Essential Role of MicroRNA-155 in Regulating Endothelium-Dependent Vasorelaxation by Targeting Endothelial Nitric Oxide Synthase

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Abstract—Nitric oxide generated by endothelial nitric oxide synthase (eNOS) plays an important role in maintaining cardiovascular homeostasis. Under various pathological conditions, abnormal expression of eNOS contributes to endothelial dysfunction and the development of cardiovascular diseases. A variety of pathological stimuli has been reported to decrease eNOS expression mainly through decreasing eNOS mRNA stability by regulating the binding of several cytosolic proteins to the cis-acting sequences within eNOS mRNA 3′ untranslated regions. However, the detailed mechanisms remain elusive. Because microRNAs inhibit gene expression through binding to the 3′ untranslated regions of their target mRNAs, microRNAs may be the important posttranscriptional modulators of eNOS expression. Here, we provided evidence that eNOS is a direct target of miR-155. Overexpression of miR-155 decreased, whereas inhibition of miR-155 increased, eNOS expression and NO production in human umbilical vein endothelial cells and acetylcholine-induced endothelium-dependent vasorelaxation in human internal mammary arteries. Inflammatory cytokines including tumor necrosis factor-α increased miR-155 expression. Inhibition of miR-155 reversed tumor necrosis factor-α–induced downregulation of eNOS expression and impairment of endothelium-dependent vasorelaxation. Moreover, we observed that simvastatin attenuated tumor necrosis factor-α–induced upregulation of miR-155 and ameliorated the effects of tumor necrosis factor-α on eNOS expression and endothelium-dependent vasodilation. Simvastatin decreased miR-155 expression through interfering mevalonate-geranylgeranyl-pyrophosphate-RhoA signaling pathway. These findings indicated that miR-155 is an essential regulator of eNOS expression and endothelium-dependent vasorelaxation. Inhibition of miR-155 may be a new therapeutic approach to improve endothelial dysfunction during the development of cardiovascular diseases. (Hypertension. 2012;60:1407-1414.) • Online Data Supplement

Key Words: endothelial nitrite oxide synthase • endothelial dysfunction • tumor necrosis factor-α • microRNA-155 • simvastatin

Nitric oxide (NO) generated and released by endothelial NO synthase (eNOS) exerts multiple beneficial functions in vessels and plays a critical role in maintaining cardiovascular homeostasis.¹ Dysregulation of NO synthesis attributable to the abnormal activity or expression of eNOS or both has been considered to be a major contributor to the pathogenesis of vascular diseases, such as hypertension and atherosclerosis.²³ eNOS expression can be modulated by a variety of physiological and pathophysiological stimuli. Previous studies have demonstrated that hypoxia, lipopolysaccharide (LPS), tumor necrosis factor-α (TNFα), and oxidized low-density lipoprotein decreased, whereas hydrogen peroxide, shear stress, vascular endothelial growth factor, and some drugs including 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) increased, eNOS expression in endothelial cells.⁴⁵ Although the expression of eNOS is regulated at multiple levels, recent growing evidence has suggested that posttranscriptional regulation plays an important role in control of eNOS expression. Our laboratory and other laboratories have reported that many of these stimuli control eNOS expression mainly through modification of eNOS mRNA stability by affecting the binding of some cytoplasmic proteins with eNOS
mRNA 3′ untranslated regions (3′-UTR), indicating 3′-UTR of eNOS mRNA is critical for its mRNA stability and thus, for eNOS expression. However, the detail mechanisms how the 3′-UTR of eNOS mRNA modulates its mRNA stability are not fully understood.

MicroRNAs (miRNAs) are a class of small, noncoding RNAs of ≈22 nucleotides that negatively regulate gene expression by targeting the 3′-UTR of specific mRNAs through induction of mRNA degradation or translational repression. Recent work in endothelial cells showed that knockdown of Dicer, the enzyme necessary for miRNA maturation, increased eNOS expression. Transfection of miR-221 and miR-222 mimics partially reversed the increases of eNOS protein attributable to the Dicer silence, suggesting miR-221 and miR-222 may be involved in the control of eNOS expression. However, eNOS mRNA might not be the direct target of miR-221 and miR-222 because miR-221 and miR-222 are not able to bind to the eNOS mRNA 3′-UTR based on the predictive algorithm analysis (Sanger miRBase Targets21 and TargetScan22). Although this study suggested that miRNAs may be crucial regulators of eNOS expression, which miRNAs are involved in the regulation of eNOS expression directly is not clear.

