MicroRNA let-7g inhibits angiotensin II-induced endothelial senescence via the LOX-1-independent mechanism

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Abstract. Endothelial senescence leads to cell dysfunction, which in turn eventually results in cardiovascular disease. Identifying factors that regulate endothelial senescence may provide insight into the pathogenesis of aging. Insulin-like growth factor (IGF) signaling has a significant role in the physiology of endothelial cells (ECs). Overactivation of IGF signaling has been implicated in promoting the aging process. Lectin-like oxidized low-density lipoprotein (oxLDL) receptor-1 (LOX-1) is a scavenger receptor that mediates the internalization of oxLDL into cells. Previous studies by our group have indicated that microRNA let-7g exerts an anti-aging effect on ECs and also suppresses LOX-1 expression. Since LOX-1 also induces the aging process, the present study we explored whether let-7g still exerts an anti-aging effect on ECs when LOX-1 is suppressed. Angiotensin II (Ang II) was used to induce senescence in ECs. It was revealed that Ang II significantly increased the expression of aging markers, including β-galactosidase, LOX-1, IGF1 and its receptor IGF1R. On the contrary, Ang II decreased the expression of the anti-aging gene sirtuin 1 (SIRT1). When LOX-1 was knocked down by small interfering RNA, let-7g still exerted an anti-aging effect on ECs. Using senescence-associated β-gal staining, it was confirmed that let-7g exerts a LOX-1-independent anti-aging effect on ECs. In conclusion, the present study demonstrated that let-7g has an anti-aging effect regardless of the presence or absence of LOX-1.

Introduction

Senescence may be caused by telomere uncapping due to replicative exhaustion, mitochondrial deterioration, oxidative stress, severe or irreparable DNA damage or selected oncogene expression (1). Endothelial senescence leads to cell dysfunction, which in turn eventually results in cardiovascular diseases, including hypertension and atherosclerosis (2). Oxidized low-density lipoprotein (oxLDL) is an important risk factor in promoting the development of atherosclerosis. oxLDL stimulates endothelial cells (ECs) to produce adhesion molecules and proinflammatory substances. The scavenger receptor lectin-like oxLDL receptor-1 (LOX-1) mediates the binding and internalization of oxLDL into cells. Accordingly, LOX-1 has a critical role in endothelial function (3), and LOX-1 has been suggested to be an aging marker (4).

Angiotensin II (Ang II) is the primary effector of the renin-angiotensin system (RAS) and is a multifunctional hormone that has a major role in regulating blood pressure and cardiovascular homeostasis (5). Mammalian cells express 2 types of Ang II receptors, Ang II type 1 receptor (AT1R) and AT2R (6,7). Ang II-induced reactive oxygen species production via AT1R promotes the onset of vascular senescence as well as functional and structural changes in blood vessels (8). Ang II signaling is considered to contribute to the pathogenesis of atherosclerosis (9). In addition, Ang II has been reported to upregulate LOX-1, which facilitates oxLDL uptake by ECs (10). However, it remains elusive whether Ang II also deteriorates EC function via a LOX-1-independent pathway.

Sirtuin 1 (SIRT1) is implicated in anti-aging processes and has a role in the tolerance of oxidative stress (11). A previous study by our group demonstrated that micro (mi)RNA let-7g increased SIRT1 expression in ECs (12). SIRT1 is highly

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expressed in blood vessels, and a decrease of SIRT1 expression may be a risk factor for atherosclerosis (13-15). The insulin-like growth factor 1 (IGF1) signal transduction pathway has been demonstrated to be associated with the aging process (16,17). Furthermore, SIRT1 expression has been negatively correlated with the level of IGF1 (18). SIRT1 has been linked to the insulin/IGF signaling pathway in Caenorhabditis elegans, drosophila and mice through its ability to deacetylate forkhead box O proteins (19-21). Treatment of cells with IGF1 may decrease their SIRT1 levels, suggesting an inverse association between SIRT1 and the insulin/IGF pathway (20).

