

Visfatin attenuates the ox-LDL-induced senescence of endothelial progenitor cells by upregulating SIRT1 expression through the PI3K/Akt/ERK pathway

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Abstract. Endothelial progenitor cells (EPCs) play an important role in aging-associated senescence, thereby potentially contributing to vascular pathologies. Visfatin, identified as a new adipocytokine, is closely associated with the senescence of human cells. However, the effects of visfatin on the oxidized low-density lipoprotein (ox-LDL)-induced senescence of EPCs has not yet been explored, to the best of our knowledge. For this purpose, in the present study, we examined the effects of visfatin in ox-LDL-stimulated EPCs as well as the underlying mechanism responsible for these effects. We found that visfatin attenuated the ox-LDL-induced senescence of EPCs by repressing β -galactosidase expression and recovering telomerase activity. Western blot analysis confirmed that visfatin induced a dose-dependent increase in sirtuin 1 (SIRT1) expression in EPCs and ox-LDL exposure decreased SIRT1 expression. Silencing SIRT1 abolished the inhibition of EPC senescence and the suppression of p53 expression induced by visfatin. Moreover, visfatin attenuated the inhibition of phosphorylation of Akt, phosphoinositide-3-kinase (PI3K) and extracellular signal-regulated kinase (ERK) induced by ox-LDL. Taken together, these findings suggest that the treatment of EPCs with visfatin markedly attenuates the ox-LDL-induced senescence of EPCs by upregulating SIRT1 expression through the PI3K/Akt/ERK pathway.

Introduction

Endothelial progenitor cells (EPCs) play an important role in vascular morphogenesis, both during embryonic development and in postnatal neovascularization, thereby contributing to tissue maintenance and repair in various pathological conditions (1,2).

Previous findings have shown that EPCs contribute to blood flow recovery in ischemic limbs and improve left ventricular function following myocardial infarction (3,4), indicating a potential role for EPCs in the cell-based therapeutic neovascularization of ischemic tissues. However, the numbers and function of EPCs decline as part of aging-associated senescence; aged EPCs are less able to contribute to vascular repair and regeneration and thereby contribute to the increased propensity toward vascular pathology (5,6). Therefore, it is critical to further elucidate the mechanism responsible for the senescence of EPCs in order to provide novel insights into therapeutic neovascularization.

Oxidized low-density lipoprotein (ox-LDL) is taken up by EPCs in a receptor-dependent manner wherein it generates oxidative stress. The level of ox-LDL increases in patients with coronary artery disease or diabetes and serves as an independent predictor for future cardiac events in these patients (7,8). Several studies have shown that ox-LDL functions as an important factor that adversely affects the growth and bioactivity of EPCs by triggering apoptosis, inducing cell ossification and inhibiting endothelialization (9-12). It has also been demonstrated that ox-LDL also accelerated the senescence of EPCs, leading to cellular dysfunction (13,14). Lai and Liu showed that ox-LDL-induced senescence of EPCs was mediated by oxidative stress and the Akt/hTERT pathway (15).

Visfatin was originally cloned in 1994 as a cytokine named pre-B cell colony-enhancing factor (PBEF) from a bone marrow cDNA library based on sequence similarities to cytokines (16). It is now well established that visfatin is expressed in adipose tissue as well as skeletal muscle, liver and immune cells. Visfatin has also been found to be produced within the myocardium localized to cardiomyocytes and fibroblasts, and within the brain localized to neuronal cells, with particularly high expression during ischemia. The widespread distribution of this molecule is indicative of the broad functions of visfatin in both health and disease. Recently, visfatin has been implicated in the pathogenesis of various cardiovascular disorders, such as atherosclerosis and myocardial failure, and in brain ischemia (16). van der Veer *et al* showed that FK866, an antagonist of visfatin, induced premature senescence in human vascular smooth muscle cells, together with enhanced resistance to oxidative stress (17). However, the effects of visfatin on the ox-LDL-induced senescence of EPCs as well as the underlying mechanism responsible

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for these effects has not yet been explored, to the best of our knowledge. In the present study, we report that visfatin is a senescence-related protein that inhibits the ox-LDL-induced senescence of EPCs by activating sirtuin 1 (SIRT1) through the phosphoinositide-3-kinase (PI3K)/Akt/extracellular signal-regulated kinase (ERK) pathway.

Materials and methods

Cell culture. EPCs were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 100 µg/ml penicillin/streptomycin (all from Invitrogen, Carlsbad, CA, USA).

Ox-LDL preparation. The fresh human plasma was collected from patients at Xiangya Hospital of Central South University (Changsha, China). Human ox-LDL was isolated from fresh human plasma by sequential ultracentrifugation, as previously described (18). Briefly, LDL was isolated by ultracentrifugation at 290,000 x g for 4 h in a sodium bromide (NaBr) gradient, and the top layer (containing LDL) was collected. To validate the observations made with copper-oxidized LDL, we performed additional experiments using L5, an electronegative and minimally oxidized LDL circulating in patients with hypercholesterolemia.

