Induction of Endothelial Nitric Oxide Synthase, SIRT1, and Catalase by Statins Inhibits Endothelial Senescence Through the Akt Pathway

Hidetaka Ota, Masato Eto, Mitsunobu R. Kano, Tomoaki Kahyo, Mitsutoshi Setou, Sumito Ogawa, Katsuya Iijima, Masahiro Akishita, Yasuyoshi Ouchi

Objective—Statins (3-hydroxy-3-methylglutaryl–coenzyme A reductase inhibitors) have pleiotropic vascular protective effects besides cholesterol lowering. Recently, experimental and clinical studies have indicated that senescence of endothelial cells is involved in endothelial dysfunction and atherogenesis. Therefore, the present study was performed to determine whether statins would reduce endothelial senescence and to clarify the molecular mechanisms underlying the antisenescent property of statins.

Methods and Results—Senescent human umbilical vein endothelial cells were induced by hydrogen peroxide (H2O2), as judged by senescence-associated β-galactosidase assay and cell morphological appearance. Atorvastatin, pravastatin, and pitavastatin inhibited the oxidative stress induced-endothelial senescence. These statins phosphorylated Akt at Ser473 and subsequently led to increased expression of endothelial nitric oxide synthase (eNOS), SIRT1, and catalase. Treatment with LY294002 or Akt short interfering RNA decreased the eNOS activation, SIRT1 expression, and antisenescent property of atorvastatin. Moreover, in streptozotocin-diabetic mice, administration of pitavastatin increased eNOS, SIRT1, and catalase expression and decreased endothelial senescence, but levels remained unaltered in Sirt1 knockout mice.

Conclusion—Our results indicate that treatment with statins inhibits endothelial senescence and that enhancement of SIRT1 plays a critical role in prevention of endothelial senescence through the Akt pathway, a direct target of statins. (Arterioscler Thromb Vasc Biol. 2010;30:2205-2211.)

Key Words: endothelium ■ nitric oxide synthase ■ SIRT1 ■ senescence ■ statin

The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, statins, are effective in lowering the plasma concentration of low-density lipoprotein cholesterol and are widely used in patients with hypercholesterolemia. Recently, experimental and clinical evidence has indicated that the pleiotropic effects of statins involve improvement or restoration of endothelial function, enhanced activity of endothelial nitric oxide synthase (eNOS), and decreased oxidative stress.1

Oxidative stress is implicated in the pathogenesis of cardiovascular diseases, such as atherosclerosis.2 Excessive production of reactive oxygen species inflicts damage on endothelial cells and leads to the onset of endothelial senescence. Senescence of endothelial cells is involved in endothelial dysfunction and atherogenesis.3 Histological study of human atherosclerotic lesions has demonstrated the existence of endothelial cells that exhibit the morphological features of senescence.4 Assmus et al have shown that statins reduce senescence and increase proliferation of endothelial progenitor cells.5

In Saccharomyces cerevisiae, the silent information regulator 2 (Sir2) family of genes governs budding exhaustion and replicative life span.6,7 Sir2 has been identified as an NAD+-dependent histone deacetylase and is responsible for maintenance of chromatin silencing and genome stability.8 Sir2 genes are conserved during evolution, and 7 homologs of sirtuins (Sirt1 to Sirt7) have been cloned in mammals. Mammalian sirtuin 1 (Sirt1), the closest homolog of Sir2, regulates the cell cycle, senescence, apoptosis, and metabolism by interacting with a number of molecules, including p53, promyelocytic leukemia (PML), and peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α).9-11 A recent study has shown that production of NO, stimulated by caloric restriction, increases SIRT1 expression; this study suggests that eNOS may be involved in regulation of the expression of SIRT1 in murine white adipocytes.12 Importantly, SIRT1 has been recognized as a key regulator of vascular endothelial homeostasis, controlling angiogenesis, endothelial senescence, and dysfunction.13-15

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The present study indicated that statins reduced oxidative stress-induced endothelial senescence, and SIRT1 played a critical role in prevention of endothelial senescence through the Akt pathway.

