ORIGINAL RESEARCH ARTICLE

Inhibition of Aberrant MicroRNA-133a Expression in Endothelial Cells by Statin Prevents Endothelial Dysfunction by Targeting GTP Cyclohydrolase 1 in Vivo

BACKGROUND: GTP cyclohydrolase 1 (GCH1) deficiency is critical for endothelial nitric oxide synthase uncoupling in endothelial dysfunction. MicroRNAs (miRs) are a class of regulatory RNAs that negatively regulate gene expression. We investigated whether statins prevent endothelial dysfunction via miR-dependent GCH1 upregulation.

METHODS: Endothelial function was assessed by measuring acetylcholine-induced vasorelaxation in the organ chamber. MiR-133a expression was assessed by quantitative reverse transcription polymerase chain reaction and fluorescence in situ hybridization.

RESULTS: We first demonstrated that GCH1 mRNA is a target of miR-133a. In endothelial cells, miR-133a was robustly induced by cytokines/oxidants and inhibited by lovastatin. Furthermore, lovastatin upregulated GCH1 and tetrahydrobiopterin, and recoupled endothelial nitric oxide synthase in stressed endothelial cells. These actions of lovastatin were abolished by enforced miR-133a expression and were mirrored by a miR-133a antagomir. In mice, hyperlipidemia- or hyperglycemia-induced ectopic miR-133a expression in the vascular endothelium, reduced GCH1 protein and tetrahydrobiopterin levels, and impaired endothelial function, which were reversed by lovastatin or miR-133a antagomir. These beneficial effects of lovastatin in mice were abrogated by in vivo miR-133a overexpression or GCH1 knockdown. In rats, multiple cardiovascular risk factors including hyperglycemia, dyslipidemia, and hyperhomocysteinemia resulted in increased miR-133a vascular expression, reduced GCH1 expression, uncoupled endothelial nitric oxide synthase function, and induced endothelial dysfunction, which were prevented by lovastatin.

CONCLUSIONS: Statin inhibits aberrant miR-133a expression in the vascular endothelium to prevent endothelial dysfunction by targeting GCH1. Therefore, miR-133a represents an important therapeutic target for preventing cardiovascular diseases.

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Clinical Perspective

What Is New?

- Compromised endothelial nitric oxide synthase functions underlie a broad range of cardiovascular anomalies. Deficiency of GTP cyclohydrolase 1, the rate-limiting enzyme for de novo tetrahydrobiopterin synthesis, is an important mechanism of endothelial nitric oxide synthase dysfunction.
- Here we show that aberrant expression of microRNA-133a, a novel epigenetic factor, may explain GTP cyclohydrolase 1deficiency in the presence of cardiovascular risk factors.
- Statin, as a traditional lipid-reducing drug, promotes GTP cyclohydrolase 1 expression and preserves arterial tetrahydrobiopterin levels by repressing microRNA-133a expression.

What Are the Clinical Implications?

- Tetrahydrobiopterin is highly unstable and is easily oxidized; thus, it is not suitable for chronic oral administration.
- Clinically, a pharmacological approach that can effectively elevate GTP cyclohydrolase 1 expression is currently lacking.
- Our findings offer a mechanistic basis for augmenting endogenous tetrahydrobiopterin levels by targeting microRNA-133a as a rational therapeutic approach to correct endothelial nitric oxide synthase dysfunction, and provide further support to the widely prescribed statin for combating endothelial dysfunction in cardiovascular diseases, including hypertension and atherosclerosis.

Endothelial dysfunction, caused by the loss of nitric oxide (NO), is an early marker of cardiovascular disease (CVD). Coupled endothelial NO synthase (eNOS) must be fully saturated with tetrahydrobiopterin (BH4) to generate NO.[1] The vital role of GTP cyclohydrolase 1 (GCH1) in eNOS function has been established as the rate-limiting enzyme for de novo BH4 biosynthesis. We have previously reported that GCH1 silencing by small interfering RNA causes BH4 deficiency and eNOS uncoupling both in vitro and in vivo,[2,3] indicating that posttranscriptional regulation plays an important role in regulating GCH1 gene expression.

MicroRNAs (miR) are ≈20-nucleotide, single-stranded RNA molecules that target the 3′-untranslated region (3′-UTR) of specific mRNAs through partial complementarity, resulting in translational repression or mRNA degradation.[4] Voellenkle et al[5] reported that 400 miRs were within a broad abundance range in human umbilical vein endothelial cells (HUVECs) as determined by RNA deep sequencing, indicating that miRs are indispensable for maintaining vascular homeostasis in endothelial cells. Furthermore, miR-126, miR-21, miR-221/222, miR-503,

and miR-92a are consistently expressed in endothelial cells and have been identified to regulate vascular endothelial growth factor signaling, angiogenesis, oxidative stress, and NO production.[6–8] However, it is not known whether GCH1 mRNA expression is determined by a specific miR.

Statin is a hydroxymethylglutaryl-coenzyme A reductase inhibitor widely used to treat hypercholesterolemia.[9] The efficacy of statin therapy for preventing CVDs has been well recognized.[10] However, increasing evidence reveals that statin prevents endothelial dysfunction independent of its cholesterol-lowering activity. Emerging data also indicate that miRs such as miR-22, miR-34a, and miR-155 are mediators that contribute to multiple activities of statins.[11–13] However, the roles of miRs in statin-improved endothelial dysfunction need to be defined.

Although statin therapy is beneficial to improve vascular endothelial function,[14] the effects of statin on skeletal or cardiac muscles are distinctly detrimental as characterized by skeletal muscle toxicity and myocardial atrophy.[15] A few miRs, particularly miR-1 and miR-133a/b, are highly expressed and play crucial roles in skeletal and cardiac muscle biology.[16] Conversely, the expression of miR-1 and miR-133a/b in vascular endothelial cells is very low under physiological conditions.[17] These differences suggest that miR-1 or miR-133a/b might be a key molecular switch to determine the differential effects of statin between the endothelium and the muscle. We hypothesized that statin may produce skeletal muscle toxicity by inhibiting miR-1 or miR-133a/b, and, alternatively, statin might inhibit their ectopic expression in endothelial cells to prevent endothelial dysfunction.

In this study, we demonstrate that GCH1 is a target of miR-133a and that its ectopic expression in endothelial cells mediates endothelial dysfunction induced by multiple CVD risk factors. Our data also suggest that statin upregulates GCH1 gene expression by inhibiting aberrant miR-133a expression to prevent endothelial dysfunction.

METHODS

An expanded Methods section is available in the online-only Data Supplement.

Establishing Animal Models of Hyperglycemia, Hyperhomocysteinemia, and Dyslipidemia

The procedures followed were in accordance with our institutional guidelines and approved by the Animal Care and Use Committee of Xinxiang Medical University. To develop the diabetic model in rats or mice, a low-dose of streptozotocin (50 mg·kg^{-1}·d^{-1} for 5 consecutive days, intraperitoneally) was used to induce persistent hyperglycemia. A single injection of native low-density lipoprotein (LDL) (4 mg/kg) was applied to induce dyslipidemia in rats. For the induction of hyperhomocysteinemia (HHCY), rats were gavaged with homocysteine thiolactone (50 mg·kg^{-1}·d^{-1}) for 8 weeks as described previously.[18]

Measurement of Endothelium-Dependent Vasorelaxation

Mice or rat aorta rings were mounted in an organ bath in Kreb buffer. Contractile response was evoked by treatment with phenylephrine (1 μmol/L). At the plateau of contraction, accumulative acetylcholine (ACh) was added to elicit endothelium-dependent vasorelaxation (EDR) as described previously.[19] The relaxation was calculated as a ratio of ACh-induced vasodilation to phenylephrine-elicited vasoconstriction, and the ratio at 1 was set as 100% of relaxation.