Telomeric repeat amplification protocol (TRAP) assay. For quantitative analysis of telomerase activity, the TRAP assay, in which the telomerase reaction product is amplified by a polymerase chain reaction (PCR), was performed using the *TeloTAGGG* PCR ELISA^{PLUS} kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions.

Construction of shRNAs. We designed and purchased three different shRNA duplexes of SIRT1 from Gene Pharma (Shanghai, China). The following primers were used: shRNA-1 (SIRT1-homo-1075) sense, 5'-CACCGCAACTATACCCAGA ACATAGTTCAAGAGACTATGTTCTGGGTATAGTTGCTTT TTTG-3' and reverse, 5'-GATCCAAAAAAGCAACTAT ACCCAGAACATAGTCTCTTGAAGTATGTTCTGGGTAT AGTTGC-3'; shRNA-2 (SIRT1-homo-1961) sense, 5'-CACCG CTTGATGGTAATCAGTATCTTTCAAGAGAAGATACTG ATTACCATCAAGCTTTTTTG-3' and reverse, 5'-GATCCA AAAAAGCTTGATGGTAATCAGTATCTTCTTGAAG ATACTGATTACCATCAAGC-3'; shRNA-3 (SIRT1-homo-2214) sense, 5'-CACCGGAGATGATCAAGAGGCAATTTC AAGAGAATTGCCTCTTGATCATCTCCT TTTTTG-3 and reverse, 5'-GATCCAAAAAAGGAGATGATCAAGA GGCAATTCTTGAAGTGCCTCTTGATCATCTCC-3.

Transfection. Twenty-four hours prior to transfection, the cells were seeded in a 6-well culture plate. SIRT1 shRNAs were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Twenty-four or forty-eight hours later, the EPCs were collected and subjected to further analysis. Total RNA or protein was extracted from the indicated cells for analysis. The experiment was performed in triplicate. More than nine wells were treated with the same type of shRNA.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from the cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. qPCR was performed using the StepOne Plus sequence detection system (Applied Biosystems, Foster City, CA, USA). To ensure the reproducibility of the results, all the genes were tested in triplicate. The following primers were used: SIRT1 sense (5'-TAGCCTTGTCAGATAAGGAAGGA-3') and antisense (5'-ACAGCTTCACAGTCAACTTTGT-3'); p53 sense (5'-ACTTGTCGCTCTTGAAGCTAC-3') and antisense (5'-GATGCGGAGAATCTTTGGAACA-3'); AKT1 sense (5'-AGCGACGTGGCTATTTGTGAAG-3') and antisense (5'-GCCATCATTCTTGAGGAGGAAGT-3'); PI3K/p85 sense (5'-ACACCACGGTTTGGACTATGG-3') and antisense (5'-GGCTACAGTAGTGGGCTTGG-3'); ERK sense (5'-ACACCACGGTTTGGACTATGG-3') and antisense (5'-GGCTACAGTAGTGGGCTTGG-3'); and β-actin sense, (5'-CATTAAGGAGAAGCTGTGCT-3') and antisense (5'-GTTGAAGGTAGTTTCGTGGA-3'). The cycling conditions were an initial denaturation for 2 min at 94°C; followed by 40 cycles of 10 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C.

Western blot analysis. Whole cell extracts were prepared with a cell lysis reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions, and then, the protein was quantified using a BCA assay (Pierce, Rockford, IL, USA). The protein samples were then separated by SDS-PAGE (10%) and detected by western blot analysis using polyclonal rabbit anti-PI3K p85 antibody (#4257; Cell Signaling Technology, Danvers, MA, USA,), rabbit anti-p53 antibody (sc-6243; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-β-galactosidase (Gal) antibody (Cell Signaling Technology, #2372), rabbit anti-Akt antibody (#9272; Cell Signaling Technology), rabbit anti-SIRT1 antibody (ab110304; Abcam, Cambridge, UK), rabbit anti-phosphorylated (p-)Akt(Ser473) antibody (ab81283; Abcam), rabbit anti-ERK antibody (#4695; Cell Signaling Technology), rabbit anti-p-ERK antibody (#9101; Cell Signaling Technology) and monoclonal mouse anti-GAPDH (SC-365062; Santa Cruz Biotechnology) as a control. Goat anti-rabbit IgG (Pierce) secondary antibody conjugated to horseradish peroxidase and ECL detection systems (SuperSignal West Femto; Pierce) were used for detection.

Statistical analysis. Each experiment was repeated at least three times. Data are shown as the means ± SD and were analyzed using SPSS 18.0. Statistical comparisons between groups were analyzed using the Student's t-test and a two-tailed p<0.05 was considered to indicate a statistically significant difference.

Results

Ox-LDL induces EPC senescence. To assess the onset of senescence, β-Gal expression and telomerase activity were detected as biochemical markers of cellular senescence. As shown in Fig. 1A, ox-LDL exposure evidently increased β-Gal expression. The acceleration of EPC senescence occurred dose dependently, with a maximal effect achieved at a concentration of 4.0 nM ox-LDL.