Here, we provided evidence that miR-155 downregulated eNOS expression through decreasing eNOS mRNA stability by binding to its 3′-UTR. Cytokines including TNFα increased miR-155 expression in human umbilical vein endothelial cells (HUVECs). Knockdown of miR-155 prevented cytokine-induced downregulation of eNOS expression, reduction of NO production, and impairment of endothelium-dependent vascular relaxation. Moreover, we found simvastatin ameliorated TNFα-induced endothelial dysfunction via inhibition of miR-155 expression.

**Results**

**eNOS Is a Direct Target of miR-155**

Computational miRNA target analysis showed that miR-155 is able to bind to the eNOS mRNA 3′-UTR, suggesting this gene may be a potential molecular target for miR-155 (Figure 1A). To examine whether miR-155 can repress eNOS expression through direct 3′-UTR interaction, we cloned eNOS 3′-UTR luciferase reporter plasmid and performed reporter analysis in Cos-7 cells. Our results demonstrated that cotransfection of pre-miR-155 with eNOS 3′-UTR reporter resulted in dose-dependent inhibition of luciferase activity (Figure 1B, n=6, *P*<0.01 versus control). However, miR-155 failed to repress the activity of eNOS 3′-UTR reporter with a mutated miR-155 seed sequence (Figure 1B, n=6). Moreover, we determined the effects of miR-155 on eNOS expression in pcDNA3-eNOS-3′-UTR overexpressed Cos-7 cell lines. In agreement with the reporter assays, overexpression of miR-155 repressed eNOS protein expression in a dose-dependent manner (Figure S1, available in the online-only Data Supplement). These data indicated that eNOS is a direct target of miR-155.

**miR-155 Downregulated Endogeneous eNOS Expression in HUVECs by Destabilizing eNOS mRNA**

To further validate whether miR-155 can downregulate eNOS expression, we tested the effects of miR-155 on endogenous eNOS levels in HUVECs infected with adenovirus harboring premature miR-155 (Ad-miR-155). Overexpression of miR-155 for 48 hours reduced eNOS protein expression in a

**Materials and Methods**

An expanded Materials and Methods section is available in the online-only Data Supplement. Human internal mammary arteries were obtained intraoperatively from 26 patients undergoing coronary artery bypass grafting, and the discarded segments were collected for our experiments. The study protocol was approved by the medical research ethics committee of Sun Yat-Sen University. Informed consent was obtained from all subjects, and the experiments were conducted according to the principles expressed in the Declaration of Helsinki. The rings were preconstricted with phenylephrine (3×10−2 mol/L), and vasorelaxation was determined by measuring the cumulative response to acetylcholine (Ach; 10−9–10−5 mol/L) or to sodium nitroprusside (10−8–10−6 mol/L).

Cell extracts prepared from HUVECs were analyzed by Western blotting as described previously. Quantitative real-time reverse transcription polymerase chain reaction was performed using SYBR green fluorescence. Adenoviruses were generated using the AdMax (Microbix) and pSilencer adenovirus (Ambion) systems as described previously. NO production was assayed by measuring the cGMP levels and nitrite plus nitrate accumulation, respectively.

All data were expressed as mean±SEM. Statistical analysis was determined by an unpaired 2-tailed Student *t* test or 1-way ANOVA followed by Bonferroni multiple comparison post hoc test with a 95% CI. Values of *P*<0.05 were considered significant.

**Figure 1**. Endothelial NO synthase (eNOS) is regulated by miR-155 directly. **A**, Sequence of human miR-155 and the predicted binding sites with miR-155 within eNOS 3′-UTRs from different species are shown. The sequence of eNOS 3′-UTR mutant used for reporter assay is also shown. **B**, Luciferase reporter constructs containing 3′-UTR (Luc-eNOS-3′-UTR) or mutant 3′-UTR (Luc-eNOS-3′-UTR-mut) of eNOS gene were cotransfected with pre-miR-155 or empty vector (pCS2) in Cos-7 cell line and the luciferase activities were assayed (n=5, *P*<0.05, *P*<0.01 vs control).
dose-dependent manner (Figure 2A). Significantly, downregulation of eNOS expression correlated with decreases of eNOS activity and NO production in HUVECs (Figure 2B and 2C).

miRs have been proposed to control gene expression at the posttranscriptional levels by degradation or translational repression of their target mRNAs. To further determine the mechanisms how miR-155 inhibited eNOS expression, eNOS mRNA stability was determined in the presence of transcriptional inhibitor 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole (20 μg/mL). HUVECs were infected with either Ad-miR-155 or Ad-LacZ for 48 hours and then 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole was added to the culture medium. Zero to 24 hours later, the total RNAs were prepared, and eNOS mRNA level was analyzed by quantitative reverse transcription polymerase chain reaction. The data demonstrated that overexpression of miR-155 in HUVECs shortened the half-life of eNOS mRNA from >24 hours to 15±2 hours (P<0.05, n=5) (Figure 2D), suggesting eNOS mRNA degradation underlies miR-155–induced eNOS downregulation.