Cellular Experiments

HUVECs were obtained from Clonetics Inc. Cells were grown in endothelial basal medium supplemented with 2% fetal bovine serum. After the treatments, cells were harvested to detect protein levels by Western blotting. RNA quantification was performed using the TaqMan microRNA Reverse Transcription Kit. Reactive oxygen species and NO production were determined by measuring dihydroethidium or diaminofluorescein fluorescence. BH4 levels were determined by high-performance liquid chromatography as described previously.[20]

Statistical Analysis

Data are reported as mean±standard error of the mean. All data were analyzed with a 1-way analysis of variance followed by Bonferroni post hoc analyses, with the exception of those obtained from the time/concentration courses, which were analyzed with repeated-measures analysis of variance. A 2-sided P value of <0.05 was considered significant.

RESULTS

GCH1 mRNA Is a Target of miR-133a/b

Computational target-scan analysis (Figure 1A) showed that miR-1, miR-206, and miR-613 could potentially bind the highly conserved target site (76–83, 5′-ACAUUCC-3′) in the longer isoform (1–2011) of the 3′-UTR of GCH1 mRNA (online-only Data Supplement Figure IA). miR-133a/b can target the site (5′-GGACCAAA-3′) at 1685 to 1692 in the longer isoform or at 707 to 714 in the shorter isoform (1–1033) of the 3′-UTR of GCH1 mRNA, suggesting that GCH1 gene expression may be posttranscriptionally regulated by miRs.

To identify the miR that represses GCH1 gene expression through 3′-UTR interaction, we cloned the GCH1 3′-UTR in a luciferase reporter plasmid and performed reporter analysis in HEK293 cells. Our results demonstrated that cotransfection of miR-1, miR-206, or miR-613 with the GCH1 3′-UTR reporter plasmid did not reduce luciferase activity, whereas cotransfection of miR-133a or miR-133b resulted in significant inhibition of luciferase activity (Figure 1B). Furthermore, miR-133a/b was unable to suppress the activity of the GCH1 3′-UTR reporter with a mutated miR-133a/b seed sequence (online-only Data Supplement Figure IB). These data indicate that GCH1 mRNA is a target of miR-133a/b.

MiR-133a Reduces GCH1 Gene Expression by Destabilizing GCH1 mRNA in HEK293 Cells

To determine whether miR-133a inhibited GCH1 gene expression by destabilizing GCH1 mRNA, the half-life of GCH1 mRNA was determined in HEK293 cells that overexpressed GCH1. As shown in Figure 1C and online-only Data Supplement Figure II, cotransfection of preliminary miR-133a (premiR), but not scrambled miR (scr-miR), for 48 hours reduced the half-life of GCH1 mRNA in GCH1-overexpressing HEK293 cells, suggesting that GCH1 mRNA stability is regulated by miR-133a.

Ectopic Expression of miR-133a in Endothelial Cells Is Induced by Cytokines or Oxidants, Which Is Inhibited by Statin

To determine the role of miR-133a/b in regulating GCH1 gene expression in endothelial cells, we examined the expression of miR-133a/b in different cells. As indicated in Figure 2A, the basal level of miR-133a in endothelial cells was particularly low, ≈1/500 to 1/100 of that in skeletal cells, cardiomyocytes, and vascular smooth muscle cells, wherein miR-133a/b was highly expressed as reported.[21–23] miR-133b was undetectable in endothelial cells. These observations suggest that miR-133a/b is not required for maintaining endothelial function under physiological conditions.

Interestingly, on stimulation, the expression of miR-133a, but not miR-133b, in endothelial cells was extremely induced by oxidized LDL (ox-LDL), interleukin 6 (IL-6), and tumor necrosis factor-α (TNFα) (Figure 2B). Lovastatin remarkably suppressed the cytokine/oxidant-induced aberrant expression of miR-133a in HUVECs. These results demonstrate that miR-133a is ectopically expressed in endothelial cells and reveal a novel function of statin as a suppressor of miR-133a expression.

Exogenous Expression of miR-133a Negates Lovastatin-Induced Upregulation of GCH1 Gene Expression in Endothelial Cells

To examine whether statin upregulates GCH1 via miR-133a inhibition, we infected HUVECs with lentiviruses harboring scr-miR or premiR-133a for 48 hours followed by 12-hour lovastatin treatment. Lentivirus-mediated forced expression of miR-133a in HUVECs was assessed in Figure 2C. Infection of premiR-133a lentivirus dramatically upregulated miR-133a expression by ≈150-fold in comparison with scr-miR, which was not inhibited by lovastatin. Lovastatin significantly increased both the mRNA and protein levels of GCH1 in HUVECs infected with lentivirus scr-miR (Figure 2D and 2E), but not in HUVECs with enforced expression of exogenous miR-133a. Interestingly, premiR-133a alone showed no effect on GCH1
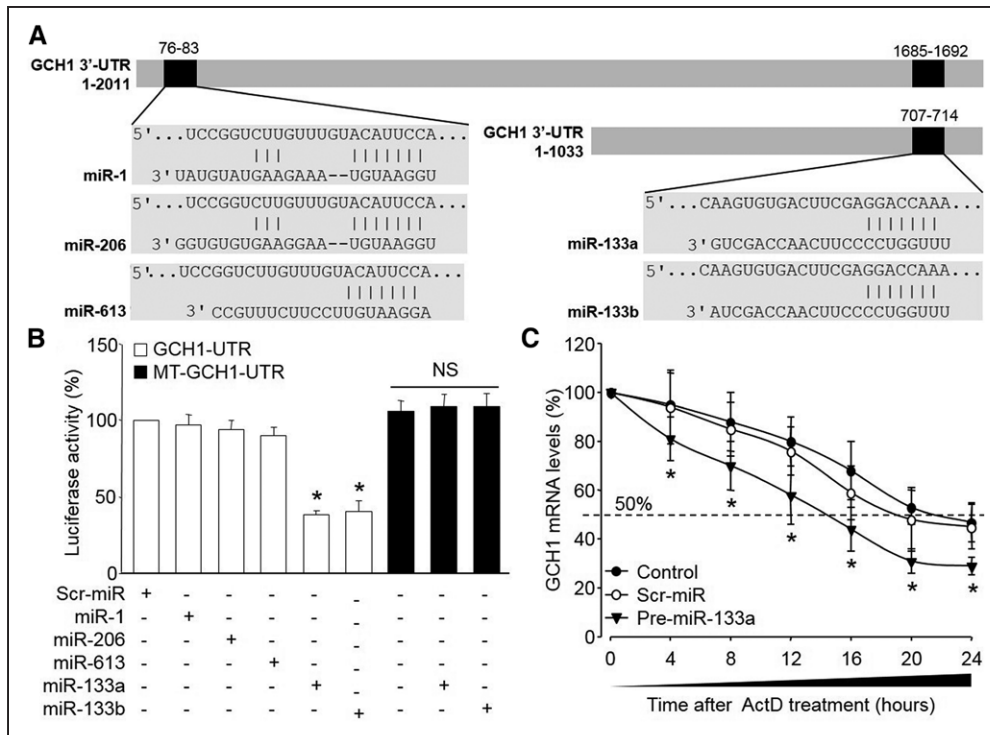
Figure 1. GTP cyclohydrolase 1 (GCH1) mRNA is a target of miR-133.
(**A**) Prediction of miRs targeting the 3′-UTR of the GCH1 mRNA by Target Scan. The GCH1 3′-UTR has 2 isoforms, the longer one (1–2011) and the shorter one (1–1033), where the shorter isoform is equal to 978 to 2011 in the longer isoform. (**B**) The luciferase reporter plasmid containing the 3′-UTR of GCH1 mRNA (GCH1-UTR) or the mutant of the GCH1-UTR (MT-GCH1-UTR) was cotransfected with scr-miR, premiR-1, premiR-206, premiR-613, premiR-133a, premiR-133b into HEK293 cells. The luciferase activity in the total cell lysates was assayed. n=5 per group. *P<0.05 versus scr-miR alone. NS indicates no significance. (**C**) HEK293 cells transfected with the plasmid harboring GCH1 cDNA were cotransfected with scr-miR or premiR-133a for 48 hours. Then, actinomycin D (ActD, 10 µg/mL) was added to the culture medium for the indicated time. The half-life of GCH1 mRNA was analyzed by RT-PCR. n=5 per group. *P<0.05 versus Scr-miR alone. miR indicates microRNA; premiR, preliminary miR-133a; RT-PCR, reverse transcription polymerase chain reaction; scr-miR, scrambled miR; and UTR, untranslated region.

gene expression in the absence of statin in HUVECs, indicating that the basal level of GCH1 in resting endothelial cells is not regulated by miR-133a.