Cellular senescence is critically influenced by telomerase, which elongates telomeres, thereby counteracting the

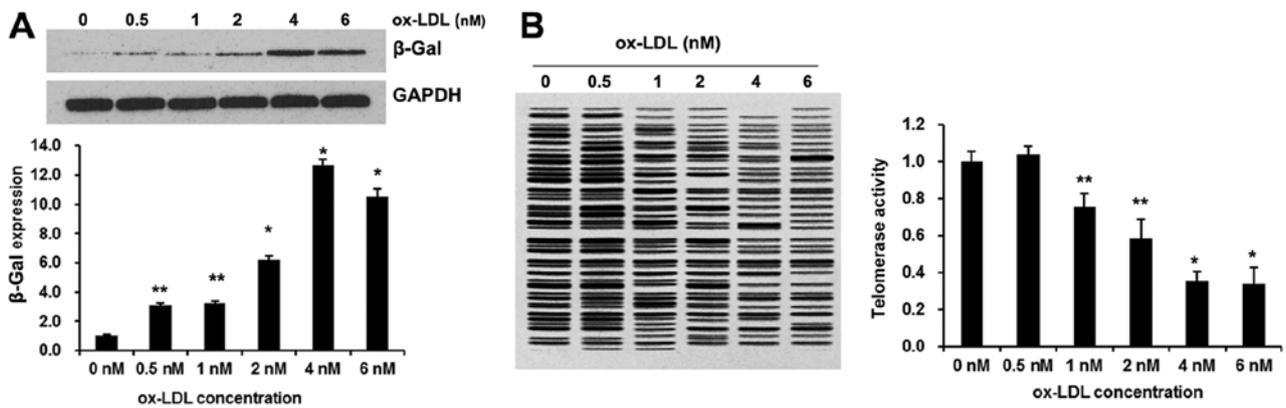


Figure 1. Dose-response relationships between oxidized low-density lipoprotein (ox-LDL) and the senescence of endothelial progenitor cells (EPCs). EPCs were treated with the indicated concentrations of ox-LDL for 24 h. (A) The effects of ox-LDL on β -galactosidase (β -Gal) expression. (B) The effects of ox-LDL on telomerase activity in EPCs. Error bars represent \pm SD and * p <0.01 versus control and 0 nM ox-LDL. ** p <0.05 vs. 0 nM ox-LDL.

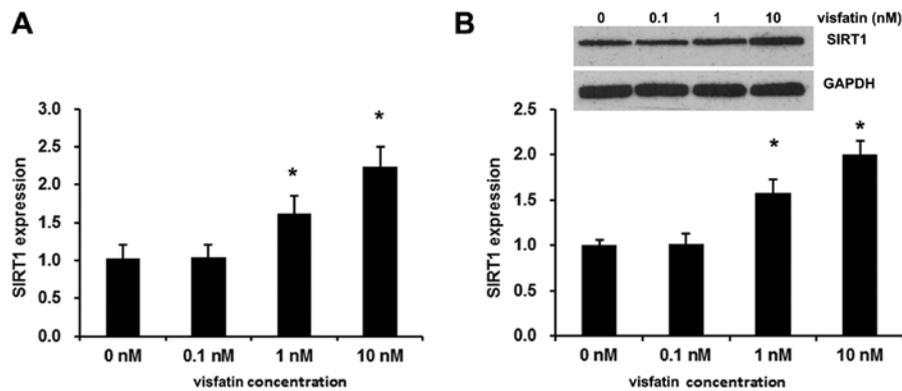


Figure 2. Visfatin affects sirtuin 1 (SIRT1) expression in endothelial progenitor cells (EPCs). EPCs were treated with the indicated concentrations of visfatin for 30 min. (A) RT-qPCR reveals the effects of visfatin on SIRT1 expression. (B) Western blot analysis reveals the effects of visfatin on SIRT1 expression. Error bars represent \pm SD and * p <0.01 versus control and 0 nM ox-LDL.

telomere length reduction induced by each cell division. Breitschopf *et al* have demonstrated that pro-atherosclerotic factors impair telomerase activity in mature endothelial cells (19). Therefore, we measured telomerase activity using the *TeloTAGGG* PCR ELISA^{PLUS} kit. As demonstrated in Fig. 1B, 4.0 nM ox-LDL significantly diminished telomerase activity to approximately 60%. These results clearly demonstrated that 4.0 nM ox-LDL significantly accelerates the senescence of EPCs.

Visfatin induces SIRT1 expression in EPCs. SIRT1 is an anti-aging molecule and SIRT1 expression and activity reduction are involved in aging-associated diseases (20,21). In the present study, exogenous visfatin evidently increased SIRT1 expression. The acceleration of the mRNA and protein expression of SIRT1 occurred dose dependently, with a maximal effect achieved at a concentration of 10 nM visfatin (Fig. 2).