**TNFα Increased miR-155 Expression in HUVECs**

To determine whether miR-155 is involved in the regulation of TNFα-induced decrease of eNOS expression, we examined miR-155 expression in HUVECs after exposure to 20 ng/mL of TNFα. Our results showed that TNFα treatment significantly increased miR-155 expression in HUVECs. The maximal expression of miR-155 was observed at 4 hours after TNFα treatment (Figure 3A). Moreover, miR-155 expression was also induced by inflammatory cytokines LPS, interleukin (IL)-1β, and interferon-γ (Figure S2).

Recently, we reported that activator protein 1 and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) are critical for LPS-induced miR-155 production. Here, we also found that TNFα-induced upregulation of miR-155 was partially blocked by SP600125 (a c-Jun N-terminal kinase inhibitor), pyrrolidine dithiocarbamate (a NF-κB inhibitor), or the combination of both inhibitors (Figure 3B), indicating that activator protein 1 and NF-κB are critical for TNFα-induced miR-155 production.

**Inhibition of miR-155 Attenuated TNFα-Induced Downregulation of eNOS Expression Through Increasing eNOS mRNA Stability**

Our results showed that eNOS is the direct target of miR-155 and TNFα can increase miR-155 expression, indicating miR-155 may contribute to TNFα-induced eNOS downregulation. To validate this hypothesis, we examined the effects of TNFα on eNOS expression in HUVECs infected with adenovirus harboring miR-155 inhibitor...
(Ad-anti-miR-155). TNFα (20 ng/mL) treatment for 48 hours decreased eNOS expression by ≈64% in HUVECs. Transfection of Ad-anti-miR-155 (100 multiplicity of infection) but not Ad-LacZ partially restored TNFα-induced downregulation of eNOS. Similar results were also observed in HUVECs after LPS and interleukin (IL)-1β treatment (Figure S3A and S3B). Moreover, Ad-anti-miR-155 infection substantially increased the basal eNOS expression (Figure 4A). In agreement with its effect on eNOS expression, Ad-anti-miR-155 also increased eNOS activity and NO production at the basal level and partially restored TNFα-induced decreases of eNOS activity and NO production (Figure 4B and 4C). Furthermore, TNFα treatment decreased eNOS mRNA stability, and Ad-anti-miR-155 partially restored TNFα-induced eNOS mRNA degradation (Figure 4D).

**MiR-155 Mediated TNFα-Induced Impairment of Endothelial-Dependent Vasodilation**

To examine whether miR-155 plays an important role in regulating endothelium-dependent vascular relaxation, the expression of eNOS and Ach-induced vascular response were measured in human internal mammary artery rings in which Ad-miR-155 or Ad-LacZ were adenovirally expressed (Figure S4). Compared with Ad-LacZ transfected controls, both eNOS expression and Ach-induced endothelium-dependent vasodilation in arteries infected with Ad-miR-155 were decreased (Figure 5A and 5B). In contrast, endothelium-independent vasorelaxation induced by sodium nitroprusside, a NO donor, was no significant difference between Ad-miR-155 and Ad-LacZ transfected rings (Figure 5C). Moreover, Ach-induced vasodilation in the presence of the NOS inhibitor NG-nitro-l-arginine methyl ester was similar in control rings and Ad-miR-155 infected rings (Figure 5B). These results suggested that miR-155 is involved in the regulation of endothelium-dependent vascular relaxation via inhibition of eNOS expression.

Because TNFα increased miR-155 expression in endothelial cells, we next determined whether miR-155 mediates TNFα-induced impairment of endothelium-dependent vasorelaxation. Consistent with the results in HUVECs, miR-155 inhibitor increased the basal eNOS expression level and prevented TNFα-induced downregulation of eNOS in internal mammary artery (Figure 5D). TNFα (20 ng/mL) treatment for 24 hours impaired endothelium-dependent vasodilation. miR-155 inhibitor itself potentiated Ach-induced vasodilation under basal conditions. Moreover, miR-155 inhibitor ameliorated TNFα-induced dysfunction of endothelium-dependent vasorelaxation (Figure 5E). Taken together, these findings indicated that the increased expression of miR-155 underlies TNFα-induced impairment of endothelium-dependent vasodilation.
Simvastatin Ameliorated TNFα-Induced Endothelial Dysfunction Through Inhibition of miR-155 Expression