Statin or miR-133a Inhibition Increases GCH1 Protein Levels in Endothelial Cells Treated with Cytokines or Oxidants

To further investigate how miR-133a regulates GCH1 gene expression, we synthesized a miR-133a antagomir with an antisense sequence to miR-133a, and a control antagomir without an antisense to any gene as described previously, as well.[24] As depicted in Figure 2F and 2G, ox-LDL, IL-6, or TNFα significantly reduced the levels of GCH1 protein in endothelial cells or in cells treated with the control antagomir, consistent with other reports.[25] Both lovastatin and miR-133a antagomir normalized the GCH1 levels in HUVECs under stimuli, demonstrating that cytokines/oxidants via miR-133a downregulate GCH1 in endothelial cells, which is inhibited by statin.

Exogenous miR-133a Abrogates Lovastatin-Induced eNOS Recoupling in Cytokine/Oxidant-Treated Endothelial Cells

Levels of BH4, an essential cofactor of eNOS, are determined by GCH1.[3] In general, eNOS generates reactive oxygen species but not NO in BH4 deficiency, a process called eNOS uncoupling.[2] Therefore, we examined the effects of lovastatin on BH4 content, NO levels, and reactive oxygen species production in HUVECs. As shown in online-only Data Supplement Figure IIIA through IIIC, ox-LDL, IL-6, and TNFα markedly decreased the BH4 levels and NO release, and increased reactive oxygen species production in HUVECs, which was reversed by lovastatin.

To study the role of miR-133a in statin-induced eNOS recoupling, we generated cells with enforced miR-133a expression by infecting cells with a lentivirus expressing premiR-133a. Although lovastatin treatment effectively normalized the BH4 levels (Figure 3A) and recoupled eNOS (Figure 3B and 3C) in ox-LDL–treated HUVECs in-
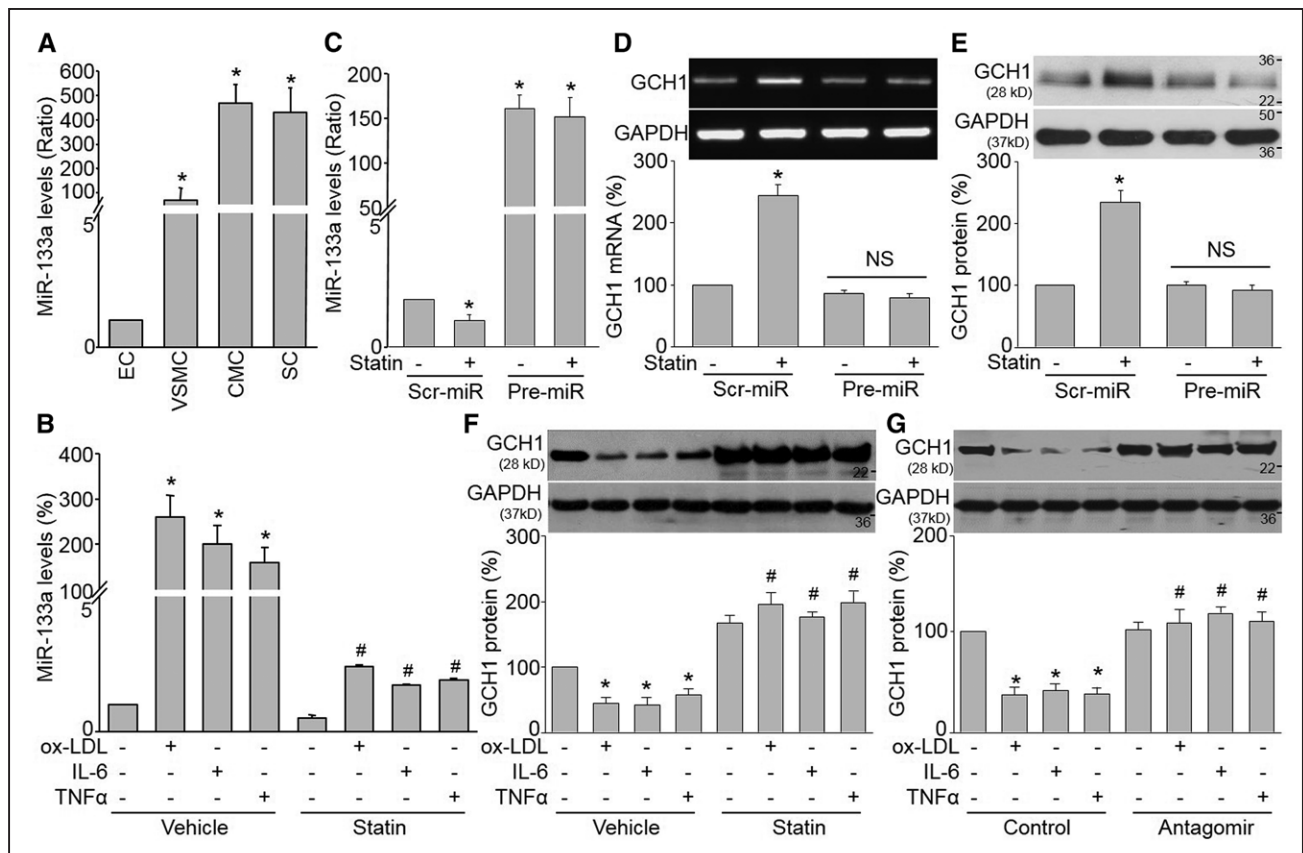
Figure 2. Cytokines/oxidants induce aberrant miR-133a expression in endothelial cells to inhibit GCH1 gene expression, which is abolished by statin.

(**A**) MiR-133a levels were measured in cultured primary cells by RT-qPCR. EC indicates endothelial cells; VSMC, vascular smooth muscle cells; CMC, cardiomyocytes; and SC, skeletal cells from hamstrings. n=5 per group. *$P<0.05$ versus EC. (**B**) HUVECs were pretreated with lovastatin (10 μmol/L) for 30 minutes followed by coincubation with ox-LDL (100 μg/mL), IL-6 (10 ng/mL), or TNFα (50 ng/mL) for 24 hours. Cells were subjected to RT-qPCR for detecting miR-133a levels. n=3 per group. *$P<0.05$ versus Vehicle alone. #$P<0.05$ versus ox-LDL, IL-6, or TNFα alone. (**C** through **E**) HUVECs were infected with lentivirus harboring the scr-miR or premiR-133a for 48 hours followed by lovastatin treatment (10 μmol/L) for 24 hours. Cells were then used for detecting miR-133a levels by RT-qPCR in **C**, GCH1 mRNA levels by RT-PCR in **D**, and GCH1 protein levels by Western blotting in **E**. n=3 per group. *$P<0.05$ versus Scr-miR alone. NS indicates no significance. (**F**) GCH1 protein levels were detected by Western blotting analysis in cells from **B**. (**G**) HUVECs were transfected with the miR-133a antagomir for 24 hours followed by coincubation with ox-LDL, IL-6, or TNFα. Cells were subjected to Western blotting analysis for GCH1 protein levels. n=3 per group. *$P<0.05$ versus Control. #$P<0.05$ versus ox-LDL, IL-6, or TNFα alone. GCH1 indicates GTP cyclohydrolase 1; HUVEC, human umbilical vein endothelial cell; IL-6, interleukin 6; miR, microRNA; ox-LDL, oxidized low-density lipoprotein; premiR-133a, preliminary miR-133a; RT-qPCR, quantitative reverse transcription polymerase chain reaction; scr-miR, scrambled miR; and TNFα, tumor necrosis factor-α.

fected with a lentivirus expressing scr-miR, it failed to increase the BH4 levels and recouple eNOS in HUVECs on exogenous miR-133a expression.

Given that statin recouples eNOS via inhibition of miR-133a, we expected that the miR-133a antagomir could mimic the effects of statin. Thus, HUVECs were transfected with the control or miR-133a antagomir. As depicted in Figure 3D through 3F, in comparison with the effects of the control antagomir, BH4 reduction and eNOS uncoupling induced by ox-LDL, IL-6, and TNFα were noticeably inhibited by the miR-133a antagomir, further suggesting that lovastatin recouples eNOS in endothelial cells via miR-133a inhibition.