Methods

Materials

Pravastatin, atorvastatin, and pitavastatin were provided by Sankyo Co Ltd, Pfizer Inc (New York, NY), and Kowa Co (Nagoya, Japan), respectively. Mevalonate, geranylgeranylprophosphate (GGPP), farnesylpyrophosphate (FPP), Y27632, and LY294002 were purchased from Sigma (St. Louis, Mo). (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)aminio] diazen-1-1M1,2 diolate (DETA-N0) was from Cayman Chemical (Ann Arbor, Mich).

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex (Walkersville, Md). Population doubling levels were calculated as described previously, and all experiments were performed at a population doubling level of 10 to 11.

Inhibition and Overexpression of SIRT1 and eNOS

Proliferating cells were washed 3 times with growth medium and exposed for 24 hours to the indicated concentrations of sirtinol (Calbiochem) or exposed for 24 hours to the indicated concentrations of sirtinol (Calbiochem) or farnesylpyrophosphate (FPP), Y27632, and LY294002 were purchased from Sigma (St. Louis, Mo). (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)aminio] diazen-1-1M1,2 diolate (DETA-N0) was from Cayman Chemical (Ann Arbor, Mich).

Antibodies, Immunoprecipitation, assay kit (Calbiochem) according to the manufacturer’s instructions.

Immunoprecipitation of eNOS and SIRT1 was carried out by using an immunoprecipitation assay kit (Calbiochem) according to the manufacturer’s instructions.

Senescence-Associated β-Galactosidase Staining

HUVEC were pretreated with vehicle (0.05% dimethyl sulfoxide), atorvastatin (50 and 100 nmol/L), pravastatin (50 and 100 nmol/L), or pitavastatin (50 and 100 nmol/L) diluted in EGM-2 medium for 1 day. HUVEC were washed 3 times with EGM-2 and then treated for 1 hour with 100 μmol/L H2O2 diluted in EGM-2. After treatment, HUVEC were trypsinized, resuspended at a density of 1×10⁶ cells per 60-mm dish, and cultured with EGM-2 containing the above compounds for 10 days. The proportion of SA-β-gal-positive cells was determined as described by Dimri et al.

Nitric Oxide Synthase Activation Assay

Nitric oxide synthase (NOS) activity was determined using an NOS assay kit (Calbiochem) according to the manufacturer’s instructions.

Antibodies, Immunoprecipitation, and Immunoblotting

Immunoprecipitation of eNOS and SIRT1 was carried out by incubating 2.5 μg of antibody with 1 mg of cell lysate overnight, followed by 40 μL of Sepharose slurry (Amersham, Piscataway, NJ) for 6 hours. After washing, immunoprecipitates were boiled in SDS-PAGE sample buffer. After blocking, the filters were incubated with the following antibodies; anti-phospho-Akt (Ser473), anti-Akt (Cell Signaling Technology), anti-eNOS, anti-SIRT1, anti-manganese superoxide dismutase (MnSOD), anti-PCG-1α (H-300), anti-catalase (N-17) (Santa Cruz Biotechnology), and anti-β-actin (Sigma).

Real-Time Quantitative Reverse Transcription

Total RNA in HUVEC was isolated with Isogen (Nippon Gene Inc, Toyama, Japan). After treatment with RNase-free DNase for 30 minutes, total RNA (50 ng/μL) was reverse transcribed with random hexamers and oligo(dT) primers. The expression levels of nuclear respiratory factor 1 (NRF-1) and mitochondrial transcription factor A (TFAM) relative to GAPDH were determined by means of staining with SYBR green dye and a LineGene fluorometric quantitative detection system (Bioflux Co, Tokyo, Japan). The following primers were used: NRF-1, forward, 5'-GATGACCACGTGCTCATTAAC-3', reverse, 5'-CTGATGCGTGGCTGTCT-3'; TFAM, forward, 5'-CATCTGTCTTGGCAAGTGTGTC-3', reverse, 5'-CCACTCGGCCCCATAAGACATC-3'; GAPDH, forward, 5'-ACCACAGTCCATGCCCATCAC-3', reverse, 5'-TCCACACACCGTGTCCTGTA-3'.

