOXO4) is expressed in EPCs and is inactivated by statins\(^{[12]}\). Therefore, other targets of Akt, such as FOXO4, might play a role in the effects of lovastatin. AMPK also phosphorylates eNOS at Ser 1177\(^{[19]}\), leading to eNOS activation. Furthermore, the activity of eNOS is regulated by caveolin-1\(^{[20]}\). Whether AMPK and caveolin-1 are involved in the effects of lovastatin on EPCs needs to be explored.

On the other hand, lovastatin up-regulated eNOS mRNA and protein expression. These findings not only suggest that activation of the Akt/eNOS pathway is involved in causing the effects of lovastatin, but also that increased gene transcription of eNOS is involved. Statins upregulate eNOS by blocking Rho geranylgeranylation and by increasing the stability of eNOS mRNA\(^{[21]}\), which could explain why lovastatin upregulated the gene transcription of eNOS.

In conclusion, the results of the present study demonstrate that treatment of EPCs with lovastatin significantly reverses the detrimental effects of oxLDL on the survival and function of EPCs through activation of the Akt/eNOS pathway and induction of eNOS gene transcription. Statins had been previously shown that atorvastatin inhibits EPC senescence and increases EPC proliferation\(^{[12]}\). Thus, statins have multiple protective effects on EPCs. These findings explain why the number and function of circulating EPCs was augmented by statin therapy in patients with stable CAD\(^{[15]}\).

![Figure 8. Effects of lovastatin on eNOS and p-eNOS protein expression. OxLDL decreased eNOS and p-eNOS protein expression, but pretreatment with lovastatin inhibited the downregulation of eNOS and p-eNOS protein expression. Representative blots of eNOS and p-eNOS protein (A, C). Summary of data of eNOS and p-eNOS protein expression (B, D). n=3. Mean±SD. \(b\) \(\text{P}<0.05\) vs control. \(e\) \(\text{P}<0.05\) vs oxLDL.](image-url)
pleiotropic protective effects on EPCs through via multiple mechanisms. Given the well-established role of EPCs in vessel repair after ischemic injury, statins might be therapeutically useful to promote the vasculogenesis in ischemic tissue, even in patients with normal cholesterol levels.

Acknowledgements

This work was supported by the National High Technology Research and Development Program of China (No 2006AA02A110).

Author contribution

Zhong-chao HAN and Feng-xia MA designed the research; Feng-xia MA, Fang CHEN and Qian REN performed the research; Fang CHEN and Qian REN contributed analytic reagents and tools; Feng-xia MA analyzed the data; Zhong-chao HAN and Feng-xia MA wrote the paper.

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