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression through degradation or translational inhibition of targeted mRNA (22). The miRNA let-7 family has a pivotal role in cell proliferation, cancer and cardiovascular diseases (23,24). A previous study by our group reported that let-7g directly targets and suppresses LOX-1 (25). Further studies by our group have reported that let-7g improves several endothelial functions by causing a decrease of senescence, inflammation and monocyte adhesion, and an increase of angiogenesis (12,25,26). The present study aimed to investigate whether let-7g also provides endothelial protection via a LOX-1-independent mechanism. Let-7g was tested for its anti-aging effect against Ang II-induced senescence in ECs while LOX-1 was suppressed.

Materials and methods

Cell culture and treatment. Human umbilical vein endothelial cells (HUVECs) were purchased as pooled primary cells from Lonza (Basel, Switzerland). The HUVECs were cultured at 37°C in an atmosphere of 5% CO₂ in EGM-2 medium (EBM medium with supplement) containing 0.5% FBS for 24 h to synchronize the cells in G0 phase. Since Ang II at 10⁷ nM was reported to increase HUVEC proliferation (27), Ang II (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 10⁻⁷ nM was initially applied to HUVECs for 24 h to determine the optimal dose for subsequent experiments.

Transfection of LOX-1 small-interfering (si)RNA. The HUVECs were synchronized via starvation for 24 h prior to each experiment. After they were arrested, the HUVECs were treated with culture media containing Ang II (10⁻⁷ nM) for 24 h. LOX-1 siRNA (25, 50 and 100 nM; cat. no. NM_002543) or control siRNA (100 nM) (both from Sigma-Aldrich; Merck KGaA) (sense, 5'-GAUCAUACGUGACUGCAUAG-3' and antisense, 5'-UCUGAUUCGUCGAUCGAUC-3') was transfected into the HUVECs for 24 h using the HiPerFect transfection reagent (Qiagen, Valencia, CA, USA). For the present time-course experiment, the HUVECs were transfected with 50 nM LOX-1 siRNA and the cells were incubated at 37°C in 5% CO₂ for set times (4, 8, 12, 24 and 48 h). For the control group, the HUVECs were transfected with the corresponding control-sequence siRNA (i.e. scrambled siRNA). After incubation for 24 h, the cells were harvested and the lysates were subjected to reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis to verify the efficacy of knockdown by siRNA.

miRNA transfection. The Ang II-treated HUVECs were first transfected with LOX-1 siRNA (50 nM) for 24 h, and then transfected with different doses of let-7g (5, 10, 25 and 50 nM) or control miRNA for 24 h using the HiPerFect transfection reagent (Qiagen). After incubation for 24 h, the cells were harvested, and the lysates were subjected to RT-qPCR or western blot analysis to verify the efficacy of let-7g transfection.

Cell viability assay. Cells grown in a 96-well plate (10⁴ cells/well) were treated with Ang II (10⁻⁷ nM) for 24 h. WST-1 reagent (Roche, Mannheim, Germany) diluted 1:10 in growth medium was added for the last 2 h according to the manufacturer's instructions. The amount of viable cells was determined via optical density measurement using a microplate reader at 450 nm, with 600 nm as a reference wavelength.

RNA isolation and RT-qPCR. Total RNA was extracted from the HUVECs using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and complementary (c)DNA was produced using 1 μg starting mRNA (Applied Biosystems; Thermo Fisher Scientific, Inc.) and random hexamers. Reverse transcription was performed according to the manufacturer's protocol (Applied Biosystems; Thermo Fisher Scientific, Inc.): 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. After the reaction was complete, samples were cooled down to 4°C. The sequences of the PCR primers are listed in Table I. The relative amount of mRNA

| Gene       | Primer                        |
|------------|-------------------------------|
| GLB1       | F: 5'-TATACCTGGCTGGATCATCGT-3'  |
|            | R: 5'-GGCAAAATTGTGCCACCTATAA-3' |
| SIRT1      | F: 5'-AAGTTGACTGGAAGCTGTA-3'   |
|            | R: 5'-TGCTACTGTCCTTACCTTGGAGG-3' |
| LOX-1      | F: 5'-TGTTCTGGGATTAGTGTA-3'    |
|            | R: 5'-GCTTCTGGGATTAGTGTA-3'    |
| IGF1       | F: 5'-GCTTCTGACATGCTGTA-3'     |
|            | R: 5'-GGCTTCTGACATGCTGTA-3'    |
| IGF1R      | F: 5'-AGGATATTGGGCTTTACAACCTG-3' |
|            | R: 5'-GAGGTAACAGAAGCTGCACTTT-3' |
| GAPDH      | F: 5'-ACACCTTGGTACCTGGAAG-3'   |
|            | R: 5'-GCCATCACCACCATTTTC-3'    |