Animal Experiments

The animal experiments were approved by our institutional review board. Twelve-week-old specific pathogen free (SPF) male wild-type B57/B6 mice (n = 40, weighing approximately 29 g) were supplied by Charles River Laboratories Inc. Twelve-week-old Sirt1 heterozygous knockout (KO) mice (provided by Dr F.W. Alt, n = 14, weighing approximately 25 g), designated Sirt1+/−, were generated in a previous study. These mice were randomly assigned to 2 treatment groups (control group, n = 20/7; pitavastatin group, n = 20/7). Each group received, by oral administration, vehicle alone or pitavastatin 3 mg/kg per day for their lifetimes. We made mice diabetic by a single intraperitoneal injection of streptozotocin (STZ) (60 mg/kg, Sigma). Tail blood glucose level and plasma insulin levels were assayed 3 days after injection using glucose test strips (Roche) and CLEIA. Blood pressure and pulse rate were measured by BP-98A (Softron Co, Tokyo, Japan). The primary antibody was purified rat anti-mouse CD31 (platelet endothelial cell adhesion molecule) monoclonal antibody from Pharmingen (San Jose, CA, USA). TOTO-3 for nuclear staining, secondary antibodies (Alexa Fluor 488 donkey anti-rat IgG and Alexa Fluor 594 donkey anti-rat IgG), and antifade reagent were from Molecular Probes (Invitrogen). Fluorescent images were analyzed using a confocal laser microscope (LSM510, Carl Zeiss MicroImaging Co Ltd).

Data Analysis

Values are shown as mean±SEM in the text and figures. Differences between the groups were analyzed using 1-way analysis of variance followed by the Bonferroni test. Probability values less than 0.05 were considered significant.

Results

Treatment With Atorvastatin, Pravastatin, and Pitavastatin Inhibits Oxidative Stress-Induced Endothelial Senescence

Endothelial senescence was induced by addition of 100 μmol/L H2O2 for 1 hour. Treatment with atorvastatin, pravastatin, or pitavastatin inhibited the senescent phenotype at 10 days (Figure 1A and 1B). Mitosis-related growth arrest and reduction of telomerase activity is a critical event for cellular senescence. In parallel with this, an increased rate of 5-bromodeoxyuridine (BrdU) (index of proliferation) incorporation and telomerase activity were restored by treatment with atorvastatin, pravastatin, and pitavastatin (Supplemental Figure 1A and 1B, available online at http://atvb.ahajournals.org). These results indicate that these statins inhibit oxidative stress-induced endothelial senescence. Statins prevent mevalonate formation and the downstream products FPP and GGPP, which finally inactivate Rho kinase. To clarify the involvement of these intermediates, we examined the influence of statins on mevalonate, FPP, and GGPP.
Treatment With Atorvastatin, Pravastatin, and Pitavastatin Increases eNOS Activity and Expression Through Akt Pathway

Recent studies have demonstrated that statins stimulate the phosphatidylinositol 3-kinase/Akt pathway, which is known to regulate eNOS activity. As shown in Figure 1D, these statins increased the phosphorylation of Akt at Ser473. To confirm the influence of treatment with these statins on eNOS activity, we examined the expression and activity of eNOS. In the presence of H$_2$O$_2$, treatment with atorvastatin, pravastatin, and pitavastatin increased eNOS expression and activity dose dependently (Figure 2A). In parallel with eNOS expression, activity of eNOS was increased by treatment with atorvastatin (Figure 2B), pravastatin, and pitavastatin (data not shown). To confirm whether a direct target of statin treatment is phosphorylation of Akt at Ser473, we treated mice with the phosphatidylinositol 3-kinase inhibitor LY294002 (10 μmol/L) for 6 hours or siRNA specifically for Akt at Ser473. D, NOS activity was measured after treatment with atorvastatin (atorva) + LY294002 or siRNA (*P<0.05, n=3). E, LY294002 or Akt siRNA reversed the effect of atorvastatin (100 nmol/L) as judged by SA-β-gal staining (*P<0.05, n=3).

Figure 1. A and B, Atorvastatin (atorva), pravastatin (prava), and pitavastatin (pitava) (50 and 100 nmol/L)-treated cells co-incubated with mevalonate (300 μmol/L), FPP (10 μmol/L), or GGPP (10 μmol/L) increased phosphorylation of Akt at Ser473. p-Akt indicates phospho-Akt.

