Diabetes mellitus (DM) is a global epidemic. Atherosclerotic vascular disease is the major cause of morbidity and mortality in DM. Vascular endothelial dysfunction precedes and promotes vascular inflammation and atherosclerosis. Hyperglycemia of DM impairs vascular endothelial function through many mechanisms. Recent evidence indicates that hyperglycemia alters the expression pattern of endothelial microRNAs. 

MicroRNAs are small noncoding RNA molecule (~22 nucleotides) found in all cell types that lead to RNA silencing and post-transcriptional regulation of gene expression, thus affecting cellular function and determining cell fate. MicroRNAs play an important role in regulating vascular function and contribute to the development of diabetic vascular pathology. MiR-34a, a p53-regulated microRNA, has emerged as a mediator of several vascular pathologies. MiR-34a promotes senescence in the vascular endothelium, causes endothelial inflammation by upregulating basal vascular cell adhesion molecule-1 and intercellular adhesion molecule-1, and impairs angiogenesis by inducing senescence of endothelial progenitor cells. However, little is known about how miR-34a is regulated in the diabetic vasculature. Moreover, what genes are targeted by miR-34a and whether their down-regulation is responsible for diabetic endothelial dysfunction have not been experimentally verified.

The NAD+-dependent lysine deacetylase sirtuin1 (Sirt1) has a vital part in governing vascular endothelial function. Sirt1 plays a salutary role in the vasculature via several mechanisms including deacetylation-induced activation of endothelial nitric oxide (NO) synthase, inhibition of macrophage

**Objective**—Diabetes mellitus causes vascular endothelial dysfunction and alters vascular microRNA expression. We investigated whether endothelial microRNA-34a (miR-34a) leads to diabetic vascular dysfunction by targeting endothelial sirtuin1 (Sirt1) and asked whether the oxidative stress protein p66Shc governs miR-34a expression in the diabetic endothelium.

**Approach and Results**—MiR-34a is upregulated, and Sirt1 downregulated, in aortic endothelium of db/db and streptozotocin-induced diabetic mice. Systemic administration of miR-34a inhibitor, or endothelium-specific knockout of miR-34a, prevents downregulation of aortic Sirt1 and rescues impaired endothelium-dependent aortic vasorelaxation induced by diabetes mellitus. Moreover, overexpression of Sirt1 mitigates impaired endothelium-dependent vasorelaxation caused by miR-34a mimic ex vivo. Systemic infusion of miR-34a inhibitor or genetic ablation of endothelial miR-34a prevents downregulation of endothelial Sirt1 by high glucose. MiR-34a is upregulated, Sirt1 is downregulated, and oxidative stress (hydrogen peroxide) is induced in endothelial cells incubated with high glucose or the free fatty acid palmitate in vitro. Increase of hydrogen peroxide and induction of endothelial miR-34a by high glucose or palmitate in vitro is suppressed by knockdown of p66shc. In addition, overexpression of wild-type but not redox-deficient p66Shc upregulates miR-34a in endothelial cells. P66shc-stimulated upregulation of endothelial miR-34a is suppressed by cell-permeable antioxidants. Finally, mice with global knockdown of p66shc are protected from diabetes mellitus–induced upregulation of miR-34a and downregulation of Sirt1 in the endothelium.

**Conclusions**—These data show that hyperglycemia and elevated free fatty acids in the diabetic milieu recruit p66shc to upregulate endothelial miR-34a via an oxidant-sensitive mechanism, which leads to endothelial dysfunction by targeting Sirt1. 

(Key Words: diabetes mellitus ■ endothelial dysfunction ■ microRNA-34a ■ p66Shc ■ sirtuin1)
foam cell formation, and prevention of hyperglycemia-induced vascular cell senescence. Caloric restriction, a well-known stimulant of Sirt1, decreases arterial blood pressure in healthy individuals and improves endothelium-dependent vasodilation in obese and overweight individuals. In addition, nutritional pharmaceuticals, such as docosahexaenoic acid, act via Sirt1-dependent deacetylation of endothelial NO synthase to increase endothelial NO production and relax blood vessels. Importantly, Sirt1 is a bona fide target of several miRNAs, including miR-34a. Moreover, serum miR-34a is upregulated in diabetics, suggesting a role for it in diabetic end-organ pathology.

P66Shc is a master promoter of oxidative stress–related pathologies. Inactivation of p66Shc confers resistance to oxidative stress and protects mice from age-associated and diabetic vascular endothelial dysfunction. Interestingly, p66Shc is regulated by Sirt1 in DM. However, whether P66Shc governs expression of Sirt1 in the diabetic vasculature and whether this expression is regulated through miR-34a are not known. Recognizing that miR-34a is an oxidative stress–induced microRNA, we asked whether P66Shc promotes miR-34a expression in the endothelium, which then leads to diabetic endothelial dysfunction by targeting Sirt1.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Upregulation of MiR-34a Causes Vascular Endothelial Dysfunction in db/db Diabetic Mice

To explore the role of miR-34a in diabetic vascular dysfunction, we first determined its expression in the vasculature of type 2 diabetic db/db mice. At 8 weeks of age, db/db mice were hyperglycemic in fed and fasted states (Figure IA and IB in the online-only Data Supplement). MiR-34a was upregulated in aortas of db/db mice, as measured by in situ hybridization and real-time quantitative polymerase chain reaction (Figure 1A and 1B). Of note, upregulation of miR-34a in nondiabetic db/+ mice did not differ from that in nondiabetic c57Bl/6j mice. Moreover, serum miR-34a is upregulated in diabetics, suggesting a role for it in diabetic end-organ pathology.