GLB1, β-galactosidase; SIRT1, sirtuin 1; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; IGF1R, insulin-like growth factor 1 receptor; F, forward; R, reverse.
of interest was normalized to GAPDH. PCR was performed using the ABI StepOnePlus™ Real-Time PCR system and SYBR-Green reagent (Applied Biosystems; Thermo Fisher Scientific, Inc.). PCR was performed in duplicate using 5 µl 2X SYBR-Green qPCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.), 0.2 µl primer sets, 1 µl cDNA and 3.6 µl nucleotide-free H2O in a 10-µl reaction system. RT-qPCR cycling conditions were as follows: 95˚C for 20 sec, followed by 40 cycles each at 95˚C for 1 sec and then 60˚C for 20 sec. The relative expression of each mRNA was calculated as the difference of cq between the expression of sample and the housekeeping gene GAPDH (28).

Western blot analysis. The HUVECs were harvested using radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.) supplemented with a protease inhibitor cocktail (Roche). The protein concentrations were determined by using the Pierce BCA Protein assay kit (Thermo Fisher Scientific, Inc.). The proteins were then transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA) and blocked with 5% non-fat dry milk for 2 h at room temperature. The membranes were incubated with primary antibodies at 4˚C for 16 h, including IGF1 (cat. no. ab9572; 0.1 µg/ml; Abcam, Cambridge, UK) and GAPDH (cat. no. GTX10011; 1:5,000 dilution; GeneTex, Inc., Irvine, CA, USA). Following three washes in PBST, the membranes were incubated with the secondary rabbit immunoglobulin G antibody conjugated to horseradish peroxidase (cat. no. GTX213110-01; 1:10,000 dilution; GeneTex, Inc.) at room temperature for 1 h. The enhanced chemiluminescence detection system was used to detect the antibody-protein complexes with the LAS-3000 imaging system (Fujifilm, Tokyo, Japan). Western blot band intensities were quantitatively measured using ImageJ 1.45s software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Statistical analysis was performed using Prism software, version 5.0 (GraphPad, Inc., La Jolla, CA, USA). Quantitative data are expressed as the mean ± standard error of the mean. Student's t-test was used to compare differences between two groups. Differences between multiple groups were analyzed using one-way analysis of variance, followed by Tukey’s post hoc multiple comparisons test. A two-sided P<0.05 was considered to indicate a statistically significant difference.

Results

Ang II increases the viability and decreases the let-7g level of HUVECs. First, different doses (ranging from 10 to 10^4 nM) of Ang II were used to test the effect on the amount of viable HUVECs. The results indicated that Ang II dose-dependently increased the amount of viable HUVECs, with the dose of 10^4 nM having the most significant effect (Fig. 1A). Therefore, the concentration of 10^5 nM was used for subsequent experiments. The endogenous let-7g levels were measured prior to and after Ang II treatment. The results indicated that
Ang II treatment reduced let-7g levels in HUVECs by ~50% (P=0.001) (Fig. 1B).

Ang II affects the expression of β-galactosidase, SIRT1 and the IGF1 pathway. The GLB1 gene encoding β-gal and the SIRT1 gene were identified to be positively and negatively associated with cell senescence, respectively. In the Ang II-treated HUVECs, a significant increase of β-gal (P<0.001) but a decrease of SIRT1 (P<0.001) mRNA expression was identified (Fig. 2A and B). In addition, IGF1 signaling was identified to be associated with the aging process. In the HUVECs, Ang II promoted LOX-1 (P<0.001), IGF1 (P<0.05) and IGF1 receptor (IGF1R; P<0.01) expression (Fig. 2C-E).