As shown in Figure 1C, the addition of mevalonate, FPP, or GGPP completely reversed atorvastatin-induced inhibition of endothelial senescence. In contrast, the senescent phenotype was not altered by treatment with Y27632, a pharmacological inhibitor of Rho kinase (Figure 1C). These results indicate that statins influence endothelial senescence through isoprenylation but independently of Rho kinase. Next, we investigated the phosphorylation of Akt because several studies have demonstrated that statins stimulate the Akt pathway, which is known to regulate senescence of endothelial cells. As shown in Figure 1D, treatment with atorvastatin, pravastatin, or pitavastatin increased the phosphorylation of Akt at Ser473.

Figure 2. A, Expression of eNOS in atorvastatin, pravastatin, and pitavastatin-treated (10, 30, or 100 nmol/L) cells. B, NOS activity was measured after treatment with atorvastatin (atorva) (*P<0.05, n=3). C, Treatment with LY294002 (LY) (10 μmol/L) for 6 hours inhibited phosphorylation of Akt at Ser473. D, NOS activity was measured after treatment with atorvastatin + LY294002 or Akt siRNA (*P<0.05, n=3). E, LY294002 or Akt siRNA reversed the effect of atorvastatin (100 nmol/L) as judged by SA-β-gal staining (*P<0.05, n=3).
or an eNOS inhibitor, L-NAME, on SIRT1 expression and to clarify the involvement of Akt/eNOS in the effect of morphological changes (data not shown). Furthermore, SIRT1 expression in a concentration-dependent manner for 10 days after treatment with H2O2 (Figure 3A). To determine whether statin treatment rescues the senescence phenotype (Figure 4E).

**Direct Interaction of SIRT1 and eNOS Increases the Protective Effect Against Endothelial Senescence**

As previously reported, SIRT1 binds to, deacetylates, and activates eNOS directly in endothelial cells. To investigate whether the interaction of SIRT1 and eNOS contributes to the protective effect against cellular senescence, we examined the effect of overexpression of SIRT1 and eNOS on the senescence-like phenotype in HEK293 cells. Because HEK 293 cells lack an endogenous eNOS, we used HEK 293 cells to estimate accurate exogenous eNOS function. As shown in Supplemental Figure IIA and IIB, overexpression of eNOS alone did not inhibit SA-βgal activity or the senescent morphological appearance. In contrast, overexpression of SIRT1 inhibited the senescence-like phenotype. Furthermore, co-overexpression of SIRT1 and eNOS significantly inhibited the senescence-like phenotype. To confirm whether SIRT1 associates closely with eNOS, we performed coimmunoprecipitation of SIRT1 and eNOS. Coimmunoprecipitation showed that SIRT1 and eNOS associated with each other in human endothelial cells (Figure 4A). In addition, double immunofluorescent staining showed that endogenous SIRT1 and eNOS colocalized in the nucleus and perinuclear cytoplasm (Figure 4B). Moreover, to verify that eNOS is a substrate of SIRT1, we induced SIRT1 expression by treatment with DETA-NO, and immunoprecipitates of eNOS protein were immunoblotted with anti-acetyllysine antibody. Induction of SIRT1 by DETA-NO decreased the acetylation status of eNOS, and SIRT1 inhibition by sirtinol or SIRT1 siRNA reversed this (Figure 4C). Likewise, we found that treatment with atorvastatin had a similar effect, decreasing eNOS acetylation (Figure 4D). These results indicate that SIRT1 and eNOS interact with each other and accelerate the protective effect against a senescent phenotype (Figure 4E).

**Statins Increase Mitochondria Biogenesis and Expression of Catalase Through Upregulation of SIRT1**

Next, to clarify the molecular mechanisms of the antioxidative effect of SIRT1 induced by statins, we evaluated mitochondria biogenesis. As shown in Figure 5A, we found that senescent endothelial cells induced by H2O2 had decreased MitoTracker Red fluorescence compared with untreated cells. In contrast, treatment with atorvastatin partially restored the MitoTracker Red fluorescence. Inhibition of SIRT1 by siRNA abrogated the effect of atorvastatin. Moreover, Akt and eNOS siRNA also abrogated the effect of atorvastatin (Figure 5A). To address whether mitochondrial transcription was increased, mRNA levels of TFAM (the principal tran-
scription factor involved in regulating mtDNA transcription) and NRF-1 were quantified by real-time polymerase chain reaction. TFAM and NRF-1 transcripts were increased by resveratrol (100 nmol/L) for 6 hours in the absence or presence of sirtinol (100 μmol/L) or SIRT1 siRNA, and immunoprecipitates of eNOS protein were immunoblotted with anti-acetyllysine antibody. D, Atorvastatin-treated (100 nmol/L) cells were lysed, and immunoprecipitates of eNOS protein were immunoblotted with anti-acetyllysine antibody. E, The SIRT1-eNOS axis modulates the protective effect of statins against endothelial senescence.