Figure 1. Vascular microRNA-34a (miR-34a) is induced in diabetes mellitus. A and B, MiR-34a is upregulated in db/db diabetic mouse aortas and suppressed by miR-34a inhibitor. A, Top: In situ hybridization for miR-34a (purple-blue) in diabetic db/db and nondiabetic db/+ mouse aortas. Mice were systemically infused with a locked nucleic acid miR-34a inhibitor (miR-34a-I) or a scrambled control locked nucleic acid (scrambled) for 4 wk. Aortas were stained with miR-34a probe or a nonspecific probe. Von Willebrand factor (vWF; brown) was stained using DAB (3,3'-diaminobenzidine) peroxidase substrate. Images were captured at ×40, and representative images are shown. Bottom: Quantification for endothelial miR-34a was performed using ImageJ. B, Real-time quantitative polymerase chain reaction (q-RTPCR) for miR-34a in whole aortas of db/db and nondiabetic db/+ mouse aortas. Mice were systemically infused with a locked nucleic acid miR-34a inhibitor (miR-34a-I) or a scrambled control locked nucleic acid (scrambled) for 4 wk. Aortas were stained with miR-34a probe or a nonspecific probe. Von Willebrand factor (vWF; brown) was stained using DAB (3,3'-diaminobenzidine) peroxidase substrate. Images were captured at ×40, and representative images are shown. Bottom: Quantification for endothelial miR-34a was performed using ImageJ. B, Real-time quantitative polymerase chain reaction (q-RTPCR) for miR-34a in whole aortas of db/db and nondiabetic db/+ mouse aortas. C and D, Upregulation of endothelial miR-34a is suppressed in endothelium-specific knockout (e-miR-34a−/−) mice. C, In situ hybridization for miR-34a (purple-blue) in aortas of miR-34afl/fl and e-miR-34a−/− mice. D, q-RTPCR for miR-34a in whole aortas of STZ-induced diabetic miR-34afl/fl and e-miR-34a−/− mice. Mice were made diabetic with streptozotocin (STZ) or given vehicle (Veh) control. D, q-RTPCR for miR-34a in whole aortas of STZ-induced diabetic miR-34afl/fl and e-miR-34a−/− mice. Data are shown as mean±SEM. *P<0.05. L indicates lumen.
db/db mouse aortas was not exclusive to endothelium (Figure 1A) because it was evident in the vascular smooth muscle layer as well. Db/db mouse aortas had impaired endothelium-dependent relaxation compared with db/+ controls (Figure 2A, left). To determine whether miR-34a is responsible for this impairment, osmotic mini pumps delivering a locked nucleic acid miR-34a inhibitor (miR-34a-I) or a scrambled control locked nucleic acid were implanted subcutaneously into mice. Systemic administration of miR-34a-I resulted in suppression of miR-34a expression in whole aortas of db/db mice (Figure 1A and 1B). The inhibitor did not selectively target endothelium because its effect on miR-34a expression was seen in both the endothelial and medial layers of mouse aortas (Figure 1A and 1B). Hyperglycemic status was not altered in db/db mice that received miR-34a inhibitor (Figure 1A and 1B in the online-only Data Supplement), indicating that miR-34a does not affect systemic glucose homeostasis. These findings indicate that systemic administration of miR-34a-I is an effective way to decrease miR-34a expression in mouse arteries in vivo.

Systemic delivery of miR-34a-I preserved endothelium-dependent vasorelaxation in aortas of db/db mice (Figure 2A, left). Although miR-34a-I decreased miR-34a expression in the medial smooth muscle cell layer (Figure 1A), this did not result in an improvement in endothelium-independent vasorelaxation in the db/db mice (Figure 2A, right). These data indicate that in db/db mice, although miR-34a is upregulated throughout the vascular wall, its effect on vasoreactivity is primarily restricted to impairing endothelium-dependent vasorelaxation.

To confirm whether miR-34a directly impairs endothelium-dependent vascular relaxation, a miR-34a oligonucleotide mimic was transfected into wild-type mouse aortas ex vivo (Figure II in the online-only Data Supplement). Transfection of the miR-34a mimic impaired endothelium-dependent vascular relaxation (Figure 2B, left) but had no effect on endothelium-independent vascular relaxation (Figure 2B, right). These findings further suggest that the effect of miR-34a on vasomotor function is principally by targeting the vascular endothelium.

**Figure 2.** MicroRNA-34a (MiR-34a) mediates impairment of endothelium-dependent vasorelaxation in diabetes mellitus. A, MiR-34a inhibitor protects against endothelial dysfunction in db/db diabetic mice. Acetylcholine (Ach)-stimulated endothelium-dependent (left) and sodium nitroprusside (SNP)-stimulated endothelium-independent (right) vasorelaxation in aortas of db/db and db/+ mice systemically administered locked nucleic acid miR-34a inhibitor (miR-34a-I) or scrambled control locked nucleic acid. #P<0.05 vs db/+ scrambled; *P<0.05 vs db/db scrambled; ¥P<0.05 vs db/+ miR-34a-I. B, MiR-34a mimic promotes endothelial dysfunction. Ach-stimulated (left) and SNP-stimulated (right) vasorelaxation in wild-type aortas transduced ex vivo with miR-34a oligonucleotide mimic (miR-34a-M) or a scrambled control miR (miR-NC). âP<0.05 vs miR-NC. C, Endothelial-specific deletion of miR-34a partially protects against endothelial dysfunction in STZ-induced diabetes mellitus. Ach-stimulated (left) and SNP-stimulated (right) vasorelaxation in STZ-induced diabetic and nondiabetic (Veh) miR-34afl/fl and e-miR-34afl/fl mice. ¥P<0.05 vs miR-34afl/fl Veh; ¥P<0.05 vs miR-34afl/fl STZ. n=8 to 10 aortic rings from 4 to 6 mice per group. Data are shown as mean±SEM.