Knockdown of LOX-1 in Ang II-treated HUVECs. To explore the effect of let-7g via the LOX-1-independent mechanism, siRNA was employed to knock down LOX-1 expression. The LOX-1-specific siRNA (doses ranging from 25-100 nM) suppressed LOX-1 levels (Fig. 3A). The concentrations of 50 and 100 nM siRNA yielded a similar effect, and therefore, 50 nM siRNA was used in the subsequent experiments unless a specific dose was otherwise mentioned. To further determine the ideal incubation time for siRNA to exert its best inhibitory effect, LOX-1 expression was measured at 5 time-points (4, 8, 12, 24 and 48 h) after transfection of 50 nM siRNA into the HUVECs. The results indicated that 90% inhibition occurred at 24 and 48 h (Fig. 3B). Accordingly, all of the subsequent
results were obtained at 24 h after siRNA transfection. When LOX-1 expression was knocked down, β-gal expression also decreased in a dose-dependent manner in the Ang II-treated HUVEcs (Fig. 3c). Furthermore, SIRT1, IGF1 and IGF1R levels in the Ang II-treated HUVEcs increased along with the elevation of the dose of LOX-1-siRNA (Fig. 3d-F).

Let-7g exerts its anti-aging effect via a LOX-1-independent mechanism. Since a previous study by our group reported that let-7g has an anti-senescence effect on ECs (12), the present study assessed whether let-7g also affects aging-associated gene expression and pathways in a LOX-1-independent manner. In the Ang II-treated and LOX-1-siRNA-transfected HUVECs, transfection of exogenous let-7g reduced mRNA expression of IGF1, IGF1R and β-gal (Fig. 4A-C), while it increased SIRT1 mRNA expression (Fig. 4D). Finally, HUVECs that had been incubated under different conditions were subjected to SA-β-gal staining (Fig. 5). The results indicated that the amount of SA-β-gal-positive HUVECs was markedly increased by Ang II (Fig. 5A and B), while transfection of LOX-1 siRNA partially abolished the effect of Ang II (Fig. 5C). The SA-β-gal staining result further supported the notion that let-7g reduces
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Discussion

Ang II has been demonstrated to be an important signaling molecule involved in atherogenic stimuli, and has been indicated to promote aging and cellular senescence (31). Ang II has been revealed to induce cardiac fibrosis and downregulation of miRNA-29 expression (32). miRNA-29 induces cellular senescence via increasing p53, upregulating β-gal (33) and suppressing SIRT1 expression (34). In the present study, it was demonstrated that Ang II attenuated let-7g expression and induced senescence in ECs. An increase of let-7g significantly upregulated SIRT1 expression and decreased β-gal expression and cellular senescence. Therefore, Ang II may increase senescence-producing miRNA and suppress anti-senescence miRNA. In Ang II-treated ECs, the expression of aging-associated markers, including β-gal, IGF1, IGF1R and LOX-1, was increased. Conversely, Ang II decreased the expression of the anti-aging marker SIRT1. In addition, LOX-1 was identified to promote the aging process, as indicated by an increase of β-gal. As the purpose of the present study was to determine the effect of let-7g on Ang II-induced senescence, all the cells were first treated with Ang II prior to further experiments. Therefore, the present study does not have data on the effect of let-7g on cells without Ang II treatment, which is a limitation. Since a previous study by our group has indicated that let-7g directly suppresses LOX-1 (25), the present study examined whether let-7g exerts a LOX-1-independent anti-aging effect. In Ang II-treated HUVECs with LOX-1 knockdown, let-7g still reduced the expression of IGF1, IGF1R and β-gal but increased the SIRT1 expression. Furthermore, using the SA-β-gal staining as an aging indicator, the anti-aging effect of let-7g via a LOX-1-independent mechanism was confirmed. Taken together, the present results suggest that anti-aging effect of let-7g on ECs proceeds via a LOX-1-independent mechanism. Along with the previous study by our group on the effect of let-7g on LOX-1 (25), its anti-aging effect determined in the present study was summarized in a hypothetical scheme (Fig. 6). The right side of Fig. 6 illustrates that the anti-senescence effect of let-7g may be mediated by suppressing the oxLDL-LOX-1 pathway, while the left side indicates that let-7g may also suppress IGF1 signaling to exert the anti-senescence effect.

LOX-1 promotes cell senescence. While LOX-1 is almost undetectable under physiological conditions, it is upregulated after exposure to several proinflammatory and proatherogenic stimuli (35). A previous study by our group confirmed that let-7g reduces EC senescence through increasing SIRT-1.