Visfatin abolishes ox-LDL-induced senescence in EPCs by upregulating SIRT1. Herein, we analyzed the effects of visfatin on SIRT1 and p53 in ox-LDL-stimulated EPCs. Firstly, 3 SIRT1 shRNAs were used to silence SIRT1 expression. As shown in Fig. 3A, shRNA2 effectively blocked the expression of SIRT1 mRNA. Western blot analysis revealed marked reductions of 82.2% with shRNA2, 72.6% with shRNA3, and 3.2% with

shRNA1 in the protein levels of SIRT1 following the transfection of EPCs with SIRT1 shRNAs, compared with the control (untransfected) group and NC (negative control) group (Fig. 3B) (p <0.01). Thus, EPCs transfected with SIRT1 shRNA2 were used for subsequent experiments. Next, 10 nM visfatin evidently increased the mRNA (Fig. 3C) and protein (Fig. 3D) expression levels of SIRT1 in ox-LDL-stimulated EPCs, which were reversed by SIRT1 shRNA. Moreover, 10 nM visfatin evidently decreased the mRNA (Fig. 3E) and protein (Fig. 3F) expression levels of p53 in ox-LDL-stimulated EPCs, which were also reversed by SIRT1 shRNA.

We then analyzed the role of visfatin in the ox-LDL-induced senescence of EPCs. The treatment of EPCs with visfatin decreased senescence-associated- β -Gal expression (Fig. 4A). Moreover, visfatin evidently reversed the ox-LDL-induced decrease in telomerase activity (Fig. 4B). These results raised the possibility that visfatin may abolish the ox-LDL-induced senescence of EPCs. We then analyzed the role of SIRT1 in the ox-LDL-induced senescence of EPCs. We found that SIRT1-shRNA abolished the effects of visfatin on the ox-LDL-induced senescence of EPCs. In cultured mesangial cells, it has been demonstrated that SIRT1 inhibits oxidative stress-related apoptosis through p53 deacetylation (22). Herein, we found that visfatin evidently decreased p53 expression in EPCs,