Inhibition of GCH1 or eNOS Bypasses the Effects of Lovastatin on Endothelial Dysfunction ex Vivo

We have reported that uncoupled eNOS contributes to endothelial dysfunction, which is defined by impairment of ACh-induced EDR.[2,3,26] To determine whether lovastatin prevents endothelial dysfunction via eNOS recoupling, an ex vivo model of endothelial dysfunction was generated by incubating isolated descending aortic rings from mice with ox-LDL, IL-6, and TNFα (Figure 4A). Treatment with lovastatin alone did not alter the ACh-induced EDR in aortic arteries. Preincubation of aortic rings with lovastatin bypassed the detrimental effects induced by ox-LDL, IL-
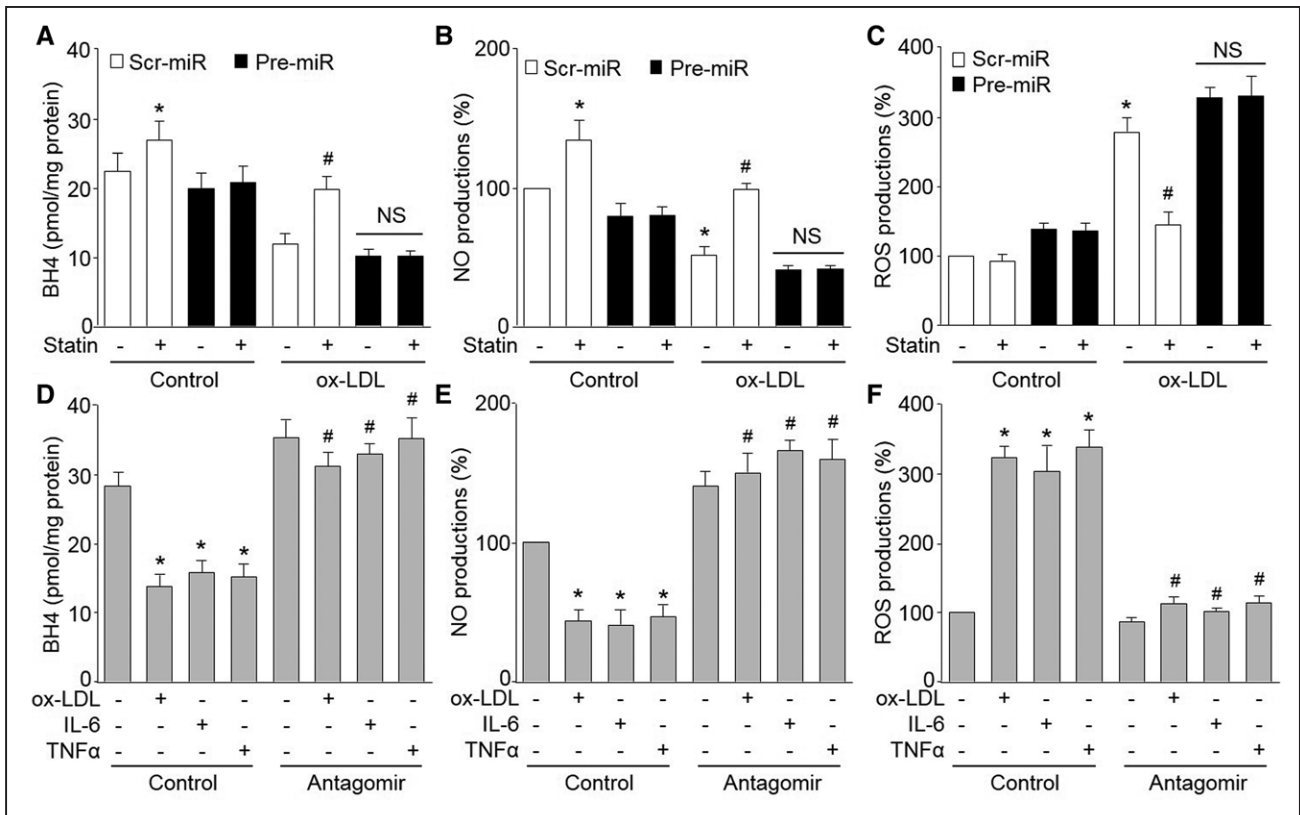
Figure 3. Lovastatin recouples eNOS in endothelial cells in a miR-133a–dependent manner.
(**A** through **C**) HUVECs infected with lentivirus containing scr-miR or premiR-133a for 48 hours followed by ox-LDL (100 µg/mL) or lovastatin treatment (10 µmol/L, 24 hours). Cell lysates were used to detect BH4 content by HPLC in **A**, NO production by measuring diaminofluorescein fluorescence intensity in **B**, and ROS production by measuring DHE fluorescence intensity in **C**. n=3 per group. *$P<0.05$ versus scr-miR alone. #$P<0.05$ versus scr-miR plus ox-LDL. NS indicates no significance. (**D** through **F**) HUVECs were transfected with the miR-133a antagomir for 24 hours followed by coincubation with ox-LDL, IL-6, or TNFα for 24 hours. Cells were used to determine BH4 content in **D**, NO production in **E**, and ROS production in **F**. n=3 per group. *$P<0.05$ versus control. #$P<0.05$ versus ox-LDL, IL-6, or TNFα alone. BH4 indicates tetrahydrobiopterin; DAF, diaminofluorescein; DHE, dihydroethidium; HPLC, high-performance liquid chromatography; HUVEC, human umbilical vein endothelial cell; IL-6, interleukin 6; miR, microRNA; NO, nitric oxide; ox-LDL, oxidized low-density lipoprotein; premiR-133a, preliminary miR-133a; ROS, reactive oxygen species; scr-miR, scrambled miR; and TNFα, tumor necrosis factor-α.

6, and TNFα (Figure 4B through 4D). Importantly, the addition of either the eNOS inhibitor N^G-nitro-L-arginine methyl ester or the GCH1 inhibitor 3-deoxy-D-arabinoheptulosonate 7-phosphate abolished the protective effects of lovastatin on improving ACh-induced EDR. Moreover, ox-LDL, IL-6, TNFα, lovastatin, N^G-nitro-L-arginine methyl ester, and 3-deoxy-D-arabinoheptulosonate 7-phosphate did not alter single-nucleotide polymorphism–induced endothelium-independent relaxation in mice aortic rings (online-only Data Supplement Figure IVA through IVD). These data reveal that lovastatin prevents endothelial dysfunction via GCH1/eNOS signaling ex vivo.

Overexpression of miR-133a Abolishes Lovastatin-Improved Endothelial Dysfunction in *Apoe⁻/⁻* Mice

Next, we investigated the role of miR-133a in lovastatin-improved endothelial dysfunction induced by hyperlipid-

emia, which is a major independent risk factor for atherosclerosis.[27] As shown in the online-only Data Supplement Figure VA and Table I, *Apoe⁻/⁻* mice were fed a high-fat diet to induce hyperlipidemia accompanied with lovastatin administration for 2 weeks. The mice were then infected with a lentivirus infection harboring the premiR-133a via tail vein injection for 4 weeks. In comparison with mice infected with the scr-miR lentivirus, infection with the premiR-133a lentivirus greatly increased the miR-133a levels in the liver, adductor skeletal muscle, heart, kidney, fat, blood (online-only Data Supplement Figure VB), and aortic arteries (online-only Data Supplement Figure VC) of mice. This indicated that infection with a lentivirus expressing premiR-133a effectively induces high and consistent expression of miR-133a in the cardiovascular system and throughout the entire body.