Figure 4. Let-7g affects aging-associated gene expression via a LOX-1-independent mechanism. Human umbilical vein endothelial cells were treated with Ang II (10^4 nM) for 24 h, followed by transfection with 50 nM small interfering RNA targeting LOX-1 for another 24 h. Subsequently, the cells were transfected with ctr microRNA or let-7g (5, 10, 25 and 50 nM) or for 24 h. The mRNA levels of (A) IGF1, (B) IGF1R, (C) β-gal and (D) SIRT1 were measured by reverse transcription-quantitative polymerase chain reaction analysis. Values are expressed as the mean ± standard error of the mean. *P<0.05, **P<0.01 and ***P<0.001 analyzed by one-way analysis of variance and Tukey's post hoc test. β-gal, β-galactosidase; SIRT1, sirtuin 1; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; IGF1R, insulin-like growth factor 1 receptor; Ang II, angiotensin II; ctr, control.
Figure 5. Let-7g suppresses Ang II-induced HUVEC senescence and IGF protein levels. (A-D) Images of SA-β-gal staining of HUVECs (scale bar, 100 μm). (A) HUVECs (normal), (B) HUVECs treated with Ang II (10^4 nM), (C) HUVECs treated with Ang II (10^4 nM) and then transfected with LOX-1 siRNA (50 nM), (D) HUVECs treated with Ang II (50 nM), followed by LOX-1 siRNA (50 nM) and then by let-7g (50 nM). Each individual incubation lasted for 24 h. (E) Percentage of SA-β-gal-positive cells. The cells from 5 randomly selected microscopic fields of view were counted. (F) Western blot results indicated that let-7g dose-dependently inhibited IGF1 protein expression via a LOX-1-independent pathway. Values are expressed as the mean ± standard error of the mean (n=3 for each group). *P<0.05, **P<0.01 and ***P<0.001 analyzed by one-way analysis of variance and Tukey’s post hoc test. LOX-1, lectin-like oxidized-low-density lipoprotein receptor-1; IGF1, insulin-like growth factor 1; HUVECs, human umbilical vein endothelial cells; SA-β-gal, senescence-associated β-galactosidase; siRNA, small interfering RNA; Ang II, angiotensin II.

Figure 6. Hypothetic scheme illustrating multiple regulatory pathways of the anti-aging effects of let-7g in human umbilical vein endothelial cells. SIRT1, sirtuin 1; LOX-1, lectin-like ox-LDL receptor-1; ox-LDL, oxidized low-density lipoprotein; IGF1R, insulin-like growth factor 1 receptor; TP53, tumor protein 53.
protein, but did not assess whether the anti-aging effect of let-7g is mediated through LOX-1 (12). The present study was specifically designed to assess the anti-aging effect of let-7g when LOX-1 was not present. The current findings imply that let-7g exerts an anti-aging effect regardless of the presence or absence of LOX-1, which further suggests that let-7g possesses an anti-aging function via multiple mechanisms.

Ang II increased the levels of LOX-1, IGFR1 and IGFR in HUVECs or microglial cells (36,37). IGF signaling has a significant role in ECs by promoting migration, tube formation and production of the vasodilator nitric oxide. The over-activation of IGF signaling, however, may suppress SIRT1 expression, contributing to the aging process. Reduced IGF signaling has also been associated with increased longevity (38). In fact, the insulin pathway coordinates growth, development, metabolic homeostasis and stress resistance, all of which affect the lifespan.

The let-7 family has a central role in nutrient homeostasis and insulin resistance (39,40). Previous studies have indicated that IGFR1 is a target of let-7g in mouse granulosa cells and colon cancer cells (29,30). The present study further indicated that let-7g reduces IGFR expression in the presence of Ang II stimulation, demonstrating a profound effect of let-7g on the IGFR1 signaling pathway.

In conclusion, the present study provided novel insight into the anti-aging effect of let-7g on ECs, which occurs regardless of the presence of LOX-1. The present results also suggest that let-7g has direct effects on multiple targets of the IGF signaling pathway. Given that healthy and young ECs are crucial for the cardiovascular system, blood brain barrier, and other systemic functions, maintenance of sufficient let-7g levels may be an important strategy to reduce the senescent process.

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Competing interests

The authors declare that they have no competing interests.

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