**Streptozotocin-Induced DM Causes Vascular Endothelial Dysfunction Through Upregulation of Endothelial MiR-34a**

The role of miR-34a upregulation in impairing vascular function was also evaluated in a streptozotocin (STZ)-induced type 1 DM mouse model. This model was established by administering a single dose of STZ (100 mg/kg) and resulted in robust increase in blood glucose over the course of 4 weeks (Figure III in the online-only Data Supplement). Although mice injected with STZ did not gain weight as their non-treated counterparts, they appeared healthy and were not emaciated (Figure IV in the online-only Data Supplement). Similar to db/db mouse aortas, miR-34a was upregulated in aortas, including the endothelium, of mice rendered diabetic by injection of STZ (Figure 1C and 1D). To tease out the role of endothelial miR-34a in DM-induced impairment of endothelial vasorelaxation, we turned to mice conditionally lacking endothelial miR-34a (e-miR-34a−/-). Knockout of miR-34a in the endothelium did not affect basal or STZ-induced blood glucose levels (Figure V in the online-only Data Supplement). E-miR-34a−/− mice were protected from STZ-induced impairment of endothelium-dependent vasorelaxation compared with miR-34afl/fl control mice (Figure 2C, left). STZ did not impair, and endothelial knockout of miR-34a had no effect on, endothelium-independent vasorelaxation (Figure 2C, right). Taken together, these data show that upregulation of endothelial miR-34a is responsible for vascular endothelial dysfunction in the STZ model of DM.

**DM, High Glucose, and FFAs Downregulate Endothelial Sirt1 via MiR-34a**

Previous studies have demonstrated that miR-34a targets Sirt1, although no studies have addressed the role of miR-34a-mediated Sirt1 downregulation in diabetic endothelial dysfunction. We, therefore, asked whether miR-34a promotes
diabetic vascular endothelial dysfunction by targeting Sirt1. We first examined whether DM affects the expression of vascular Sirt1. Sirt1 was downregulated in aortas of db/db and STZ-induced diabetic mice (Figure 3A through 3C). This downregulation was evident in both vascular smooth muscle cells and endothelial layers (Figure 3B and 3C). To determine whether miR-34a induced by DM is responsible for downregulation of Sirt1, we assessed Sirt1 expression in aortas of db/db mice systemically infused with miR-34a-I. MiR-34a-I rescued vascular Sirt1 expression in db/db mice (Figure 3A and 3B). This was particularly evident with regard to endothelial Sirt1 (Figure 3B). We also asked whether the same is true in a STZ-induced model of DM. Vascular Sirt1 was downregulated throughout the aortic wall of miR-34a−/− mice rendered diabetic with STZ (Figure 3C). In contrast, whole vascular and endothelial Sirt1 downregulation by STZ was mitigated in e-miR-34a−/− mice (Figure 3C). Thus, upregulation of miR-34a in the endothelium results in downregulation of endothelial Sirt1.

We next asked whether hyperglycemia per se leads to miR-34a-mediated downregulation of Sirt1 in the endothelium. To answer this question, we performed in vitro studies in endothelial cells incubated with medium supplemented with high glucose. Human umbilical vein endothelial cells (HUVEC) and aortic endothelial cells isolated from mice (miR-34a−/− mice) cultured in high-glucose (HG; 30 mmol/L) medium showed upregulation of miR-34a and downregulation of Sirt1 (Figure 4A through 3C). Decrease of Sirt1 by HG was rescued when HUVEC were pretreated with miR-34-I (Figure 4B). Complementary studies in aortic endothelial cells isolated from e-miR-34a−/− mice showed that HG failed to upregulate miR-34a (Figure 4C) and downregulate Sirt1 (Figure 4D). Conversely, miR-34a mimic downregulated, and miR-34-I upregulated, basal Sirt1 in HUVECs (Figure 4E).

In addition to increase in serum glucose, DM also results in increase of circulating free fatty acids (FFA). We, therefore, tested the effect of palmitate, a saturated FFA, on miR-34a and Sirt1 expression in endothelial cells. Palmitate (500 µmol/L) stimulated miR-34a expression in mouse (miR-34afl/fl mice) aortic endothelial cells (Figure 4F). In addition, palmitate decreased Sirt1 expression in these cells (Figure 4G; Figure VI in the online-only Data Supplement). In contrast, Sirt1 expression was preserved in mouse endothelial cells derived from e-miR-34a−/− mice (Figure 4G). These findings underscore that (1) miR-34a targets Sirt1 in endothelial cells, (2) high glucose and FFA induce miR-34a in endothelial cells, and (3) downregulation of endothelial Sirt1 by high glucose and FFA is mediated by miR-34a.

Reconstitution of Sirt1 Restores Endothelium-Dependent Vasorelaxation in MiR-34a–Transfected Vessels

Next, we probed for a causal relationship between downregulation of Sirt1 and endothelial dysfunction induced by miR-34a. MiR-34a mimic transfected into mouse aortas ex vivo led to marked upregulation of miR-34a expression (Figure 5A) and impairment of endothelium-dependent vasorelaxation (Figure 5B, left) but not endothelium-independent vasorelaxation (Figure 5B, right). This upregulation of miR-34a was accompanied by downregulation of vascular Sirt1 (Figure 5C and 5D). Reconstitution of Sirt1 expression with an adenovirus encoding Sirt1 lacking the 3′-UTR (AdSirt1) rescued impaired endothelium-dependent vasorelaxation triggered by miR-34a (Figure 5B, left). These data show that downregulation of Sirt1 is the proximate cause of endothelial dysfunction induced by miR-34a.
Endothelial p66Shc Is Activated by DM, HG, and FFAs and Promotes Vascular Oxidative Stress and Diabetic Endothelial Dysfunction