The localization of miR-133a in the vessel sections was determined by fluorescence in situ hybridization.
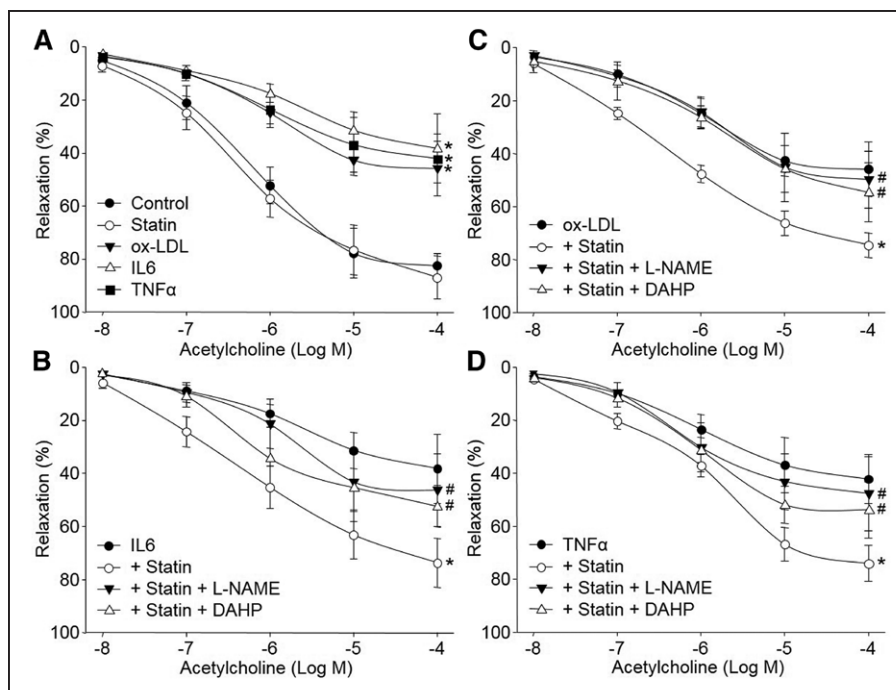
Figure 4. Inhibition of GCH1 or eNOS bypasses the protective effects of lovastatin on endothelial dysfunction ex vivo.
(**A**) Isolated mice aortic rings were incubated with lovastatin (10 μmol/L), ox-LDL (100 μg/mL), IL-6 (10 ng/mL), or TNFα (50 ng/mL) for 6 hours. ACh-induced EDR in aortic rings was assayed using an organ chamber. n=5 per group. *$P<0.05$ versus control. (**B** through **D**) Aortic rings isolated from mice were preincubated with L-NAME (1 mmol/L) or DAHP (10 mmol/L) for 30 minutes and then exposed to lovastatin (10 μmol/L) for 6 hours in the presence of ox-LDL in **B**, IL-6 in **C**, or TNFα in **D**. Vasorelaxation was elicited by ACh in an organ bath. n=5 per group. *$P<0.05$ versus ox-LDL, IL-6, or TNFα alone. #$P<0.05$ versus lovastatin treatment. Ach indicates acetylcholine; DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate; EDR, endothelium-dependent vasorelaxation; GCH1, GTP cyclohydrolase 1; IL-6, interleukin 6; L-NAME, N^G-nitro-L-arginine methyl ester; ox-LDL, oxidized low-density lipoprotein; and TNFα, tumor necrosis factor-α.

As indicated in Figure 5A, the basal expression of miR-133a in the aortic artery was mainly located in the vascular medium, other than the endothelium. However, miR-133a expression was clearly detected in the vascular endothelium and was inhibited by lovastatin in hyperlipidemic mice infected with lentiviruses harboring scr-miR. Infection with lentiviruses expressing the premiR-133a dramatically increased the expression of miR-133a in both the vascular endothelium and in the medium.

Moreover, high-fat diet reduced the vascular levels of GCH1 protein and mRNA (Figure 5B), inhibited ACh-induced EDR (Figure 5C), and uncoupled eNOS (online-only Data Supplement Figure VD through VF) as indicated by the reduction of serum NO levels and superoxide dismutase activities, and the enhancement of serum malondialdehyde levels in high-fat diet–fed Apoe$^{-/-}$ mice. Lovastatin remarkably reversed these phenotypes in hyperlipidemic mice infected with the scr-miR lentivirus, but not in mice with miR-133a overexpression. Collectively, these data provide compelling evidence that ectopic induction of miR-133a in endothelial cells contributes to endothelial dysfunction in vivo.

GCH1 Deficiency Attenuates the Effects of Lovastatin on Endothelial Dysfunction in *Apoe*$^{-/-}$ Mice

We further determined the role of GCH1 in lovastatin-improved endothelial dysfunction in Apoe$^{-/-}$ mice. Loss of GCH1 function was induced by infecting mice with a lentivirus harboring the GCH1 short hairpin RNA (Figure 6A). As indicated in Figure 6B, ACh-induced EDR in the aortic artery was improved dramatically by lovastatin treatment in Apoe$^{-/-}$ mice infected with the control short hairpin RNA–expressing lentivirus. However, lovastatin did not improve the ACh-induced EDR in mice infected with the lentivirus expressing GCH1 short hairpin RNA.

The effects of GCH1 deficiency on lovastatin-recoupled eNOS in vivo were also examined. As indicated in Figure 6C through 6F, lovastatin increased BH4 biosynthesis, serum NO levels, and superoxide dismutase activity, but reduced serum malondialdehyde levels in mice infected with the control short hairpin RNA lentivirus. All lovastatin-induced beneficial effects were reversed on GCH1 knockdown, further suggesting that GCH1 is required for lovastatin-mediated recoupling of eNOS in vivo.
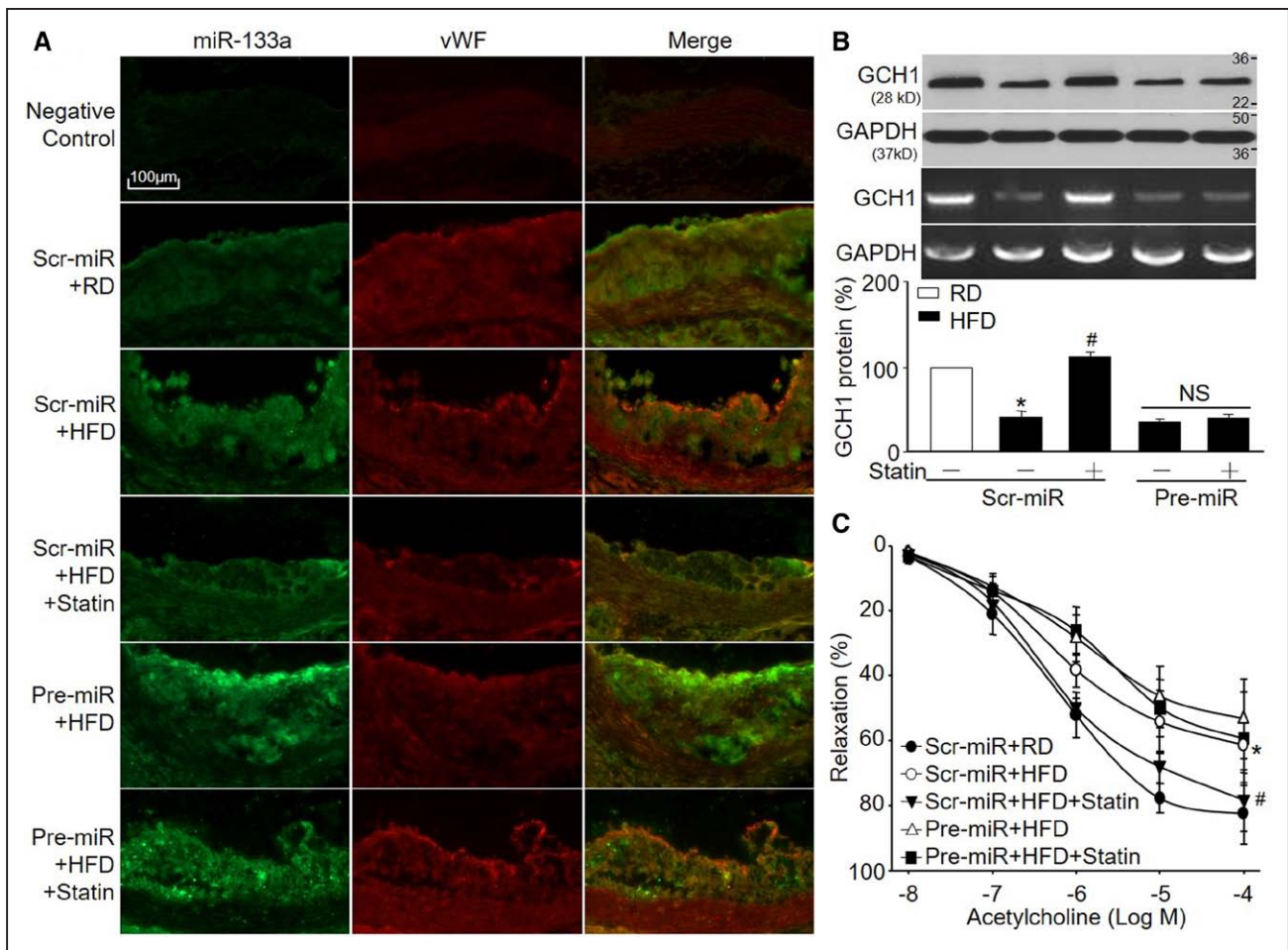
Figure 5. Overexpression of miR-133a abrogates lovastatin-preserved endothelial function in *Apoe*−/− mice fed with a high-fat diet (HFD).
The protocol and experimental designs are described in the online-only Data Supplement Methods and Figure VA. (**A**) The miR-133a expression in the aortic arteries was detected by fluorescence in situ hybridization. Green, miR-133a; Red, vWF. (**B**) Western blotting analysis of GCH1 protein and RT-PCR analysis of GCH1 mRNA in aortic tissues. (**C**) ACh-induced vasorelaxation was assayed in descending aortic arteries using an organ chamber. n=10 to 15 per group. *$P<0.05$ versus scr-miR plus RD (regular diet). #$P<0.05$ versus scr-miR plus HFD. Ach indicates acetylcholine; GCH1, GTP cyclohydrolase 1; miR, microRNA; NS, no significance; premiR, preliminary miR-133a; RT-PCR, reverse transcription polymerase chain reaction; scr-miR, scrambled miR; and vWF, von Willebrand factor.