Figure 4. A, Coimmunoprecipitation of eNOS and SIRT1 in HUVEC. SIRT1 and eNOS were overexpressed, and whole-cell lysates were immunoprecipitated (IP) with anti-SIRT1 or anti-eNOS antibodies. Immunoprecipitates were immunoblotted (IB) with anti-SIRT1 and anti-eNOS antibodies. B, Double immunofluorescence for endogenous SIRT1 (green) and eNOS (red) in HUVEC. 4′,6-Diamidino-2-phenylindole (DAPI, blue) shows nuclear staining. C, SIRT1 expression was induced by treatment with DETA-NO (100 μmol/L) for 6 hours in the absence or presence of sirtinol (100 μmol/L) or SIRT1 siRNA, and immunoprecipitates of eNOS protein were immunoblotted with anti-acetyllysine antibody. D, Atorvastatin-treated (100 nmol/L) cells were lysed, and immunoprecipitates of eNOS protein were immunoblotted with anti-acetyllysine antibody. E, The SIRT1-eNOS axis modulates the protective effect of statins against endothelial senescence.

Figure 5. A, MitoTracker Red fluorescence was evaluated in atorvastatin (100 nmol/L)-treated cells at 10 days after addition of H2O2. Inhibition of SIRT1, eNOS, and Akt by siRNA abrogated the effect of atorvastatin. B, mRNA levels of TFAM and NRF-1 were quantified by real-time polymerase chain reaction. GAPDH was used as the internal control (*P<0.05). C, Expression of PGC-1α, MnSOD, and catalase were assessed by Western blot analysis. D, Knockdown of PGC-1α and catalase reversed the inhibitory effect on senescence of atorvastatin (100 nmol/L, *P<0.05, n=5).

Administration of Pitavastatin Inhibits Vascular Endothelial Senescence in STZ-Diabetic Mice

To investigate whether statins have a protective effect against vascular endothelial senescence in vivo, we used STZ-diabetic mice, in which endothelial senescence has been documented.27 We considered STZ-diabetic mice suitable for investigation of clinical settings. STZ-treated mice with and without pitavastatin administration had elevated plasma glucose associated with decreased plasma insulin level compared with control mice (Supplemental Figure IV A). Body weight, blood pressure, and pulse rate were unaltered in STZ-treated mice with and without pitavastatin (Supplemental Figure IVB). We resected the thoracic aorta of these mice and compared the senescent phenotype with and without pitavastatin administration (Figure 6A and 6B). The number of SA-βgal-stained cells was significantly increased in the
Figure 6. A, SA-βgal staining of thoracic aorta from C57BL6 wild-type mice or Sirt1-heterozygous knockout mice receiving pitavastatin (3 mg/kg per day) at 7 days after a single intraperitoneal injection of STZ (60 mg/kg). B and C, Number of SA-βgal-stained cells in pitavastatin-treated thoracic aorta. SA-βgal-positive cells were mostly located on the luminal surface and stained for CD-31, a marker of vascular endothelial cells. D, The thoracic aortas were lysed, and Western blot was performed. Pitavastatin increased eNOS and catalase expression in the thoracic aorta of wild-type mice, but expression was unaltered in Sirt1 KO (+/−) mice. E, Immunofluorescent staining for SIRT1 (green), platelet endothelial cell adhesion molecule 1 (red), and TOTO-3 (blue).

