antibody showed localization and its translocation in HSMC before or after stimulation of Pi alone (2.6 mM) or AngII alone (10 pmol/L). Nucleus was detected by DAPI stain.

Statistical analysis

All results are presented as mean ± standard error (SE). Differences between the groups were analyzed using ANOVA, followed by Fisher's PLSD test. A value of $P < 0.05$ was considered to be significant. All *in vitro* experiments were performed at least three times.

Figure legends

Supplemental figure 1. Deterioration of osteoblastic transition by SIRT1 knockdown under high-dose Pi stimulation

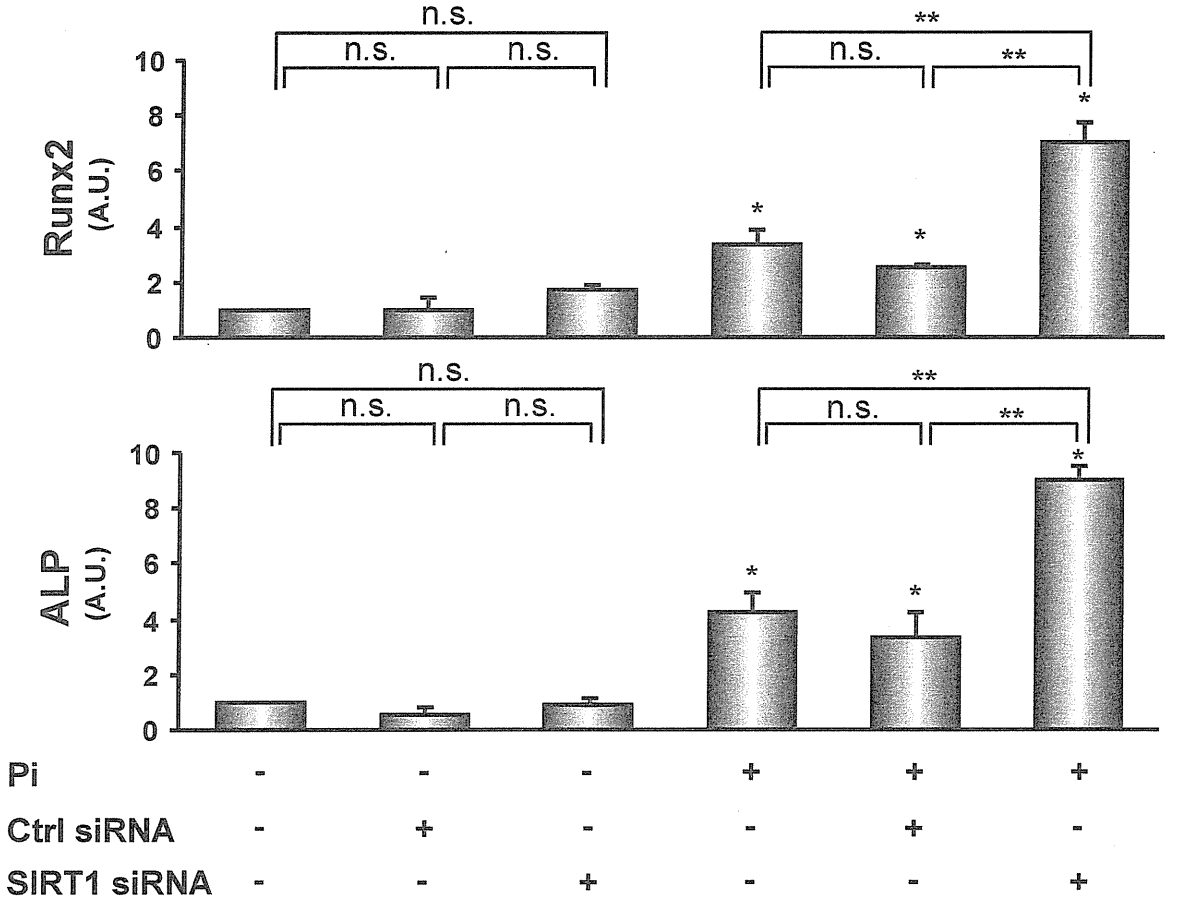
The effect of SIRT1 knockdown on osteoblastic markers, Runx2 and ALP, in the condition of normal Pi or high-dose Pi was examined. Complete knockdown of SIRT1 showed significantly augmented expression of both osteoblastic markers, Runx2 and ALP, in a high-dose Pi condition; however, augmentation was not found in a normal Pi condition. These data suggest that intracellular Pi influx by Pi stimulation is essential to