MiR-133a Antagonism Recouples eNOS and Prevents Endothelial Dysfunction in Diabetic Mice

If statin prevents endothelial dysfunction via inhibition of miR-133a, miR-133a antagonism might produce effects similar to those produced by statin in vivo. To test this notion, streptozotocin-induced diabetic C57B16 mice received in vivo delivery of miR-133a antagomir through Alzet osmotic minipumps for 4 weeks to establish a loss-of-function model for miR-133a (online-only Data Supplement Figure VIA and Table II). In comparison with those in control mice, hyperglycemia reduced the levels of GCH1 mRNA and protein (Figure 7A), and the BH4 content (Figure 7B) in aortic arteries, which were normalized by the miR-133a antagomir. Functional analysis indicated that hyperglycemia impaired the ACh-induced

EDR, decreased serum NO levels and superoxide dismutase activities, and increased serum malondialdehyde levels in mice infused with the control antagomir, as well (Figure 7C through 7F). Importantly, the miR-133a antagomir rescued these abnormal phenotypes in diabetic mice. These data suggest that antagonism of miR-133a upregulates GCH1 expression and rescues endothelial function in diabetes mellitus.

Lovastatin Inhibits Aberrant miR-133a Expression in the Vascular Endothelium in Rat Models of Diabetes Mellitus, Dyslipidemia, and HHCY

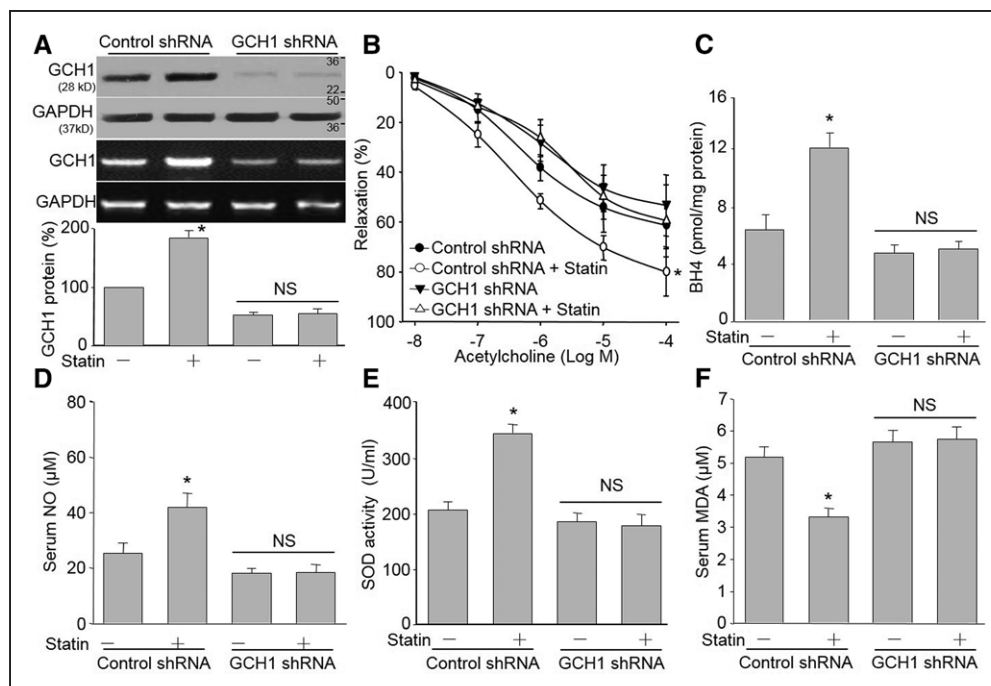The inhibitory effect of statins on the ectopic miR-133a expression in vascular endothelium was further con-

Figure 6. GCH1 deficiency abolishes lovastatin-improved endothelial dysfunction in *Apoe*^-/- mice.
The protocol and experimental designs are described in online-only Data Supplement Methods and Figure VA. (**A**) Western blotting analysis of GCH1 protein and RT-PCR analysis of GCH1 mRNA in aortic tissues. (**B**) ACh-induced vasorelaxation in descending aortic artery was assayed using an organ chamber. (**C**) The BH4 content in aortic tissues was determined by HPLC. Serum NO levels (**D**), serum SOD activities (**E**), and serum MDA levels (**F**) were measured in mice. n=10 to 15 per group. *$P<0.05$ versus control shRNA alone. Ach indicates acetylcholine; BH4, tetrahydrobiopterin; GCH1, GTP cyclohydrolase 1; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; NO, nitric oxide; NS, no significance; RT-PCR, reverse transcription polymerase chain reaction; shRNA, short hairpin RNA; and SOD, superoxide dismutase.

firmed by determining the subcellular distribution of miR-133a in the vascular walls from diabetic rats. To this end, a rat model of type 1 diabetes mellitus was induced by injection of streptozotocin and was treated orally with lovastatin (online-only Data Supplement Figure VIB and Table III). Endothelial cells and vascular smooth muscle cells were stained for von Willebrand factor and α-smooth muscle actin, respectively. Vascular expression of miR-133a was determined by fluorescence in situ hybridization. As shown in Figure 8A, miR-133a was dominantly expressed in the vascular smooth muscle cell layer but not in the endothelial layer in normal rats. Lovastatin alone reduced the basal expression of miR-133a in the vascular medium. Hyperglycemia dramatically induced ectopic miR-133a expression in the vascular endothelium, which was remitted by lovastatin treatment in rats. Quantitative analysis of miR-133a by quantitative reverse transcription polymerase chain reaction also indicated that the miR-133a levels in the vascular walls were significantly higher in diabetic rats than those in nondiabetic rats (Figure 8B). Enhancement of miR-133a induced by hyperglycemia was normalized by lovastatin treatment. Importantly, the inhibition of miR-133a by lovastatin was recapitulated in the rat models of other recognized cardiovascular risk factors, including dyslip-

idemia and HHCY (online-only Data Supplement Figure VIC and VID, Tables IV and V, and Figure 8B). In summary, these results demonstrate that statin can inhibit ectopic miR-133a expression in the vascular endothelium under pathophysiological conditions.