P66shc is a master regulator of the redox state and promotes endothelial dysfunction by inducing oxidative stress. We determined the status of p66Shc, and its role in endothelial dysfunction, in our models of DM. Because p66Shc is activated by serine phosphorylation,15 we first assessed the phosphorylation state of p66Shc in diabetic vasculature. Phosphorylation on serine 36, as well as expression, of p66Shc was increased in db/db diabetic mouse aortas compared with db/+ nondiabetic controls (Figure 6A). Similarly, endothelial p66Shc was phosphorylated on serine 36 in the STZ model of DM (Figure 6B, top; p66-N mice). To assess the role of p66Shc in diabetic oxidative stress and endothelial dysfunction, we used mice expressing a shRNA targeting p66Shc (p66-T) in which p66Shc is globally knocked down.16 Phosphorylation of p66Shc on serine 36 was significantly reduced in p66-T mice compared with p66-N nontransgenic littermates (Figure 6B). Consistent with the phosphorylation status of p66Shc, staining for 8-hydroxy-deoxyguanosine, a marker of oxidative DNA damage, was significantly lower throughout the aorta, including the endothelium, of diabetic p66-T mice, compared with their p66-N nontransgenic controls (Figure 6C). P66-T mice were partially protected from...
Figure 6. Endothelial p66Shc is upregulated and phosphorylated by diabetes mellitus and promotes vascular oxidative stress and endothelial dysfunction. A–D, Endothelial p66Shc is phosphorylated on serine 36 in diabetes mellitus and promotes vascular oxidative stress and endothelial dysfunction. A, Immunoblots for p66Shc and phospho-S36 p66Shc (P-S36-p66Shc) in whole aortas of db/db diabetic and db/+ nondiabetic mice. Immunohistochemistry for (B) P-S36-p66Shc (red) and (C) the oxidative stress marker 8-hydroxy-deoxyguanosine (8-OHdG; red) in aortas of transgenic mice expressing shRNA targeting p66Shc (p66-T) or their wild-type nontransgenic littermates (p66-N), treated with vehicle control (Veh) or rendered diabetic with streptozotocin (STZ). L indicates lumen. Graphs at bottom show quantification of endothelial P-S36-p66Shc and 8-OHdG expressed relative to nondiabetic (Veh-treated) p66-N mice. D, p66-T mice are protected from diabetic endothelial dysfunction. Endothelium-dependent (left) and endothelium-independent (right) vasorelaxation in aortas of p66-T or p66-N mice treated with vehicle (Veh) or rendered diabetic with STZ. $P<0.05$, p66-N-Veh vs P66-T-STZ; #P<0.05, p66-T-STZ vs P66-N-STZ; %P<0.05, p66-T-STZ vs P66-N-Veh; **P<0.05, p66-T-Veh vs P66-N-Veh. n=8 to 10 from 4 to 5 mice per group.

E, p66Shc silencing in HUVECs abrogates high glucose (HG)–induced oxidative stress (H2O2). H2O2 was quantified by Amplex red fluorescence.

F, p66Shc knockdown in aortic endothelial cells abrogates HG-induced oxidative stress. Aortic endothelial cell isolated from p66-T mice or p66-N littermates were treated with HG for 24 h. H2O2 was quantified as above.

G, Overexpression of p66Shc in HUVECs induces oxidative stress (H2O2). p66Shc knockdown in aortic endothelial cells abrogates palmitate-induced oxidative stress. Aortic endothelial cell isolated from p66-T mice or their p66-N littermates were treated with 500 µmol/L for 12 h. H2O2 was quantified as above. In all panels, representative immunoblots or immunohistochemistry images are shown. Data are shown as mean±SEM. n=3 to 5 for all data except vasorelaxation and *P<0.05.
P66Shc Downregulates Vascular Sirt1

Finally, we asked whether vascular Sirt1 downregulation in DM is mediated by p66Shc. Under basal conditions, aortic Sirt1 was higher in p66-T mice compared with non-transgenic p66-N controls (Figure 7K). Increase in aortic Sirt1 was evident in the tunica media and the endothelium (Figure 7K). In addition, although STZ-induced DM downregulated endothelial Sirt1 in aortas of p66-N mice, endothelial Sirt1 expression was essentially unchanged in diabetic p66-T mice (Figure 7K). These data show that p66Shc promotes downregulation of vascular Sirt1 expression in DM.

Discussion

Sirt1 was first described as an important determinant of vascular endothelial function by targeting endothelial NO synthase for deacetylation and increasing endothelium-derived NO. Since then, numerous studies have shown that Sirt1 plays a protective role in the vasculature. Sirt1 exerts its vasoprotective effect not only through direct actions on the vasculature but also indirectly such as through its stimulation of reverse cholesterol transport mechanisms.

By using 2 models of DM (db/db and STZ), our present data show the vital part miR-34a plays in impairing endothelial function in DM in vivo. Furthermore, these data corroborate the previously appreciated relationship between miR-34a and Sirt1 in the context of diabetic endothelial dysfunction: Sirt1 is the principal functionally relevant target of miR-34a in the diabetic endothelium, as rescue of Sirt1 restores endothelium-dependent vasorelaxation. Although we did not look at other metrics of endothelial dysfunction such as vascular inflammation and eventual atherosclerosis, given that impairment of endothelium-dependent vasorelaxation is one of the earliest manifestations of endothelial dysfunction that predicts the development of atherosclerotic disease, it would be safe to say that the p66shc–miR-34a–Sirt1 axis is also likely to be relevant to the development of accelerated atherosclerosis in DM.