Thoracic aorta of untreated mice, but it was decreased in the thoracic aorta of pitavastatin-treated mice (Figure 6C). However, in the haploinsufficient Sirt1 KO (+/−) mice, the number of SA-βgal-stained cells was not completely restored in the thoracic aorta from pitavastatin-treated STZ-diabetic mice (Figure 6C). Cross-sections of aorta stained with SA-βgal showed that positive cells were mostly located on the luminal surface and stained for CD-31, indicating that blue staining originated from vascular endothelial cells and not from the extracellular matrix (Figure 6B). Consistent with in vitro studies, pitavastatin administration increased eNOS and catalase expression in the thoracic aorta of wild-type mice, but we observed unaltered eNOS and catalase expression in the haploinsufficient Sirt1 KO (+/−) mice (Figure 6D). Immunostaining of sections for SIRT1 showed that SIRT1 expression in aortic endothelial cells was increased by treatment with pitavastatin (Figure 6E).

Discussion

The results of this study demonstrated that statins inhibit oxidative stress-induced endothelial senescence and that, subsequently, upregulation of SIRT1 plays a critical role in prevention of senescence through Akt pathway.

The mechanisms by which statins stimulate the expression and activation of eNOS appear to involve the geranylgeranylated pathway, because mevalonate, GGPP, and FPP reversed the inhibitory effect of statins on senescence. It is well known that inhibition of geranylgeranylation leads to inactivation of Rho kinase. However, pharmacological inhibitors of Rho kinase did not affect endothelial senescence, which indicated that the inhibitory effect of statins on senescence was not mediated by inhibition of Rho kinase. Moreover, treatment with statins increased the phosphorylation of Akt at Ser473. Treatment with Akt siRNA or LY294002, which inhibited phosphorylation of Akt at Ser473, abrogated the eNOS activation and antisenescent property of atorvastatin. These results demonstrate that statins activate the phosphatidylinositol 3-kinase/Akt pathway via isoprenylation, resulting in enhancement of eNOS expression and activation.

The free-radical theory of aging proposes that degenerative senescence is largely the result of the cumulative effect of reactive oxygen species.28 Previous studies have shown that overexpression of SIRT1 antagonizes cellular senescence through acetylation of p53 with localization of the PML body.10 In addition, SIRT1 binds to and targets eNOS for deacetylation at lysines 494 and 504 in human endothelial cells.26 Recently, we reported that SIRT1 overexpression prevented the development of oxidative stress-induced premature senescence in human endothelial cells.14 Although NO is known to be involved in reducing oxidative stress and the progression of atherosclerosis, the present study suggests that the interaction of SIRT1 with eNOS plays an important role in augmentation of the protective effect of statins against endothelial senescence (Figure 4E).

In this study, we examined the effect of pitavastatin on endothelial senescence, using STZ-diabetic mice as a clinical oxidative condition. Pitavastatin, a lipophilic 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, is categorized as a strong statin. Pitavastatin was chosen for this in vivo study because it is hardly metabolized by the cytochrome P450 system in the liver. The haploinsufficient Sirt1 KO (+/−) mice did not show a senescent phenotype of aorta without STZ treatment (Supplemental Figure IV C). In contrast with wild-type mice, the haploinsufficient Sirt1 KO (+/−) mice showed a senescent phenotype of aorta with STZ treatment, and pitavastatin did not recover it. These findings indicate that the maintenance of SIRT1 expression is important in developing stress tolerance.

It is now apparent that mitochondrial dysfunction is causal in many disease states, and improvement of mitochondria function could be an important therapeutic target. In this study, we observed that treatment with statins increased mitochondria biogenesis in SIRT1-dependent manner. In accordance with our results, it has been shown that overexpression or activation of SIRT1 regulates mitochondrial function and attenuates mitochondrial reactive oxygen species (mtROS) production and cellular H₂O₂ level in human coronary arterial endothelial cells.29 We observed that expression of MnSOD and catalase were increased. In addition, previous study reported that resveratrol, an activator of
SIRT1 increases mitochondrial content in the vascular endothelium. According to the mitochondrial theory of aging, mitochondria biogenesis reduces the flow of electrons per unit mitochondria; thus, statin-induced mitochondria biogenesis may be attributable to a reduction of oxidative stress in human endothelial cells.

Our results indicated that 100 nmol/L levels of statins are sufficient to exert protective effects against endothelial senescence. Considering that a 1 nmol/L level of statins was hardly able to prevent endothelial senescence under oxidative conditions in this study (data not shown), it becomes apparent that effective concentrations of statins are likely to be slightly higher. The use of statins is relatively safe, with few side effects. However, it should be noted that myopathy is the most common side effect, with symptoms ranging from fatigue, weakness, and pain to rhabdomyolysis.