Lovastatin Reverses GCH1 Levels and Recouples eNOS in Rat Models of Multiple Cardiovascular Risk Factors

We then sought to determine whether miR-133a–mediated downregulation of GCH1/eNOS signaling is a common mechanism in endothelial dysfunction and could be recapitulated in rats in the presence of multiple cardiovascular risk factors. Thus, GCH1 gene expression, BH4 content, and eNOS function in the aortic artery were determined in rat models of dyslipidemia, HHCY, and diabetes mellitus. The levels of GCH1 protein and mRNA (Figure 8C) and BH4 content (Figure 8D) were decreased in these models. In addition, these risk factors reduced the serum NO levels and superoxide dismutase activities, and increased the malondialdehyde levels in serum and in carotid arteries from rats (online-only Data Supplement Figure VIIA through VIID). Notably, administration of lovastatin abolished these alterations in rats,

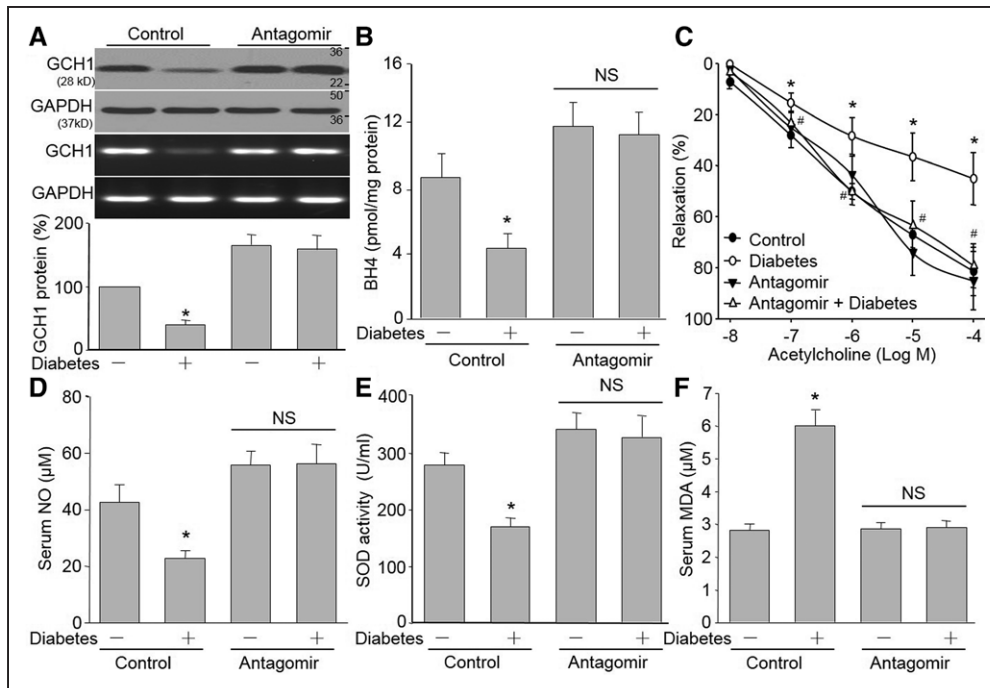*Circulation.* 2016;134:1752–1765. DOI: 10.1161/CIRCULATIONAHA.116.017949

Figure 7. In vivo antagonism of miR-133a improves endothelial dysfunction in STZ-induced diabetic mice.
The protocol and experimental designs are described in online-only Data Supplement Methods and Figure VIA. (**A**) Western blotting analysis of GCH1 protein and RT-PCR analysis of GCH1 mRNA in aortic tissues. (**B**) BH4 content in aortic tissues was determined by HPLC. (**C**) ACh-induced vasorelaxation in descending aortic artery was assayed using an organ chamber. Serum NO levels (**D**), serum SOD activities (**E**), and serum MDA levels (**F**) were measured in mice. n=10 to 15 per group. *$P<0.05$ versus control mice alone. #$P<0.05$ versus diabetic mice. Ach indicates acetylcholine; BH4, tetrahydrobiopterin; GCH1, GTP cyclohydrolase 1; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; miR, microRNA; NO, nitric oxide; NS, no significance; RT-PCR, reverse transcription polymerase chain reaction; SOD, superoxide dismutase; and STZ, streptozotocin.

implying that the miR-133a/GCH1/BH4 signaling pathway not only mediates the protective effects of lovastatin, but is also a common mechanism of endothelial dysfunction in CVDs.

Lovastatin via GCH1 Upregulation Improves Endothelial Dysfunction in Rats With Hyperglycemia, HHCY, and Dyslipidemia

Finally, to determine whether lovastatin prevents endothelial dysfunction in a GCH1-dependent manner, we treated rats with lovastatin or 3-deoxy-D-arabinoheptulosonate 7-phosphate. As shown in online-only Data Supplement Figure VIIE, aortic rings from rats with dyslipidemia, HHCY, or hyperglycemia presented significant inhibition of ACh-induced EDR, in comparison with that in control rats. Treatment with lovastatin dramatically rescued the abnormal phenotypes of endothelial dysfunction induced by diabetes mellitus, dyslipidemia, and HHCY in rats (Figure 8E through 8G). As expected, cotreatment with 3-deoxy-D-arabinoheptulosonate 7-phosphate completely abolished the beneficial effects of lovastatin on endothelial dysfunction in these rats, suggesting that lovastatin improves endothelial dysfunction in rats with CVD risk factors, in a GCH1-dependent manner.

DISCUSSION

The major finding of this study was that GCH1 mRNA is a target of miR-133a. The miR-133 family has 2 members, namely, miR-133a and miR-133b, located on different chromosomes.[28] Functionally, miR-133a/b was identified to regulate cell specification, differentiation, and proliferation by targeting a number of genes including SFR, HDAC4, SP1, and cyclin D2.[21,29–32] In epithelial cells, miR-133a is upregulated by neurotensin to mediate colonic inflammation.[33] In this study, we further demonstrated that GCH1 mRNA is a target of miR-133a in endothelial cells. Although GCH1 gene expression is regulated posttranslationally, such as by the ubiquitin-proteasome pathway and phosphorylation,[25,34,35] this study is the first to ascertain that GCH1 mRNA stability is determined by miR-133a. This discovery not only uncovers a novel mechanism of GCH1 gene regulation, but also broadens the biological functions of miR-133a.

