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 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, geranylgeranylporphosophate (GGPP), farnesylpyrophosphate (FPP), Y27632, and LY294002 were purchased from Sigma (St. Louis, Mo). (Z)-1-[2-(2-aminoethyl)-N-(2-aminoethyl)amino] diazen-1-IM1,2 diolate (DETA-NO) 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 N\(^{-}\)nitro-L-arginine methyl ester hydrochloride (L-NAME, Sigma) diluted in medium. Proliferating cells were trypsinized, reseeded at a density of 1\(^{10}\) cells per 60-mm dish, and cultured with EGM-2 containing the above compounds. After washing, cells were treated with 100 \(\mu\)M H\(_2\)O\(_2\) for 1 hour. Treatment with atorvastatin, pravastatin, and pitavastatin (Supplemental Figure IA and IB, available online at http://atvb.ahajournals.org) with atorvastatin, pravastatin, and pitavastatin were considered significant.

Results

Treatment With Atorvastatin, Pravastatin, and Pitavastatin Inhibits Oxidative Stress-Induced Endothelial Senescence
Endothelial senescence was induced by addition of 100 \(\mu\)M H\(_2\)O\(_2\) 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 IA and IB, 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.
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.23 As shown in Figure 1D, treatment with atorvastatin, pravastatin, or pitavastatin increased the phosphorylation of Akt at Ser473. p-Akt indicates phospho-Akt.

**Figure 1.** A and B, Atorvastatin (atorva), pravastatin (prava), and pitavastatin (pitava) (50 and 100 nmol/L)-induced endothelial senescence as judged by SA-β-gal-positive cells (%). C, Treatment with LY294002 (LY) (10 μmol/L)-treated cells coincubated with mevalonate (300 μmol/L), FPP (10 μmol/L), or GGPP (10 μmol/L) and Y27632 (10 μmol/L)-treated cells as judged by SA-β-gal staining. D, Treatment with atorvastatin, pravastatin, or pitavastatin (100 nmol/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.

**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.24 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 H₂O₂, treatment with atorvastatin, pravastatin, and pitavastatin increased eNOS expression 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) (100 nmol/L) for 6 hours inhibited phosphorylation of Akt at Ser473. E, LY294002 or Akt siRNA reversed the effect of atorvastatin (100 nmol/L) as judged by SA-β-gal staining.

**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) (100 nmol/L) for 6 hours inhibited phosphorylation of Akt at Ser473. D, NOS activity was measured after treatment with atorvastatin (atorva) (100 nmol/L) for 6 hours inhibited phosphorylation of Akt at Ser473. E, LY294002 or Akt siRNA reversed the effect of atorvastatin (100 nmol/L) as judged by SA-β-gal staining.

**Treatment With Atorvastatin, Pravastatin, and Pitavastatin Increases SIRT1 Expression Through Activation of eNOS**

In our previous study, we found that after treatment with either an NO donor (such as DETA-NO or SNAP), a cAMP
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Statins, we examined the effect of Akt siRNA, eNOS siRNA, to clarify the involvement of Akt/eNOS in the effect of 3).

In atorvastatin (100 nmol/L)-treated cells (* P<0.05, n=11005/11021 siRNA on SIRT1 expression (C) and endothelial senescence (D) was confirmed by Western blotting (Supplemental Figure IIC).

Figure 3. A, SIRT1 expression was dose-dependently increased by treatment with atorvastatin, pravastatin, or pitavastatin (10, 30, 100 nmol/L). B, Inhibition of SIRT1 by sirtinol or SIRT1 siRNA reversed the atorvastatin (100 nmol/L)-induced reduction of SA-βgal-positive cells (P<0.05, n=3). C and D, Effect of L-NAME (5, 10, 20 μmol/L) for 24 hours, eNOS siRNA, or Akt siRNA on SIRT1 expression (C) and endothelial senescence (D) in atorvastatin (100 nmol/L)-treated cells (P<0.05, n=3).

Analog (8-Br-cAMP), or a cGMP analog (8-bromo [Br]-cGMP), expression of SIRT1 protein was markedly higher than that in untreated HUVEC. Therefore, we hypothesized that an increase in eNOS activation caused by statins could promote the longevity gene, SIRT1. We found that atorvastatin, pravastatin, and pitavastatin significantly increased SIRT1 expression in a concentration-dependent manner for 10 days after treatment with H2O2 (Figure 3A). To determine the role of endogenous SIRT1 in premature senescence, HUVEC were treated with a SIRT1 chemical inhibitor, sirtinol, or SIRT1 siRNA. Knockdown of SIRT1 with siRNA was confirmed by Western blotting (Supplemental Figure ID). As shown in Figure 3B, SIRT1 inhibition abrogated the effect of atorvastatin on SA-βgal activity and specific senescent morphological changes (data not shown). Furthermore, to clarify the involvement of Akt/eNOS in the effect of statins, we examined the effect of Akt siRNA, eNOS siRNA, or an eNOS inhibitor, l-NAME, on SIRT1 expression and endothelial senescence. As shown in Figure 3C, treatment with Akt siRNA decreased SIRT1 expression. As shown in Figure 3C and 3D, treatment with eNOS siRNA or l-NAME decreased SIRT1 expression and the inhibitory effect of statins on senescence. These results indicate that SIRT1 could play an important role in the protective effect of statins against a senescent phenotype and that the Akt pathway is a direct target of statins to increase SIRT1 expression through eNOS activation. As previously reported, sirtinol or SIRT1 siRNA itself promotes endothelial senescence,14 and we investigated whether statin treatment rescues the senescence induced by sirtinol alone or l-NAME alone. As shown in Supplemental Figure IC, atorvastatin did not reverse sirtinol- or l-NAME-induced senescence. In addition, we examined whether statin treatment itself affects eNOS and SIRT1 expression without oxidative stress. Treatment with atorvastatin increased eNOS and SIRT1 expression in HUVEC (Supplemental Figure IIC).

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 antioxi- dative 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 treatment with atorvastatin, and SIRT1 inhibition by siRNA abrogated the effect of atorvastatin. 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=3).

**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 IV B). 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
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-β-gal-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, 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).

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 geranylgeranyl 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. Previous studies have shown that overexpression of SIRT1 antagonizes cellular senescence through acetylation of p53 with localization of the PML body. In addition, SIRT1 binds to and targets eNOS for deacetylation at lysines 494 and 504 in human endothelial cells. Recently, we reported that SIRT1 overexpression prevented the development of oxidative stress-induced premature senescence in human endothelial cells. 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 IVC). 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. 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 raises the flow of electrons per mitochondria biogenesis reduces the flow of electrons per mitochondria; thus, statin-induced mitochondria biogenesis may be attributable to a reduction of oxidative stress in human endothelial cells.

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DISCLOSURES
None.

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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-ßgal 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-ß-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. Control eNOS SIRT1 SIRT1+eNOS

B. 

Supplementary Figure II.

C. SIRT1 eNOS Catalase β-actin

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Sirt1

eNOS

β-actin

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NOS activity (%)

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SA-β-gal positive cells (%)

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+PGC-1α siRNA, +catalase siRNA

D. 

Supplementary Figure III.
Supplementary Figure IV.

A. Blood glucose (mg/dl) and Plasma insulin (ng/ml) levels in different conditions.

B. Summary of BW, sBP, dBP, and PR across different groups.

C. Image showing Wt and Sirt1 KO mice with STZ and Pitavastatin treatment marks.