In summary, we have shown that statins inhibit oxidative stress-induced endothelial senescence and that, subsequently, enhancement of SIRT1 expression through the Akt pathway plays a critical role in the inhibition of a senescent phenotype in human endothelial cells.

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Disclosures
None.

References
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Supplemental Material

BrdU Incorporation Assay

BrdU incorporation was analyzed using a commercial kit (Roche, Indianapolis, USA).

Telomerase Assay

Telomerase activity was measured with 2 µg protein using a telomerase PCR-ELISA kit according to the manufacturer’s instructions (Chemicon, Temecula, CA, USA).

Supplementary Figure Legends

Supplementary Figure I. Atorvastatin (atorva), pravastatin (prava), and pitavastatin (pitava) (50, 100 nmol/L) inhibited H$_2$O$_2$ (100 µmol/L)-induced endothelial senescence as judged by BrdU incorporation (A) and telomerase activity (B) at 10 days after addition of H$_2$O$_2$ (\*p<0.05, N=3). C. Atorvastatin (100 nmol/L) did not inhibit sirtinol (100 µmol/L) or L-NAME (20 µmol/L)-induced endothelial senescence as judged by SA-βgal staining (\*p<0.05, N=3), n.s: not significant. D. Knock down of SIRT1 by siRNA was confirmed by Western blotting analysis.

Supplementary Figure II. Overexpression of SIRT1 (10 µg) and eNOS (10 µg) inhibited oxidative-stress induced senescence-like phenotype in HEK293 cells. Expression of SIRT1 and eNOS were detected by western blotting analysis (A). For detection of a senescence-like phenotype, senescent morphological appearance and SA β-gal staining (B) (\*p<0.05, N=3) were used. C. Expression of SIRT1, eNOS, and catalase were detected by western blotting analysis. Treatment with atorvastatin
increased SIRT1, eNOS, and catalase expression for 6, 12, and 24 hrs, respectively in HUVEC.

**Supplementary Figure III.** A. Treatment with resveratrol (10, 30, 100 µmol/L) increased SIRT1 and eNOS expression. B. Treatment with resveratrol (res) (100 µmol/L) increased eNOS activity (\(^*p<0.05, N=3\)). C. Knockdown of PGC-1α and catalase reversed the inhibitory effect on senescence of resveratrol (100 nmol/L, \(^*p<0.05, N=3\)). D. The molecular mechanism of anti-senescence by which statin treatment upregulates eNOS (2), SIRT1 (3), and catalase expression through phosphorylation of Akt (1).

**Supplementary Figure IV.** A. Plasma glucose and plasma insulin levels in streptozotocin (STZ)-diabetic mice (\(^*p<0.05, N=3\)). B. Body weight (BW), blood pressure (BP), and pulse rate (PR) of STZ-diabetic mice with and without pitavastatin (3 mg/kg/day). C. Sirt1 KO (+/-) mice did not show a senescent phenotype of aorta without treatment with STZ.
Supplementary Figure I.
A. 

![Western blot images](Sirt1, eNOS, β-actin)

H\textsubscript{2}O\textsubscript{2} 0 10 30 100 
+ + + + 
Resvaratrol

B. 

![Graph](NOS activity (%))

H\textsubscript{2}O\textsubscript{2} - + + + 
Res 0 0 100

C. 

![Graph](SA-β-gal positive cells (%))

H\textsubscript{2}O\textsubscript{2} Res 0 10 30 60 100 100 100 
+ + + + + + 
+PGC-1α siRNA +catalase siRNA

D. 

![Flowchart](Statin treatment)

1. P-akt
2. eNOS
3. SIRT1
(1) Mitochondria biogenesis
(2) MnSOD/Catalase
(3) Endothelial senescence

Supplementary Figure III.
Supplementary Figure IV.

A. Blood glucose (mg/dl) and plasma insulin (ng/ml) levels in mice treated with STZ and Pitava.

B. Summary of BW, sBP, dBP, and PR measurements in different mouse groups.

C. Image of Wt and Sirt1 KO mice treated with STZ.