Simvastatin prevented TNFα-induced downregulation of eNOS expression as well as NO production in a concentration-dependent manner (0.1–1.0 μmol/L) in HUVECs (Figure S5A and S5B). In internal mammary arteries, 1 μmol/L of simvastatin pretreatment ameliorated TNFα-induced impairment of endothelium-dependent vasorelaxation (Figure S5C). To determine the molecular mechanism underlying the beneficial effect of simvastatin on TNFα-induced endothelial dysfunction, we examined the effect of simvastatin on miR-155 expression in HUVECs. Our results showed that, in basal condition, simvastatin (1 μmol/L) inhibited miR-155 expression in HUVECs in a time-dependent manner (Figure 6A). Moreover, simvastatin concentration dependently (0.1–1.0 μmol/L) decreased TNFα-induced upregulation of miR-155 expression in HUVECs (Figure 6B). These results implied that downregulation of miR-155 expression mediates the beneficial effect of simvastatin on endothelial dysfunction.

Figure 5. Involvement of miR-155 in tumor necrosis factor-α (TNFα)-induced dysfunction of endothelium-dependent relaxation in human internal mammary artery. A, Overexpression of miR-155 inhibited endothelial nitric oxide synthase (eNOS) expression in human internal mammary artery. The arteries were transfected with Ad-miR-155 or Ad-LacZ at 6×10¹⁰ viral particles per milliliter ex vivo. Twenty-four hours later, the tissue lysates were collected for Western blotting. n=4, **P<0.01 vs Ad-LacZ. B, miR-155 inhibited endothelium-dependent vasorelaxation. The arterial rings were infected with Ad-anti-miR-155 or Ad-LacZ at 6×10¹⁰ viral particles per milliliter ex vivo. Twenty-four hours later, 20 ng/mL TNFα was added for another 24 hours and then the tissue lysates were collected for Western blotting. n=4, **P<0.01 vs control, #P<0.01 vs TNFα only group. E, miR-155 inhibitor prevented TNFα-induced impairment of endothelium-dependent vasodilation. The arterial rings were pretreated as depicted and endothelium-dependent vasodilation was determined by measuring acetylcholine (Ach)-induced relaxation in rings precontracted with phenylephrine. n=5, *P<0.05, **P<0.01.

Figure 6. Simvastatin inhibited tumor necrosis factor-α (TNFα)-induced miR-155 expression. A, Simvastatin concentration-dependently decreased TNFα-induced upregulation of miR-155 in human umbilical vein endothelial cells (HUVECs). HUVECs were pretreated with simvastatin (Sim, 0.1 to 1.0 μmol/L). Four hours later, 20 ng/mL of TNFα was added for another 24 hours and the expression of miR-155 was determined by real-time reverse transcription polymerase chain reaction (RT-PCR). n=5, **P<0.01 vs control, #P<0.05, ##P<0.01 vs TNFα only group. B, Time-dependent effect of simvastatin on miR-155 expression. n=4, *P<0.05, **P<0.01 vs control.
Simvastatin Attenuated miR-155 Expression by Interfering Mevalonate-RhoA Pathway

As shown in Figure 7A, pretreatment with 100 μmol/L of mevalonate (MVA), 10 μmol/L of geranylgeranylphosphate (GGPP), or 10 μmol/L of farnesylphosphate (FPP) but not cholesterol abolished simvastatin-induced inhibition of miR-155 expression. MVA, GGPP, FPP, or cholesterol was added 1 hour before simvastatin (Sim). **P<0.01 vs tumor necrosis factor-α (TNFα) only group, ###P<0.01 vs TNFα+Sim group, n=5 to 6. B, Effects of Rho A inhibitor C3 exoenzyme (C3) and Rho kinase inhibitor Y-27632 on TNFα-induced miR-155 expression. Cells were preincubated with C3 exoenzyme (0.25 μg/mL) or Y-27632 (10 μmol/L) for 4 hours before TNFα treatment. **P<0.01 vs TNFα only group, n=5. DMSO indicates dimethyl sulfoxide.

Discussion

MiR-155, the product of B-cell integration cluster gene, was first identified as an oncogenic miRNA.

Upregulation of miR-155 has been linked to the development of different kinds of cancers. Recent growing evidence has further demonstrated that miR-155 is implicated in regulating a variety of cellular functions including hematopoiesis, immunologic response, inflammation, viral infection, and macrophage-derived foam cell formation. To date, a few direct targets of miR-155, such as suppressor of cytokine signaling 1, SH2-containing inositol 5′-phosphatase, Sma- and Mad-related protein 2, hexokinase 2, casein kinase-1α, and human germinal center-associated lymphoma, have been identified by luciferase report assay.