MiR-34a was induced both in the endothelium and media of aortas from both db/db and STZ-treated mice. Moreover, knockdown of p66Shc (which, in the p66-T mice, is global and therefore would be expected to be present in both the endothelial and smooth muscle layers) suppressed miR-34a expression throughout the thickness of diabetic aortas. This finding suggests that expression of miR-34a in DM, at least in the vasculature, is governed by molecular mechanisms that are common to all major cell types comprising the vascular wall. However, despite upregulation of miR-34a in the entire vascular wall, the fact that miR-34a deletion exclusively in the endothelium was sufficient to rescue endothelial function in STZ mice indicates that it is endothelial miR-34a that is responsible for diabetic vascular dysfunction, at least as it pertains to endothelium-dependent vasorelaxation. This conclusion is corroborated by the finding that miR-34a mimics impaired endothelium-dependent but not endothelium-independent vasorelaxation. Further supporting this conclusion is data that although endothelium-independent relaxation was impaired in db/db mice, downregulation of miR-34a with miR-34a inhibitor in the whole vessel did not improve vasorelaxation. However, one cannot completely exclude the possibility that miR-34a upregulation in the medial smooth muscle layer also contributes to impaired vascular function of DM. Such function...
Figure 7. P66Shc stimulates endothelial microRNA-34a (miR-34a) and suppresses endothelial sirtuin1 (Sirt1), in diabetes mellitus. A, MiR-34a is not induced in diabetic p66-T mice. Left: In situ hybridization for miR-34a (purple-blue) in aortas of p66-T or p66-N mice treated with vehicle control (Veh) or streptozotocin (STZ). L indicates lumen. Right: Graphs showing quantification of endothelial miR-34a relative to nondiabetic Veh-treated p66-N aortas. B, C, and E, P66Shc promotes miR-34a expression in endothelial cells. B, P66Shc silencing in human umbilical vein endothelial cells (HUVEC) prevents high glucose (HG)–induced upregulation of miR-34a. q-RTPCR for miR-34a in HUVECs transfected with p66Shc siRNA and treated with HG. C, p66Shc knockdown in aortic endothelial cells prevents HG-induced upregulation of miR-34a. Aortic endothelial cell isolated from p66-T and p66-N mice were treated with HG for 24 h. miR-34a levels were quantified using real-time quantitative polymerase chain reaction (q-RTPCR). D, p66Shc knockdown in aortic endothelial cells prevents palmitate-induced upregulation of miR-34a. Aortic endothelial cell isolated from p66-T and p66-N mice were treated with 500 µmol/L palmitate for 12 h. miR-34a levels were quantified using q-RTPCR. E, Overexpression of wild-type p66Shc induces miR-34a. Top: q-RTPCR for miR-34a in HUVECs transfected with plasmid encoding wild-type p66Shc. Bottom: Representative immunoblots for p66Shc. F and G, The nonphosphorylatable redox-deficient S36A mutant of p66Shc is impaired in its capacity to induce miR-34a. F, Top: q-RTPCR and (G) Top: promoter activity for miR-34a in HUVECs overexpressing wild-type (WT) or redox-deficient (S36A) p66Shc. F and G bottom: Representative immunoblots for p66Shc. H and I, P66Shc induces endothelial miR-34a through oxidative stress. Q-RTPCR for miR-34a in HUVECs expressing WT p66Shc treated with the cell-permeable antioxidants (H) N-acetylcysteine (NAC, 500 µmol/L, 24 h) or (I) polyethylene glycol catalase (PEG-C, 500 U/mL, 24 h). Control HUVECs were treated with Veh or polyethylene glycol (PEG). J, MiR-34a is induced by exogenous oxidants. Q-RTPCR for miR-34a in HUVECs treated with H2O2 (50 µmol/L, 24 h). K, P66Shc suppresses endothelial Sirt1 expression in diabetes mellitus. Left: Immunofluorescence for Sirt1 (red) in aortas of p66-T or p66-N mice treated with Veh or STZ. L indicates lumen. Right: Quantification of Sirt1 immunofluorescence in endothelium relative to nondiabetic Veh-treated p66-N mice. All values are shown as mean±SEM. n=3 to 5. *P<0.05.
may take other forms not captured by vasorelaxation studies. For example, previous work has shown that miR-34a causes senescence and upregulation of proinflammatory markers in vascular smooth muscle cells.23

Although our studies show that overexpression of Sirt1 rescues miR-34a–induced endothelial dysfunction, it would be naive to conclude that miR-34a is acting exclusively via Sirt1. MicroRNAs target a network of genes, and it is likely that miR-34a’s effect on vascular function is mediated by many up- and downregulated genes. The full spectrum of gene targets of miR-34a in the endothelium is presently unknown. In addition, by suppressing Sirt1, an epigenetic modifier, miR-34a may also indirectly regulate endothelial gene expression.

Our data indicate that endothelial miR-34a expression in DM is induced by p66Shc, that the redox property of p66Shc is essential for this induction, and that reducing oxidative stress in endothelial cells suppresses miR-34a expression. Furthermore, they show that endothelial p53 is upregulated by high glucose, and p53 participates in miR-34a induction. These findings are consistent with miR-34a being a tumor suppressor microRNA induced by p53.3 They are also consistent with the part p66Shc plays in p53-induced oxidative stress and DNA damage.17 It is notable that p66shc is also transcriptionally upregulated by p53 in endothelial cells.16 Thus, p53 may conspire with p66shc to induce miR-34a expression in a redox-dependent fashion. It is also noteworthy that miR-34a’s effect on vascular function is mediated by activating endothelial nitric oxide synthesis.

Disclosures

None.

References


**Highlights**

- Vascular endothelial microRNA-34a (miR-34a) is upregulated in type 1 and 2 diabetes mellitus.
- MiR-34a mediates diabetic vascular endothelial dysfunction.
- MiR-34a promotes diabetic endothelial dysfunction by downregulating sirtuin1.
- Activation of p66shc in diabetes mellitus stimulates endothelial miR-34a expression.
- Elevated glucose and free fatty acids in diabetes mellitus stimulate endothelial miR-34a via increase of p66shc-mediated reactive oxygen species.
- Tumor suppressor p53 is obligatory for endothelial miR-34a induction by elevated glucose.
P66Shc-Induced MicroRNA-34a Causes Diabetic Endothelial Dysfunction by Downregulating Sirtuin1
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Supplemental Materials

**P66Shc-induced MicroRNA-34a Causes Diabetic Endothelial Dysfunction by Downregulating Sirtuin1**

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Supplemental Figure Legends

**Supplemental Figure I.** Systemic administration of miR-34a inhibitor does not affect blood glucose. A, Fasting blood glucose (overnight) and B, random blood glucose in db/db and db/+ mice systemically infused with a locked nucleic acid miR34a inhibitor (miR-34a-I) or a scrambled control locked nucleic acid (scrambled) for 4 weeks. Data are shown as mean ± SEM. n=6-8, * P<0.05.