induce SMC calcification in association with osteoblastic phenotypic change, and the osteoblastic transition may be correlated with NPC, a cotransporter of Pi.

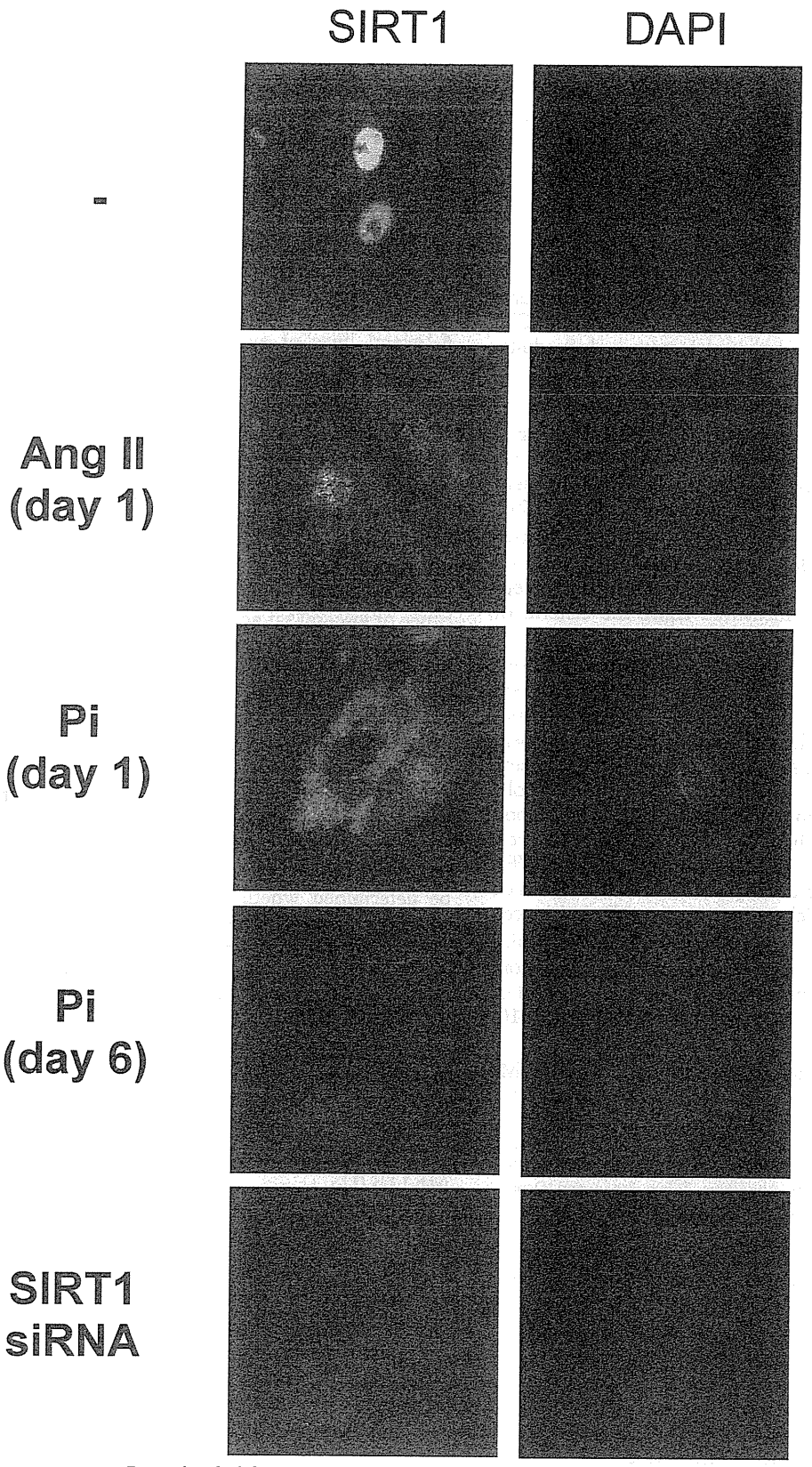
Supplemental figure 2. Translocation of SIRT1 in HASMC is induced by Pi, but not AngII.