In endothelial cells, type 1 angiotensin II receptor, BACH1, and cysteine-rich protein 61 have been validated to be regulated by miR-155. Notably, previous work showed that the level of circulating miR-155 is remarkably reduced in patients with coronary heart disease. Moreover, miR-155 is drastically upregulated in human atherosclerotic plaques and oxidized-low-density lipoprotein-treated macrophages. These findings indicated that miR-155 may be an essential regulator of cardiovascular functions. Here, our data demonstrated that eNOS is a direct target of miR-155. Overexpression of miR-155 decreased eNOS expression and NO production in HUVECs and impaired Ach-induced endothelium-dependent vasorelaxation in human internal mammary arteries. miR-155 downregulated eNOS expression mainly through decreasing eNOS mRNA stability by binding to its 3′-UTR. Because NO exerts multiple beneficial functions in vessels, including regulation of vascular tone and blood pressure, prevention of the expression of adhesion molecules and inflammation, reduction of platelet activation, and inhibition of vascular smooth muscle cell proliferation, our results suggested that miR-155 may play a critical role in regulation of cardiovascular homeostasis through modification of eNOS expression.

It has been shown that inflammatory cytokines including TNFα, LPS, or interferon-γ could increase miR-155 expression in macrophages, monocytes, human mesangial cells, and human coronary artery endothelial cells. Consistent with these studies, our present results showed that miR-155 was upregulated in HUVECs after treatment with TNFα, LPS, interleukin (IL)-1β, or interferon-γ. Our previous work has demonstrated that the promoter of B-cell integration cluster/miR-155 gene contains activator protein 1 and NF-κB binding motifs, and these 2 transcription factors contribute to LPS-induced expression of miR-155. Here, we also showed that inhibitors of activator protein 1 and NF-κB significantly inhibited TNFα-induced upregulation of miR-155 in HUVECs. More importantly, we found that pretreatment of miR-155 inhibitor reversed TNFα-induced downregulation of eNOS expression, reduction of NO production in HUVECs, and impairment of Ach-induced endothelium-dependent vasorelaxation in human internal mammary arteries, suggesting that upregulation of miR-155 underlies, at least in part, the inhibitory effect of TNFα on eNOS expression. Moreover, we found that miR-155 inhibitor increased eNOS expression in HUVECs and potentiate endothelium-dependent vasorelaxation in human internal mammary arteries in basal conditions, indicating miR-155 is implicated in regulation of eNOS expression in physiological status.

Recent studies have demonstrated that some clinically used drugs can modulate miRNA expression, indicating that miRNAs may be involved in the therapeutic effects of these agents. Statins, the widely used lipid-lowering drugs in clinic, have pleiotropic actions in the cardiovascular system including inhibition of vascular smooth muscle cell proliferation,
improvement of endothelial function, increase of NO availability, reduction of inflammatory response, and oxidative stress production.\textsuperscript{39} Statins improve eNOS expression mainly through increasing eNOS mRNA stability.\textsuperscript{4,5} Although previous reports have shown that statins increased eNOS mRNA half-life through modification of the binding of some cytosolic proteins to the 3′-UTR of eNOS mRNA\textsuperscript{5,7,9} the detailed mechanisms remain elusive. Here, we showed that simvastatin can decrease miR-155 expression in both nontreated and TNFα-treated HUVECs. Pretreatment with MVA, GGPP, or farnesylpyrophosphate but not cholesterol abolished the inhibitory effect of simvastatin on TNFα-induced miR-155 expression. Moreover, RhoA and Rho kinase inhibitors also remarkably attenuated TNFα-induced upregulation of miR-155. These results indicated that simvastatin reversed TNFα-induced miR-155 expression via inhibition of MVA-GGPP-RhoA signal pathway, and the downregulation of miR-155 plays an important role in simvastatin-induced increase of eNOS expression.

**Perspectives**

The present work demonstrated that miR-155 is involved in regulating NO production and endothelium-dependent vaso-relaxation by targeting eNOS. Upregulation of miR-155 contributes to TNFα-induced decreases of eNOS expression and impairment of endothelium-dependent relaxancy. Simvastatin reduced miR-155 expression and reversed TNFα-induced endothelial dysfunction. Our data suggested that miR-155 plays an essential role in regulating the physiological functions of the cardiovascular system. Inhibition of miR-155 expression may be a novel therapeutic intervention to ameliorate endothelial dysfunction and, thus, to prevent cardiovascular diseases.

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**Disclosures**

None.