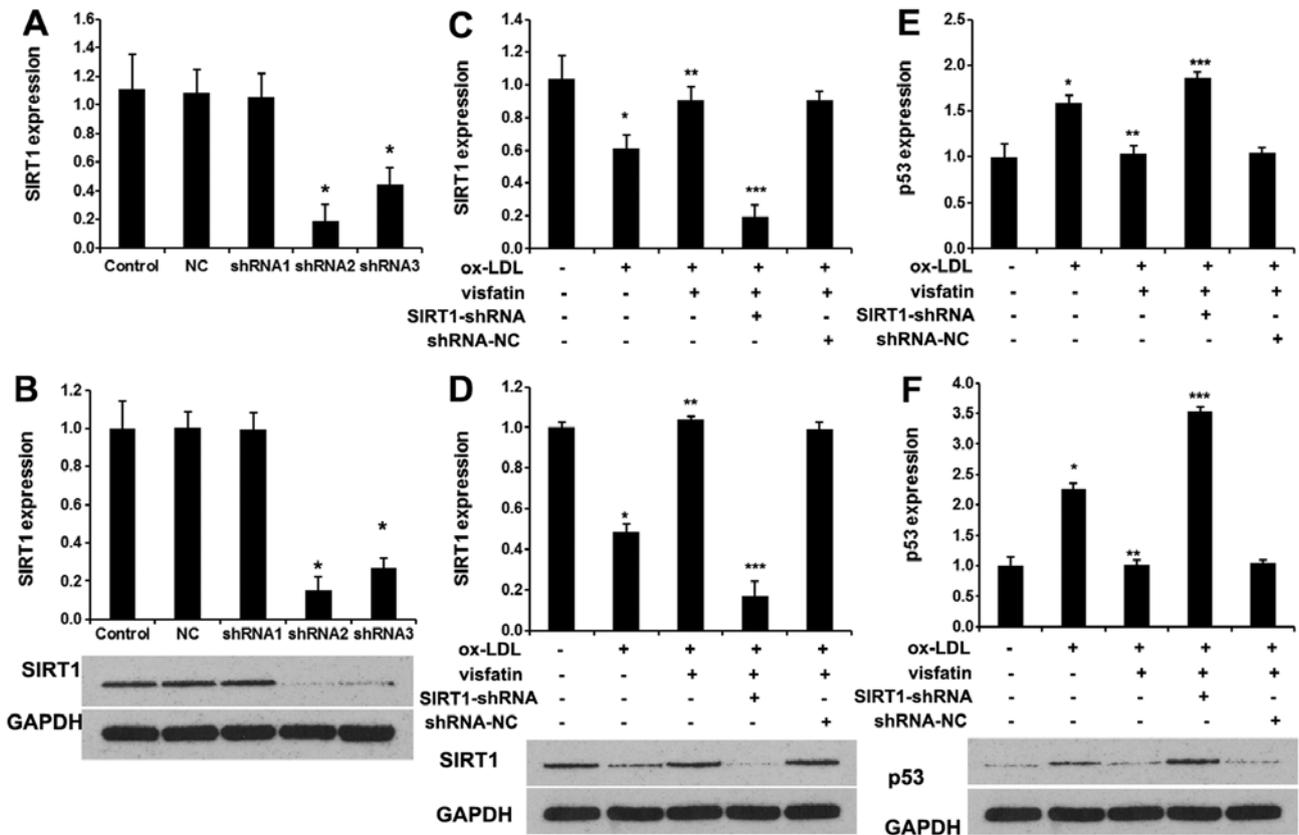


Figure 3. Visfatin affects sirtuin 1 (SIRT1) and p53 expression in oxidized low-density lipoprotein (ox-LDL)-stimulated endothelial progenitor cells (EPCs). EPCs were treated with 4.0 nM ox-LDL, 10 nM visfatin and SIRT1shRNA. (A) RT-qPCR and (B) western blot analysis revealed the effects of SIRT1 shRNAs on SIRT1 expression in EPCs. (C) RT-qPCR and (D) western blot analysis revealed SIRT1 expression in EPCs. (E) RT-qPCR and (F) western blot analysis revealed p53 expression in EPCs. Error bars represent \pm SD. * $p < 0.01$ versus control group. ** $p < 0.01$ vs. ox-LDL treatment group. *** $p < 0.01$ vs. ox-LDL and visfatin treatment group.

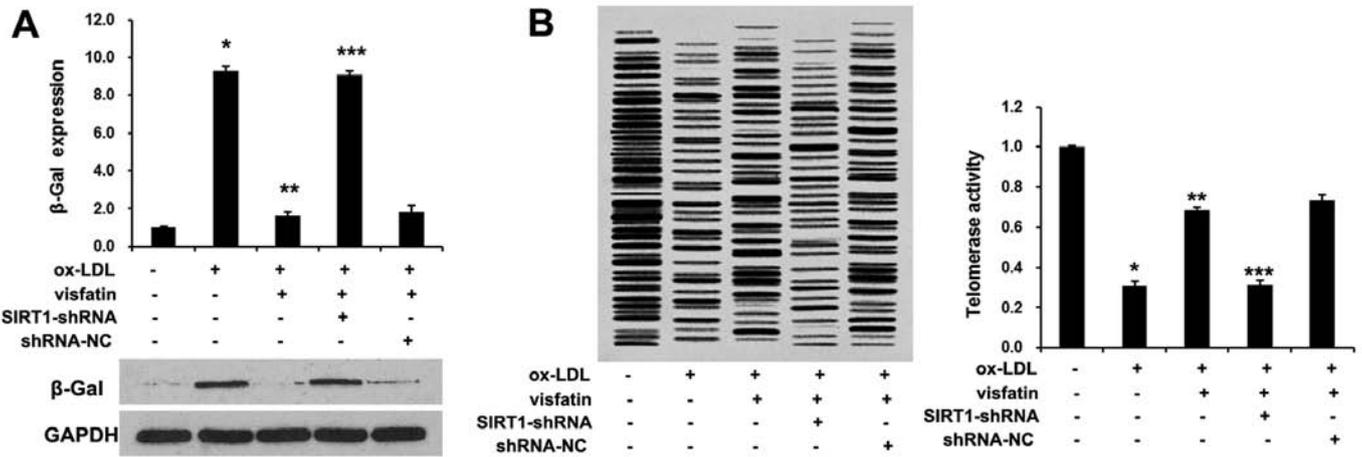


Figure 4. Visfatin abolishes oxidized low-density lipoprotein (ox-LDL)-induced senescence in endothelial progenitor cells (EPCs). EPCs were treated with 4.0 nM ox-LDL, 10 nM visfatin and SIRT1shRNA. EPC senescence was evaluated by (A) β -galactosidase (β -Gal) expression and (B) telomerase activity of EPCs. Error bars represent \pm SD. * $p < 0.01$ versus control group. ** $p < 0.01$ versus ox-LDL treatment group. *** $p < 0.01$ versus ox-LDL and visfatin treatment group.

which was reversed by SIRT1-shRNA. In conclusion, these results indicated that visfatin abolished the ox-LDL-induced senescence of EPCs by upregulating SIRT1 expression.