**Supplemental Figure II.** *Ex vivo* transfection of miR-34a mimic increases miR-34a abundance in whole aortas. Aortas isolated from wild-type C57BL/6 mice were transfected with miR-34a oligonucleotide mimic (miR-34a-M) or a scrambled control locked nucleic acid (miR-NC). Aortic miR-34a was quantified by real time qPCR. Data are shown as mean ± SEM. n=3, * P<0.05.

**Supplemental Figure III.** STZ-induced hyperglycemia in mice. MiR-34afl/fl and e-miR-34a-/- mice, 8 weeks old, were injected one dose of STZ (100 mg/kg) or vehicle (Veh). Random blood glucose was measured at baseline, and 21 and 28 days after injections. Data are shown as mean ± SEM. n=4, * P<0.05.

**Supplemental Figure IV.** Body weight is maintained after STZ induced diabetes in mice. MiR-34afl/fl and e-miR-34a-/- mice were injected with vehicle (Veh) or STZ. Body weight was measured at baseline, and 28 days after injection. Data are shown as mean ± SEM. n=4, * P<0.05.

**Supplemental Figure V.** Deletion of endothelial miR-34a does not affect glucose homeostasis. Fasting (overnight) blood glucose in miR-34afl/fl and e-miR-34a-/- mice 28 days after injection with Veh or STZ. Data are shown as mean ± SEM. n=4-6, * p<0.05.

**Supplemental Figure VI.** Palmitate time-dependently downregulates Sirt1 and upregulates p53 expression in mouse aortic endothelial cells. Aortic endothelial cells were isolated from WT mouse aorta, and treated with 500 uM palmitate. Sirt1 and p53 expression was measured by immunoblots at times indicated. Representative immunoblots are shown.

**Supplemental Figure VII.** P66Shc knockdown prevents induction of miR-34a in whole aortas of diabetic mice. p66Shc shRNA transgenic (p66 -T) and control non-transgenic mice (p66-N) mice were injected with vehicle (Veh) or STZ. MiR-34a expression was measured 28 days after STZ injection using real time qPCR. Data are shown as mean ± SEM. n=4, * p<0.05.

**Supplemental Figure VIII.** Co-dependent relationship between P53 and miR-34a in vascular endothelial cells. A, Silencing of p53 using siRNA. Human umbilical vein endothelial cells (HUVEC) were transfected with p53 siRNA for 24 h, and p53 expression was measured by immunoblotting. B, Silencing of p53 prevents its induction by HG. HUVECs were transfected with p53 or scrambled siRNA for 24 h followed by the treatment with mannitol or high glucose (HG) at 30 mM for 24 h. p53 mRNA levels was measured by real time qPCR. Values are expressed relative to scrambled siRNA. C, Silencing of p53 suppresses HG-induced miR-34a in endothelial cells. MiR-34a was quantified in HUVECs transfected with p53 or scrambled siRNA for 24 h followed by the treatment with mannitol or high glucose (HG) at 30 mM for 24 h. Values are expressed relative to scrambled siRNA. D, Deletion of miR-34a prevents upregulation of p53 by HG. Aortic endothelial cells were isolated from miR-34afl/fl and e-miR-34a-/- mice and treated with mannitol or HG at 30 mM for 24 h. P53 expression was determined using immunoblotting, and data, normalized to miR-34afl/fl, are shown as mean ± SEM. n=4-6. * p<0.05.
Supplemental Figure IX. Characterization of mouse aortic endothelial cells. A&B, Immunocytochemistry and immunoblotting for endothelial markers vWF and eNOS, and smooth muscle cell marker α-actin, showing enrichment of endothelial cells. C, Real time qPCR showing knockdown of miR-34a in aortic endothelial cells (EC) isolated from e-miR-34a⁻/⁺ compared to miR-34a⁻⁻/⁻ mice. Data are shown as mean ± SEM and normalized to miR-34a⁻⁻/⁻ mice. n=4-5. * p<0.05.
**Supplemental Figure IV**

- Bars represent body weight (g) for different groups over 28 days.
- Key: Veh, STZ, miR-34a^{fl/fl}, e-miR-34a^{-/-}.
- Significant differences indicated with asterisks (*).
- ns denotes no significant difference.

**Supplemental Figure V**

- Bars represent fasting blood glucose (mg/dl) for different groups.
- Key: Veh, STZ, miR-34a^{fl/fl}, e-miR-34a^{-/-}.
- Significant differences indicated with asterisks (*).
- ns denotes no significant difference.

**Supplemental Figure VI**

- Western blot showing effects of Palmitate (500µM) on Aortic Endothelial Cell.
- Proteins: Sirt1, p53, GAPDH.
- Molecular weights: 100KD, 50KD, 37KD.

**Supplemental Figure VII**

- Bar chart showing miR-34a/RNU6 levels.
- Key: Veh, STZ, P66-N, P66-T.
- Significant differences indicated with asterisks (*).
Supplemental Figure IX