**References**


**Novelty and Significance**

### What Is New?
- MicroRNA-155 regulates endothelium-dependent vasorelaxation by modulating the expression of endothelial NO synthase through directly binding to the 3′ untranslated regions of endothelial NO synthase mRNAs.
- Upregulation of miR-155 expression underlies tumor necrosis factor-α–induced decrease of endothelial NO synthase expression and impairment of endothelium-dependent vasorelaxation.
- Simvastatin inhibits miR-155 expression through interfering mevalonate-geranylgeranyl-pyrophosphate-RhoA signaling pathway.

### What Is Relevant?
- Endothelial dysfunction contributes to the development of hypertension.

### Increase of miR-155 induces, whereas inhibition of miR-155 ameliorates, endothelial dysfunction.

**Summary**

These findings indicate that miR-155 is an essential regulator of endothelial NO synthase expression and endothelium-dependent vasorelaxation. Our results provide new insights into the mechanisms underlying the endothelial dysfunction in pathological conditions, suggesting that inhibition of miR-155 may be a new therapeutic target to improve endothelial dysfunction and cardiovascular diseases.
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Essential role of microRNA-155 in regulating endothelium-dependent vasorelaxation by targeting endothelial nitric oxide synthase

Sun et al. MicroRNA-155 and eNOS mRNA stability

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SUPPLEMENTAL METHODS

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were cultured in medium 199 (Gibco BRL/Invitrogen, Carlsbad, CA, USA) containing endothelial cell growth supplement, 20% fetal bovine serum, and antibiotics (50 IU/mL penicillin and 100 µg/mL streptomycin). Cos-7 cell line was cultured in DMEM supplemented with 10% fetal bovine serum and 50 IU/mL of penicillin and 50 µg/mL of streptomycin.

Western blotting

HUVECs (80% confluent) were cultured in medium 199 with 20% fetal bovine serum and infected with adenovirus at the indicated multiplicity of infection (MOI). 12 hr after transduction, the viral suspension was removed and the cells were grown in medium 199 containing 20% FBS for another 36 hr. Forty-eight hours after transduction, cells were then treated with or without of TNF-α (20 ng/mL) for another 24 hr. Cell extracts prepared from HUVECs were analyzed by western blotting as previously described1, 2. Briefly, cells were rinsed with ice-cold PBS (pH 7.4) and lysed with lysis buffer containing: 50.0 mmol/L Tris pH=7.6, 150.0 mmol/L NaCl, 0.1% SDS, 1.0% NP-40, Protease Inhibitor Cocktail, Phosphatase Inhibitor Cocktail (Sigma, St. Louis, MO, USA)). Protein concentrations were determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of total proteins (30 µg) were separated on a 8% SDS-polyacrylamide gel and transferred onto Polyvinylidene Fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Immunoblotting was performed with the primary antibody against eNOS (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA). α-tubulin (1:5000 dilutions; Sigma, St. Louis, MO, USA) was measured as an internal control. Immunodetection was accomplished using a goat anti-rabbit secondary antibody and rabbit anti-mouse secondary antibody and the enhanced chemiluminescence kit (Amersham Biosciences Corp., Piscataway, NJ, USA).

Adenovirus construction

Adenoviruses construction was performed as previously described2. Adenoviruses harboring a 409–base pair DNA fragment encompassing the has-mir-155 gene (Ad-miR-155) and the adenoviruses harboring anti-miR-155-specific stem-loop oligonucleotides (5'-TCGAGCCCTATCACGATTAGCATTATTCAAGAGATTAATGCTATGAGGGGA-3' and 5'-CTAGTCCCCTATCACGATTAGCATTAATCTCTTGAATTAATGCTATCGTGATAGGGGC-3', Ad-anti-miR-155) were generated using the AdMax (Microbix) and pSilencer™ adeno 1.0-CMV (Ambion) systems according to the manufacturers’ recommendations. An adenovirus bearing LacZ (Ad-LacZ) was obtained from Clontech. Viruses were packaged and amplified in HEK293A cells and purified using CsCl banding followed by dialysis against 10 mmol/l Tris-buffered saline with 10% glycerol. Titering was performed on HEK293A
cells using the Adeno-X Rapid Titer kit (BD Biosciences Clontech, Palo Alto, CA, USA) according to the manufacturer’s instructions.