Visfatin regulates SIRT1 expression through the PI3K/Akt/ERK pathway. To examine the role of the PI3K/Akt/ERK pathway in the visfatin-mediated senescence of EPCs, the cells were treated with ox-LDL and then visfatin, finally the inhibi-

tors, LY294002, SCH772984. SCH772984 (Active Biochem, Maplewood, NJ, USA) was used to inhibit ERK and LY294002 (Selleck Chemicals, Houston, TX, USA) was used to inhibit PI3K. Consistent with previous findings (18), ox-LDL disrupts the PI3K/Akt signaling pathway by reducing the p-Akt/Akt and p-PI3K/PI3K (p85) ratio in EPCs (Fig. 5), which was reversed by visfatin treatment. Furthermore, LY294002 abolished visfatin-induced PI3K phosphorylation and Akt

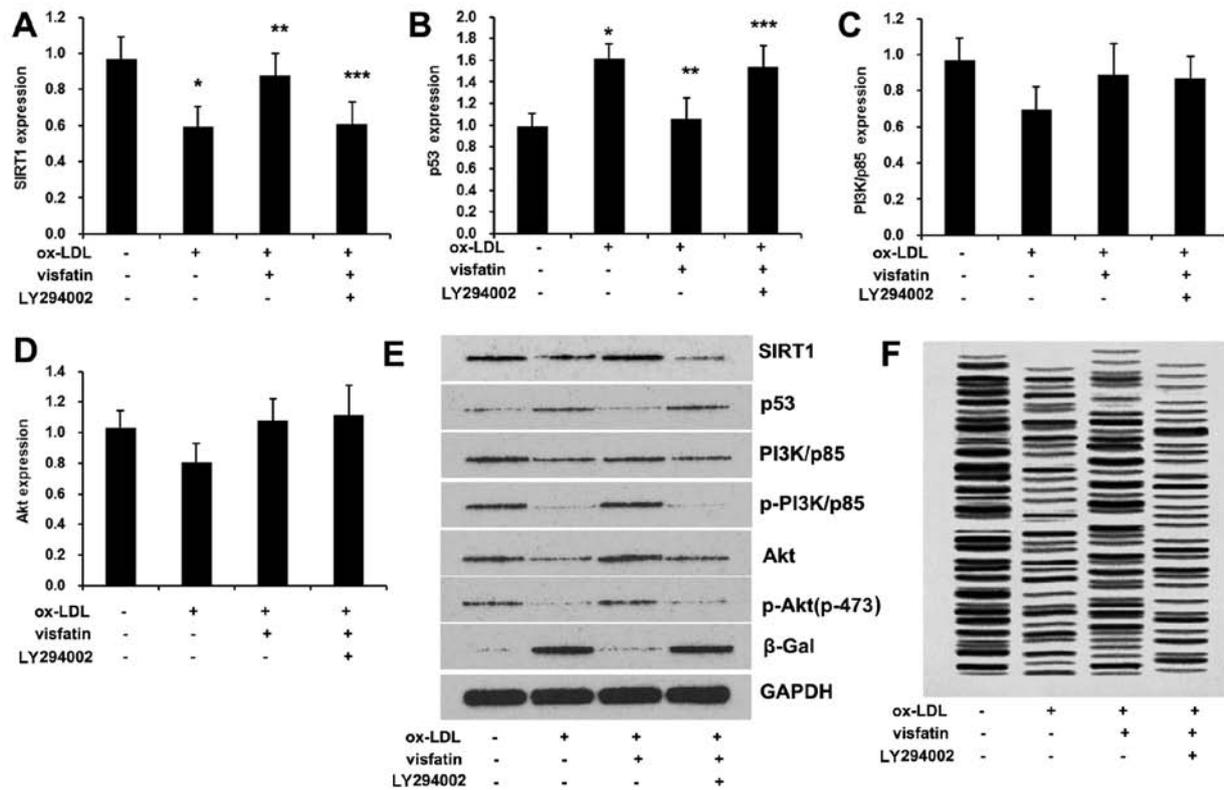


Figure 5. Visfatin regulates sirtuin 1 (SIRT1) expression through the PI3K/Akt pathway. Endothelial progenitor cells (EPCs) were treated with 4.0 nM oxidized low-density lipoprotein (ox-LDL), 10 nM visfatin and PI3K inhibitor (LY294002). RT-qPCR reveals (A) SIRT1 expression, (B) p53 expression, (C) PI3K expression and (D) Akt expression in EPCs. (E) Western blot analysis reveals SIRT1, p53, PI3K/p85, phosphorylated (p)-PI3K/p85, Akt, p-Akt(p-473) and β -galactosidase (β -Gal) expression in EPCs. (F) Telomerase activity of EPCs. Error bars represent \pm SD. * $p < 0.01$ versus control group. ** $p < 0.01$ vs. ox-LDL treatment group. *** $p < 0.01$ versus ox-LDL and visfatin treatment group.

phosphorylation (Fig. 5E). In addition, LY294002 abrogated the visfatin-induced increase in the expression levels of SIRT1, as well as the visfatin-induced decrease in the expression of p53 (Fig. 5A, B and E), and diminished telomerase activity (Fig. 5F). These results suggested that the PI3K/Akt pathway was regulated by visfatin in order to mediate SIRT1 expression and ox-LDL-induced senescence of EPCs. Moreover, ox-LDL also reduced the p-ERK/ERK ratio in EPCs (Fig. 6D), which was reversed by visfatin treatment. Pre-treatment with SCH772984 abolished the visfatin-induced increase in ERK phosphorylation (Fig. 6D). It should be noted that no significant effects were observed on ERK mRNA expression (Fig. 6C). In addition, SCH772984 abolished the visfatin-induced increase in the expression levels of SIRT1, as well as the visfatin-induced decrease in p53 expression (Fig. 6A, B and D), and diminished telomerase activity (Fig. 5F), suggesting that visfatin-regulated SIRT1 expression and ox-LDL-induced senescence of EPCs involved ERK phosphorylation. In conclusion, visfatin regulates SIRT1 expression in ox-LDL-induced EPC senescence through the PI3K/Akt/ERK pathway.