We further illustrated that ectopic expression of miR-133a in endothelial cells was inhibited by statin. MiR-133a has been classified as a muscle-specific miRNA and is necessary for proper development and function of skeletal and cardiac muscles,[16,28,36] but it has not been tested in other tissues.[17] In this study, quantitative reverse
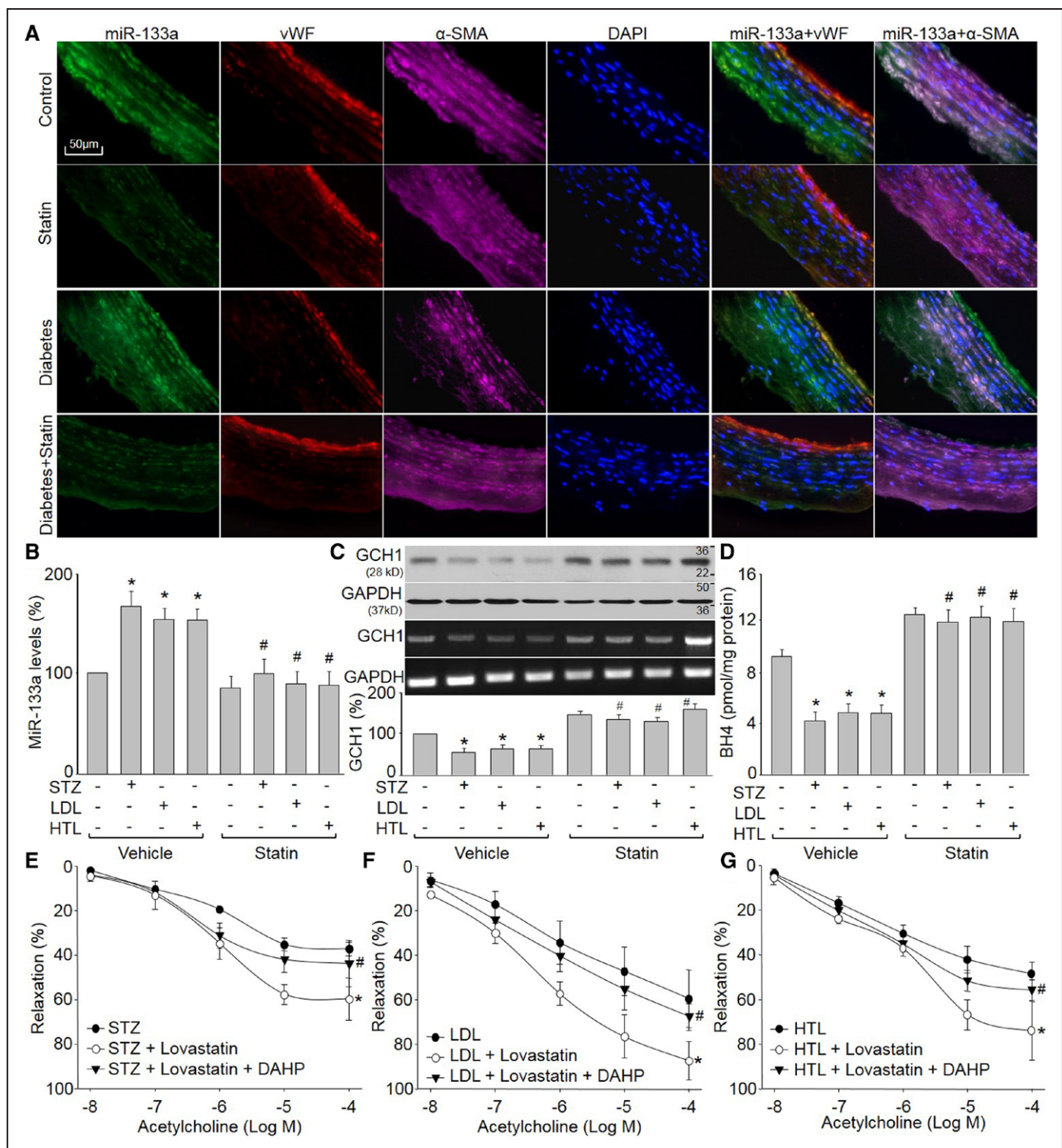
Figure 8. Lovastatin inhibits miR-133a ectopic expression, increases GCH1 protein levels, and prevents endo-thelial dysfunction in rat models of dyslipidemia, HHCY, and hyperglycemia.

The protocol and experimental designs are described in online-only Data Supplement Methods and Figure VIB through VID. (A) The carotid artery was subjected to fluorescence in situ hybridization to detect miR-133a expression in rats. Green, miR-133a; Red, vWF; Purple, α-SMA; Blue, DAPI. (B through D) Aortic tissues from rats were subjected to RT-qPCR analysis for miR-133a expression in B, Western blotting analysis of GCH1 protein and RT-PCR analysis of GCH1 mRNA in C, and HPLC analysis of BH4 content in D. n=10 to 15 in each group. *$P<0.05$ versus control rats. #$P<0.05$ versus rats treated with n-LDL, STZ, or HTL. (E) ACh-induced EDR in diabetic rats treated with lovastatin or plus DAHP. n=10 to 15 per group. *$P<0.05$ versus STZ group. #$P<0.05$ versus STZ plus lovastatin group. (F) ACh-induced EDR in dyslipidemic rats with lovastatin or plus DAHP. n=10 to 15 per group. *$P<0.05$ versus LDL group. #$P<0.05$ versus LDL plus lovastatin group. (G) ACh-induced EDR in HHCY rats with lovastatin or plus DAHP. n=10 to 15 per group. *$P<0.05$ versus HTL group. #$P<0.05$ versus HTL plus lovastatin group. Ach indicates ace-tylcholine; BH4, tetrahydrobiopterin; DAHP, 3-deoxy-ᴅ-arabinoheptulosonate 7-phosphate; DAPI, 4′,6-diamidino-2-phenylindole; (Continued)

transcription polymerase chain reaction analysis indicated that the basal level of miR-133a was very low, whereas miR-133b was undetectable in endothelial cells. This is partially consistent with reports from Voellenkle et al[5] that both miR-216 and miR-21 are highly abundant, whereas miR-133a/b are not detected in HUVECs by RNA deep sequencing. We also observed that miR-133a was ectopically expressed in endothelial cells by pathophysiological factors. This is similar to the expression of miR-130a, which is very low in quiescent endothelial cells, but can be activated by serum.[37,38] Furthermore, aberrant expression of miR-133a and subsequent endothelial dysfunction in vascular endothelium were normalized by treatment with statin. Traditionally, statin is considered as a lipid-lowering drug to suppress the progress of atherosclerosis.[39] In the present study, we uncovered an hydroxymethylglutaryl-coenzyme A reductase–independent, miR-133a–mediated action of statin to improve endothelial function in CVDs.

The study also demonstrated that miR-133a–mediated GCH1 downregulation in endothelial cells might be a common mechanism observed at the beginning of CVDs. The available data suggest that oxidant stress mediates endothelial dysfunction in diabetic mice and in angiotensin II–induced hypertensive or aneurysmal mice.[40,41] How oxidative stress is produced as an early and common pathogenic phenomenon in cardiovascular risk factors remains to be established. In this study, we found that upregulation of miR-133a and consequent GCH1 reduction was observed in several rat models, because GCH1 deficiency has been demonstrated to cause endothelial dysfunction in animal models.[2,3]

It is interesting to note that miR-133a overexpression had no effect on GCH1 mRNA in the absence of statin (Figure 2D), and statin had no effect on GCH1 protein in the presence of miR-133a overexpression (Figure 2E). These data clearly indicated that both the basal level and the statin-mediated increased level of GCH1 in resting endothelial cells are not regulated by endogenous miR-133a. Forced expression of miR-133a negated the effect of statin on GCH1 mRNA, suggesting that statin had no effect on the miR-133a molecules per se, but likely affected signaling events upstream of miR-133a transcription. In terms of function, statin can inhibit endogenous expression but not the exogenous expression of miR-133a in endothelial cells. Our in vivo results also demonstrated that statin increased GCH1 protein in normal rats without significant reduction in miR-133a (Figure 8B and 8C), supporting that the effects of statin on basal GCH1 are independent of miR-133a inhibition. We thought that

statin might increase the GCH1 protein levels under physiological conditions through gene transcription as suggested by the results in Figure 2D, wherein statin remarkably upregulated GCH1 mRNA. We previously reported that GCH1 protein can be degraded via ubiquitin-dependent proteasomes in endothelial cells[3] and that AMP-activated protein kinase activation inhibits proteasome activity in endothelial cells.[27] Statin is known to activate AMP-activated protein kinase[42] and inhibit proteasomal activity in endothelial cells.[43] Thus, we speculated that protein stabilization is possibly involved in statin-induced upregulation of GCH1 protein.

In summary, this study uncovered a common mechanism by which multiple CVD risk factors induce endothelial dysfunction (online-only Data Supplement Figure VIII). Statin, as a lipid-lowering drug, inhibits the aberrant expression of miR-133a in endothelial cells and upregulates GCH1 gene expression, resulting in restoration of BH4 and consequent eNOS recoupling. Thus, statin prevents the endothelial dysfunction induced by dyslipidemia, HHCY, and diabetes mellitus. In conclusion, targeting miR-133a should be an attractive strategy to clinically improve endothelial function in CVD patients.

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DISCLOSURES

None.

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Figure 8. **Continued** EDR, endothelium-dependent vasorelaxation; GCH1, GTP cyclohydrolase 1; HHCY, hyperhomocysteinemia; HPLC, high-performance liquid chromatography; HTL, homocysteine thiolactone; LDL, low-density lipoprotein; n-LDL, native low-density lipoprotein; RT-PCR, reverse transcription polymerase chain reaction; RT-qPCR, quantitative reverse transcription polymerase chain reaction; α-SMA, α-smooth muscle actin; STZ, streptozotocin; and vWF, von Willebrand factor.

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FOOTNOTES

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