**Quantitative real-time PCR**

Total RNA was isolated from HUVECs using Trizol reagent according to the manufacturer’s instructions. Two micrograms of total RNA were reverse transcribed in a total volume of 20 µl, and real-time PCR was performed using SYBR green fluorescence. To detect miR-155 expression, complementary DNA was synthesized using a miR-155-specific stem-loop primer: 5'-GTCGTATCCAGTGACGGGTC-CGAGGTATTCGCACTGGGATACGACCCCCTA-3’. Quantitative polymerase chain reaction (PCR) analysis used the following primers: forward, 5’-CTGTTATGCTAATCGTAGAG-3’; reverse, 5’-GCAGGGTCCCGAGGT-3’. Small unclear RNA U6 (U6) small RNA was used as internal control with the following primers: forward, 5’-CTCGCTTCGGCAGCACA-3’; reverse, 5’-AACGCTTCACGAATTTTGCGT-3’. The reverse primer of U6 was used for reverse transcription. To analyze the effect of has-mir-155 on eNOS mRNA stability, HUVECs (70% confluent) were infected with AdLacZ and Ad-miR-155 at MOI of 100 for 48 hours, then 20µg/mL 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB, Sigma, St. Louis, MO, USA) was added. Total RNAs were prepared 0-24 hours thereafter. The specific primers used for 18S rRNA detection were 5’-CGGCTACCACATCCAAGGAA-3’ and 5’-CTGGATATTCCGCGGCT-3’, eNOS primers were 5’-CCCTTCAGTGGCTGGTACAT-3’ and 5’-CACGGTGACTTTTGCGT-3’. The fold change in expression of each gene was calculated using the $2^{-\Delta\Delta CT}$ method with 18S rRNA or U6 as an internal control.

**Transient transfection and luciferase reporter assay**

Based on the human eNOS mRNA sequence deposited in the GenBank database (accession no NM_000603), fire luciferase cDNA fused with eNOS mRNA 3’-UTR (420nt, 3906 to 4325) was amplified and cloned into the pcDNA3.0 vector. A 409–base pair DNA fragment encompassing the has-mir-155 gene was amplified from genomic DNA by PCR and inserted into the EcoR I and XhoI sites of pCS2-6XMT vector. Preconfluent (60 to 70%) Cos7 cells in 6-well plates were transfected with 300 ng of firefly luciferase reporter plasmid (Luc-eNOS-3’UTR), indicated pCS2-miR-155 constructs and 20 ng of Renilla luciferase reporter plasmid pRL-RSV (Promega) using the FuGENE 6 transfection reagent. After 48 h, cell lysates were assayed for luciferase activity using the Luciferase Assay System (Promega) as measured with a luminescence counter (Centro XS3 LB 960, Berthold Technologies) according to the manufacturer’s instructions. Firefly luciferase activity was
normalized for transfection efficiency by the corresponding Renilla luciferase activity. All transfection experiments were performed at least 5 times.

**Measurement of nitrite and nitrate released by HUVECs.**

Nitrite (NO$_2^-$) and nitrate (NO$_3^-$) in cell culture supernatants were examined using a commercially available kit (Nitrate/Nitrite Fluorometric Assay kit; Cayman Chemicals, Ann Arbor, MI) according to the manufacturer’s recommendations. Total nitrite and nitrate levels were normalized to total proteins of HUVECs.

**Measurement of cGMP contents.**

After treatment, NO levels produced from HUVECs were determined by measuring the accumulation of cGMP contents in the cultured medium, using a commercially available kit (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer’s recommendations. Total cGMP levels in cell culture supernatants were normalized to total proteins of HUVECs.

**Arterial ring preparation, ex vivo adenovirus infection and vascular tension recording**

Human internal mammary arteries were obtained intra-operatively from 26 patients undergoing coronary artery bypass grafting and the discarded segments were collected for our experiments. The study protocol was approved by the Medical Research Ethics Committee of Sun Yat-sen University. Informed consent was obtained from all subjects and the experiments were conducted according to the principles expressed in the Declaration of Helsinki. The vessels were cleaned of adventitia and excess fat and were cut into 2 or more rings. The rings were then infected with appropriate adenovirus at 6×10$^{10}$ viral particles per ml at 37 °C for 24 hours. Control rings were infected with Ad-LacZ adenovirus. After treatment, the isolated rings were suspended in organ bath containing 3 ml modified Krebs solution (mmol/L: NaCl 137, KCl 5.4, CaCl$_2$ 2.0, MgCl$_2$ 1.1, NaH$_2$PO$_4$ 0.4, NaHCO$_3$ 11.9, Glucose 5.6, pH=7.2) at 37°C and gassed with 95%O$_2$/5%CO$_2$ continuously. After mounting, the vessel segments were equilibrated for 2 hours in bath medium under a resting tension of 1.5 g. The rings were washed with fresh oxygenated solution every 20 to 30 minutes. Tension was recorded using a high-sensitivity isometric force transducer and the data was stored in a personal computer connected to an analogue-to-digital converter (Powerlab, AD Instruments, Australia). The results were analyzed using Chart version 5.4.1 software program (Powerlab, AD Instruments, Australia). Before experiment, aortic rings were contracted with 100 mmol/L KCl to verify the vessel viability. The rings were preconstricted with phenylephrine (3×10$^{-7}$ mol/L) and endothelium-dependent vasorelaxation was determined by measuring the cumulative response to acetylcholine (10$^{-9}$-10$^{-5}$ mol/L). Endothelium-dependent eNOS-independent vasodilation was assessed by measuring the cumulative response to acetylcholine in rings pretreated with the eNOS inhibitor L-NAME (10$^{-4}$ mol/L). Endothelium-independent vasodilation was measured by addition
of cumulative sodium nitroprusside \((10^{-10}-10^{-6} \text{ mol/L})\). Vasorelaxation was expressed as percent contraction determined by the percentage of inhibition to the preconstricted tension.