Discussion

Previous findings suggest that circulating EPCs serve as a biological marker for vascular function (23) and that the number of EPCs is a significant predictor of future cardiovascular events (24,25). EPCs also play an important role in post-ischemic vascular repair (26,27). Ox-LDL, as one of the most important

risk factors for cardiovascular disease, has been revealed to exert a detrimental effect on the growth and bioactivity of EPCs by triggering apoptosis, inducing cell ossification and inhibiting endothelialization (9-12). For example, Ji *et al* showed that ox-LDL significantly decreased the proliferation, migration and adhesion capacity of EPCs, whereas ROS production and NADPH oxidase expression was significantly increased (28). Ox-LDL stimulates p53-dependent activation of pro-apoptotic Bax leading to apoptosis of differentiated EPCs (29). Ox-LDL induced dose- and time-dependent activation of p38 MAPK, which plays a critical role in regulating the numbers and functions of EPCs (30). Previous findings have demonstrated that ox-LDL accelerates the onset of EPC senescence, which may be related to telomerase inactivation, leading to the impairment of proliferative capacity and network formation (13). Consistent with this study (13), the present study found that ox-LDL induces EPC senescence by evidently increasing β -Gal expression and diminishing telomerase activity.

Visfatin has been implicated in the pathogenesis of various cardiovascular disorders including atherosclerosis and myocardial failure, and in brain ischemia (16,31). Previously, van der Veer *et al* showed that FK866, an antagonist of visfatin, induced premature senescence in human vascular smooth muscle cells, indicating visfatin is a potential anti-senescence molecule (17). In this study, we found that 10 nM visfatin evidently abolished ox-LDL-induced EPC senescence, thereby providing a novel therapeutic target for the reversal of EPC dysfunction in neovascularization.