A

vWF-red
DAPI-blue

HUVECs  Aortic ECs

B

eNOS
α-Actin
GAPDH

C2C12  Aortic EC  HUVEC

C

miR-34a/cel-miR-39

miR-34a<sup>fl/fl</sup>  e-miR-34a<sup>−/−</sup>
Materials and Methods

Animals
Experiments were performed on 8–12 week-old 1) C57Bl/6 mice, 2) endothelium–specific miR34a knockout mice, 3) p66ShcRNAi transgenic mice, and 4) db/db (BKS.Cg-Dock7m +/+ Lepr<sup>db</sup>/J) homozygous and heterozygous mice. The endothelium–specific miR-34a knockout mice (E-miR-34a<sup>−/−</sup>) were generated by crossing miR34a<sup>fl/fl</sup> mice with cadherin-5-Cre mice, in which the Cre recombinase is driven by an endothelium–specific cadherin-5 promoter. MiR-34a<sup>fl/fl</sup> mice were used as controls. P66shcRNAi transgenic mice (p66<sup>−T</sup>) were created in a B6SJL background. The transgene consists of a short hairpin sequence 5' involvement CT GTC ATC GCT G TTC AAG AGA C AGC GAT GAC 3' directed to nucleotides 239 to 257 of p66Shc mRNA, transcribed by the U6 RNA polymerase III promoter. Nucleotides 239 to 257 in p66Shc mRNA are in the coding region for the CH<sub>2</sub> domain of p66Shc. This domain is conserved in human and mouse, and not found in p46/52shc RNA. Mice were genotyped for the presence of the transgene in tail genomic DNA by PCR. Non-transgenic littermates (p66<sup>−N</sup>) were used as controls. Mice were fed standard chow (Research Diets Inc., New Brunswick, NJ) containing (kilocalories): 10% fat, 70% carbohydrate, and 20% protein (D12450B). Terminal experiments were performed after mice were anesthetized (2–5% isoflurane). The entire aorta was isolated and used for immunoblotting, real-time qPCR, and vascular function. All protocols were approved by the institutional animal care and use committee of University of Iowa.

Immunohistochemistry
Aortic sections were de-paraffinized with xylene, followed by antigen retrieval by heating in citrate buffer (10 mM). Sections were probed with appropriate primary antibodies. Sirt1 antibody (Santa Cruz Biotech, Dallas, TX), p-S36-p66Shc antibody (Abcam, Cambridge, MA), von Willebrand Factor antibody (Abcam), and 8-hydroxydeoxyguanosine (8-OHdG) antibody (Abcam) were used at a 1∶50 to 1∶200 dilution followed by a biotinylated secondary antibody, streptavidin peroxidase solution, DAB peroxidase substrate, and hematoxylin counterstain or by an AlexaFluor®647-conjugated anti-mouse IgG (1/500) as the secondary antibody. Sections were digitally imaged with Olympus BX-61. Endothelial layer was quantified using ImageJ.

Mouse Vascular Reactivity
Male mice, 8–12 weeks old, were anesthetized and euthanized by rapid cardiac excision. The whole aorta was carefully excised and placed in ice-cold Krebs buffer (118.3 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 1.2 mM MgSO<sub>4</sub>, 11 mM glucose, 0.0026 mM CaNa<sub>2</sub>EDTA). The aortas were cleaned of excess fat, cut transversely into 5–10 rings (1.8–2.0 mm wide). The arterial rings were placed in oxygenated chambers (95% O<sub>2</sub>/5% CO<sub>2</sub>) super fused with Krebs buffer solution and maintained at 37°C and pH 7.4. Each ring was suspended between two wire stirrups in a 6 ml organ chamber of a four-chamber myograph system (DMT). One stirrup was connected to a three-dimensional micromanipulator and the other to a force transducer. The contractile force was recorded electronically. All rings were stretched to 2, 000 mg in 500 mg increments over a 1 h period to optimize the contractile response to KCl. One dose of KCl (60 mM) was added to verify vascular smooth muscle viability. Cumulative dose–response curve for phenylephrine (PE) (10<sup>−9</sup> to 10<sup>−5</sup> M) was obtained by administering the drug in one-half log doses. Endothelium–dependent vasodilation was determined by generating dose–response curves to acetylcholine (Ach) (10<sup>−9</sup> to 10<sup>−7</sup> M). Vasorelaxation evoked by acetylcholine was expressed as the percent of maximal contraction. Endothelium–independent vasodilation was measured by the vasorelaxation evoked by cumulative sodium nitroprusside (SNP) in rings pre-constricted with phenylephrine (10<sup>−7</sup> M) and was also expressed as percent maximal contraction.
**Isolation and culture of mouse aortic endothelial cells**

Mouse aortic endothelial cells were isolated as described previously\(^1\). Male mice, 8–12 weeks old, were anesthetized and euthanized by rapid cardiac excision. The whole aorta was carefully excised and placed in DMEM supplied with 20% FBS. The aortas were cleaned of excess fat and connecting tissues. A 24 gauge cannula was inserted into one end of aorta and tied using surgical sutures. After washing with serum-free DMEM, the aorta was filled with collagenase II (Sigma, 2mg/ml) through the cannula, and incubated for 45 min at 37 °C. The aorta was then flushed several times with DMEM. The endothelial cells in the flushed medium were spun down at 1,200 rpm for 5 min, and gently resuspended in endothelial growth medium (EGM-2, Lonza, Walkersville, MD, USA) supplied with 10% FBS. Penicillin at 100μg/ml was added. The cells were seeded in a 12-well plate (1 mouse aorta/well). The medium was changed after 4 h. In pilot experiments the purity of endothelial cell preparations was tested using vWF and eNOS (endothelial markers) and α-smooth muscle actin (Supplemental Figure IXA, B & C).

**Immunocytochemistry**

Aortic endothelial cells were fixed in 4% formaldehyde for 15 min at room temperature, followed by blocking and permeabilization (PBS with 5% goat serum and 0.2% Triton X-100) for 30-60 min at room temperature. Cells were incubated with an antibody against von Willebrand Factor (vWF) (Abcam, 1:200 dilution) overnight at 4 °C. After washing with PBS 3 times, the cells were incubated with an Alexa 647 anti-sheep secondary antibody (Abcam, 1:400 dilution) at room temperature for 1 h. The cells were washed 3 times with PBS, and the slides were mounted with an anti-fade reagent with DAPI and photographed with confocal microscopy.

**Cell culture, plasmid/siRNA transfections, and adenoviral infections**

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA USA) and cultured in endothelial growth medium (EGM-2, Lonza, Walkersville, MD USA). Cells were transfected with plasmids, validated siRNA-p66Shc (Invitrogen, Carlsbad, CA), siRNA-p53 (Santa Cruz Biotechnology, USA), or negative control siRNA purchased from Invitrogen with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were infected with 6×10\(^{11}\) viral particles per ml of the control AdLacZ or the AdSIRT1 adenoviral stocks, and incubated at 37°C for 24 h. For high glucose experiments, cells were incubated in EGM-2 supplemented with 25 mM glucose. Cells incubated in 25 mM mannitol were used as controls.