**Supplemental references**


SUPPLEMENTAL FIGURES

Figure S1. MiR-155 inhibited eNOS expression. pcDNA3-eNOS plasmid with 3'-UTR of the eNOS gene were cotransfected with pre-miR-155 or empty vector (pCS2) in Cos-7 cell line. 24 hours later, the eNOS expression was performed by western blot. (n=6, p<0.05, p<0.01 vs control).

Figure S2. Inflammatory cytokines induced miR-155 expression in HUVECs. HUVECs were treated with 20 ng/ml TNFα, 1 µg/ml LPS, 20 ng/ml IL-1β or 20 ng/ml IFNγ for 4 hours, miR-155 expression was then examined by real-time PCR. n=3, *p<0.05, **p<0.01 vs control.
Figure S3. Inhibition of miR-155 prevented LPS (A) and IL-1β (B) induced downregulation of eNOS in HUVECs. HUVECs were infected with Ad-anti-miR-155 or Ad-LacZ at 100 MOI. 48 hours later, 1 µg/ml LPS or 20 ng/ml IL-1β was added into the cell culture medium for another 24 hours and then the cell lysates were collected for western blotting. n=5, **p<0.01 vs control, #p<0.05 vs LPS or IL-1β only group.

Figure S4. Ad-miR-155 infection increased miR-155 expression in human internal mammary arteries. The arteries were infected with adenovirus at $6 \times 10^{10}$ viral particles per ml at 37 °C for 24 hours. Control rings were infected with Ad-LacZ adenovirus. After treatment, the RNA was isolated and were reverse transcribed in a total volume of 20 µl, and quantitative real-time PCR was performed using SYBR green fluorescence.
Figure S5. Simvastatin ameliorated TNFα-induced downregulation of eNOS and endothelial dysfunction. A, simvastatin prevented TNFα-induced downregulation of eNOS expression in a concentration-dependent manner. HUVECs were pretreated with simvastatin (0.1-1 µmol/L). 4 hours later, 20 ng/ml TNFα was added for another 24 hours and then the cell lysates were collected for western blotting. n=5, **p<0.01 vs control, #p<0.05, ##p<0.01 vs TNFα only group. B, simvastatin attenuated TNFα-induced reduction of NO production. HUVECs were treated as depicted in A and the cGMP level in cultured medium was determined as described in Materials and Methods. n=6, **p<0.01 vs control, #p<0.05, ##p<0.01 vs TNFα only group. C, simvastatin ameliorated TNFα-induced endothelial dysfunction. The rings from human internal mammary arteries were pretreated with or without simvastatin (1 µmol/L). 4 hours later, 20 ng/ml TNFα was added for another 24 hours and then endothelium-dependent vasodilation was determined by measuring acetylcholine (Ach) induced relaxation in rings precontracted with phenylephrine. n=5, *p<0.05, **p<0.01, ##p<0.01.
Figure S6. Simvastatin restored TNFα-induced downregulation of eNOS expression through MVA–RhoA pathway. A, MVA (100µmol/L), GGPP (10µmol/L), FPP (10µmol/L) but not cholesterol abolished simvastatin-induced upregulation of eNOS expression. MVA, GGPP, FPP or cholesterol was added to the culture medium 1 hour before simvastatin (Sim) treatment. **p<0.01 vs TNFα only group, ###p<0.01 vs TNFα+Sim group, n=4. B, effects of Rho A inhibitor C3 exoenzyme (C3) and Rho-kinase inhibitor Y-27632 on eNOS expression. Cells were pre-incubated with C3 exoenzyme (0.25 µg/ml) or Y-27632 (10 µmol/L) for 4 h before TNFα treatment. **p<0.01 vs TNFα only group, n=4.