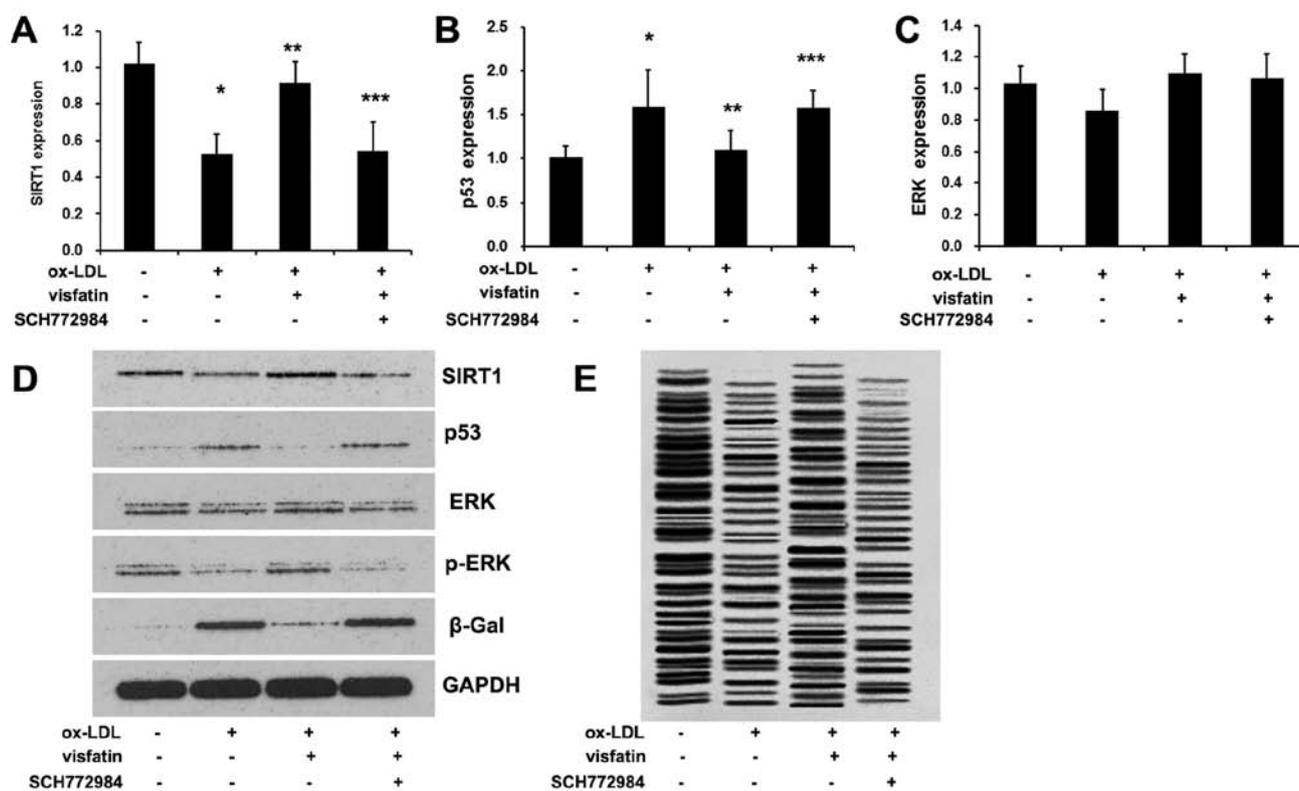


Figure 6. Visfatin regulates sirtuin 1 (SIRT1) expression through the ERK pathway. Endothelial progenitor cells (EPCs) were treated with 4.0 nM oxidized low-density lipoprotein (ox-LDL), 10 nM visfatin and ERK inhibitor (SCH772984). RT-qPCR reveals (A) SIRT1 expression, (B) p53 expression and (C) ERK expression in EPCs. (D) Western blot analysis reveals SIRT1, p53, ERK, phosphorylated (p-)ERK and β -galactosidase (β -Gal) expression in EPCs. (E) Telomerase activity of EPCs. Error bars represent \pm SD. * $p < 0.01$ versus control group. ** $p < 0.01$ versus ox-LDL treatment group. *** $p < 0.01$ versus ox-LDL and visfatin treatment group.

The family of sirtuin proteins are a class of NAD⁺-dependent protein deacetylases which are proteins regulating key cellular processes including cell cycle regulation, apoptosis, metabolic regulation and inflammation (20). SIRT1 is reported to be a regulator of ageing and its increased expression or activation prolonged lifespan in lower forms of animals (32). For example, Braidy *et al* demonstrated that SIRT1 expression increases with age, concurrently with increased acetylated p53 levels in all brain regions investigated (21). Kume *et al* showed that SIRT1 attenuates oxidative stress-induced mesangial cell apoptosis through p53 deacetylation (22). A previous study showed that SIRT1 is a critical modulator of EPC dysfunction during alterations of glucose metabolism (33), indicating that the modulation of SIRT1 activity and/or expression may provide an opportunity for counteracting the impairment of EPC levels and functionality associated with the ageing of EPCs. Moreover, dimethylarginine dimethylaminohydrolase 2 (DDAH)/asymmetric dimethylarginine (ADMA) is involved in the accelerated senescence of EPCs in diabetes, which is associated with the activation of SIRT1 (34). In this study, we found that down-regulated SIRT1 expression is associated with ox-LDL-induced EPC senescence. We further analyzed the association between visfatin and SIRT1. We found that visfatin significantly up-regulated SIRT1 expression and silenced SIRT1 evidently abolished the inhibition of senescence in EPCs and the suppression of p53 expression induced by visfatin. In conclusion, these results indicated that visfatin abolished the ox-LDL-induced senescence of EPCs by upregulating SIRT1 expression.

The PI3K/Akt signaling pathway is critical for the function and survival of EPCs (35), based largely on the downstream activation of endothelial nitric oxide synthase (eNOS) and subsequent production of NO (9,36). Imanishi *et al* (11) reported that ox-LDL impairs EPC function through effects on Akt. Imanishi *et al* showed that the ox-LDL effects on Akt within EPCs are mediated indirectly, by an upstream disruption of PI3K, specifically via nitrosylation of the p85 subunit and subsequent dissociation of the p85 and p110 subunits (13). In the present study, LY294002, a PI3K inhibitor, significantly reversed the inhibition of ox-LDL-induced senescence in EPCs as well as SIRT1 expression induced by visfatin, indicating that the PI3K/Akt signaling pathway was regulated by visfatin in order to mediate SIRT1 expression and ox-LDL-induced senescence of EPCs. The ERK pathway is often associated with SIRT1 expression in various types of cell (37). In the present study, we further analyzed whether the ERK signaling pathway is also important in ox-LDL-induced EPC senescence. We found that ox-LDL induced a decrease in the p-ERK/ERK ratio, which was reversed by visfatin. SCH772984, a selective inhibitor of ERK, significantly reduced the inhibition of ox-LDL-induced EPC senescence and the increase in SIRT1 expression induced by visfatin.

In conclusion, for the first time to the best of our knowledge, we demonstrate the anti-senescence effects of visfatin in ox-LDL-stimulated EPCs, and show that visfatin markedly attenuates ox-LDL-induced EPC senescence by upregulating SIRT1 expression through the PI3K/Akt/ERK pathway,

providing a novel therapeutic strategies for the reversal of EPC dysfunction.

Acknowledgements

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