**Quantitative Real time PCR**

Total RNA was isolated by the acid guanidinium thiocyanate/phenol/chloroform method. Total RNA from cultured cells, serum, or tissues of mice, was isolated by the TRIZOL (Invitrogen) method. Real-time PCR was performed using the Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) with the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen). The following primers (EXIQON) were used. Mouse Sirt1 forward 5′-AAT GCT GGC CTA ATA GAC TTG CA-3′, reverse 5′-CCG TGG AAT ATG TAA CGA TTT GG-3′; human Sirt1 forward 5′-TCGCAACTATACCCAGAACATAGACA-3′, reverse 5′-CTGTTGCAAAGGAACCATGACA-3′; human p53 forward 5′-TGGCACTGTTGCTTGGCGCT-3′, reverse 5′-CTGTTGCAAAGGAACCATGACA-3′; human GAPDH: forward 5′-ATG ACA TCA AGA AGG TGG TG-3′; reverse 5′-TCTGTTGCAAAGGAACCATGACA-3′; Mouse GAPDH 5′-GGC AAA TTC AAC GGC ACA-3′; 5′-CGC GCC TAC TGG AAC AAG ATG GTG AT-3′. A universal reverse primer (cDNA synthesis kit, Quanta Biosciences) was used for quantification of miRs. Mouse GAPDH and RNU6 (Quanta Biosciences) were used as internal controls or an external spike-in synthetic oligonucleotide cel-miR-39 (Norgen Biotek, Canada) was used as a control for mRNA and miR quantification, respectively.
Ex vivo infections with adenoviruses and transfections with a miR-34a mimic
MiR-34a mimic (5′-UGGCAGUGUCUUAGCUUGUUGU-3′, Ambion Life Technologies) and scrambled control (Ambion) or AdLacZ and AdSIRT1 were transfected into freshly isolated aortas from mice. MiR-34a mimic was mixed with Lipofectamine 2000 followed by addition to the medium. After 4 h, aortic rings were moved to fresh medium and further incubated for 24 h. After 24 h of miR-34a mimic transfection, AdLacZ or AdSIRT1 adenovirus was directly added to the medium and incubated for another 24 h.

In vivo inhibition of miR-34a
Locked nucleic acid miR-34a inhibitor (5′-AGCTAAGACACTGCC-3′,) or a scrambled nucleotide (5′-ACG TCT ATA CGC CCA-3′) was administered to db/db and db/+ mice using an ALZET osmotic pumps that were implanted subcutaneously in mice. Each mouse received a total dose of 400 µg of miR-34a inhibitor or scrambled nucleotide over 28 days.

STZ-induced mouse model of diabetes
Streptozocin (STZ) in sterile 0.1M citrate buffer was injected at 100 mg/kg intraperitoneally into mice starved for 6 hrs. Vehicle buffer was used in control mice. STZ-injected mice were monitored for body weight and blood glucose.

Luciferase reporter assays
The miR-34a promoter luciferase reporter plasmid was obtained from Addgene (Cambridge, MA). P66shc-WT and S36A mutant plasmids have been previously described. The reporter was co-transfected with a renilla luciferase plasmid driven by a constitutive promoter reporter into cells. Firefly and renilla luciferase luminescence were measured using a Dual Luciferase reporter kit (Promega, Madison WI) as per manufacturer’s recommendations. Firefly/renilla ratio was calculated to normalize for variations in transfection efficiencies.

Western blotting
Immunoblotting was performed as previously described. Chemiluminescent signal was developed using Super Signal West Femto substrate (Pierce, Grand Island, NY), blots imaged with a Gel Doc 2000 Chemi Doc system (BioRad, Hercules, CA), and bands quantified using Quantity One software (BioRad).

ROS measurements
Diffusible H2O2 produced by cells was measured in conditioned medium using an Amplex Red probe, as previously described.

In situ hybridization (ISH) and quantification of miR-34a
Aortic sections were de-paraffinized with xylene, followed by Proteinase K treatment (10 µg/ml for 5 min). ISH buffer (EXIQON, production # 90000) was added with miR-34a probe (EXIQON, 5′ −3′ /5DigN/AACAACCAGCTAAGACACTGCCA/3Dig-N/) or with a scramble-miR probe (EXIQON, 5′ −3′ /5DigN/TTGTACACGTCTATACGCCCA/3Dig-N/) at 20 nM or 40 nM, and incubated for 72 h at 56°C. After washing, the aortic sections were incubated in blocking solution for 15 min (5 ml PBS + 50 mg BSA + 100 ul Sheep serum + 2.5 ul Tween 20), followed by incubation with anti-DIG-FAB overnight (1:800 in Antibody dilution solution). Slides were dipped in a solution containing BCIP/NBT and incubated at 30°C for 48 h. The aortic sections were stained with nuclear fast red followed by dehydration. The slides were mounted with DPX and observe under the microscope. ISH was quantified using ImageJ.
Free fatty acid preparation

Palmitate solution was prepared as reported previously\(^5\). Palmitate (Sigma) was dissolved in 0.1 M NaOH at 72 °C for 30 min. The solution was then diluted in 20% fatty acid-free BSA (Sigma) pre-heated to 50 °C and incubated for additional 10 min at 50 °C. Fatty acid-free BSA was used as vehicle. Palmitate was used at 500 uM. Pilot experiments showed no significant decrease in cell viability at this concentration.

Statistical analysis
Statistical analysis was performed using GraphPad Prism (Version 6.0) statistical software. Significance of difference between two groups was evaluated using t-test. For multiple comparisons, one-way ANOVA was used and post-hoc analysis was performed with Tukey's test. Date are expressed as Mean ± SEM and considered significant if \( P \) values ≤ 0.05. All shown data is representative of at least three independent experiments.
References


DIABETES

Hyperglycemia
Free Fatty Acids

p66Shc

ROS

p53

miR-34a

Sirt1 mRNA

Sirt1 protein

Endothelial Dysfunction