To address a difference in senescent induction by Pi or AngII, immunohistological assessment of SIRT1 in HASMC was examined. SIRT1 was predominantly localized in nucleus without Pi. Dynamic translocation of SIRT1 to cytoplasm was observed after Pi stimulation (2.6 mM) for 24 hr and its expression disappeared in both areas on day 6. In contrast, AngII alone (10 pmol/L) did not show the dynamic translocation. SIRT1 siRNA shows complete knockdown of SIRT1 expression. DAPI shows nuclear stain.

Supplement Material Figure I



Supplement Material Figure Ⅱ



Visceral Fat Accumulation and Metabolic Risk Factor Clustering in Older Adults

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OBJECTIVES: To examine the relationship between visceral fat area (VFA) evaluated using computed tomography (CT) scans and the number of metabolic risk factors in older adults.

DESIGN: Cross-sectional study

SETTING: A community clinic in Tokyo, Japan.

PARTICIPANTS: Two hundred eighteen individuals aged 65 and older without impairments in activities of daily living who underwent geriatric health examination (63 men, mean age 74.5 ± 7.1; 155 women, mean age 75.3 ± 6.7).

MEASUREMENTS: VFA was obtained from a cross-sectional image at umbilical level in the supine position using CT scanning. Metabolic syndrome components except waist circumference were measured using the criteria of the International Diabetes Federation.

RESULTS: There was a positive correlation between VFA and number of metabolic risk factors in men and women. Multiple regression analysis demonstrated that only VFA was significantly correlated with number of risk factors in men, whereas age and VFA were significantly correlated in women; body mass index was not correlated with number of metabolic risk factors in men or women. Dyslipidemia and high blood glucose were associated with higher VFA, but high blood pressure was not. There was a negative correlation between VFA and serum adiponectin level and a positive correlation between VFA and homeostasis model assessment of insulin resistance.

CONCLUSION: Visceral fat accumulation is associated with metabolic risk factor clustering even in the elderly population. These results have clinical implications for the management of obesity in older adults. **J Am Geriatr Soc 58:1658–1663, 2010.**

Key words: visceral fat; metabolic syndrome; elderly; BMI

Several lines of evidence have suggested that visceral fat accumulation is associated with metabolic abnormalities such as high blood pressure (BP), high serum triglycerides, low serum high-density lipoprotein cholesterol (HDL-C), and high blood glucose through insulin resistance and abnormal secretion of adipocytokines.[1–4] Thus, visceral fat obesity has been established as a cause of cardiovascular disease,[5,6] although most of the subjects of studies delineating the relationship between visceral fat accumulation and metabolic abnormalities have consisted of middle-aged adults.[7–9] Therefore, the clinical significance of visceral fat accumulation in older adults is unclear in relation to metabolic abnormalities.

Aging is generally associated with a relative increase in visceral fat mass.[10,11] This is considered to be mainly due to decreased basal metabolism caused by loss of muscle mass, low physical activity, and an increase in carbohydrate intake.

Nevertheless, the prevalence of each metabolic syndrome component increases with age, and accordingly, elderly patients tend to have a higher number of metabolic abnormalities than other adults,[12–14] although it remains to be determined whether metabolic risk factor clustering, which is often observed in older adults, is attributable to visceral fat accumulation. It was assumed that visceral fat might affect this increase in the number of metabolic abnormalities with aging, through insulin resistance and abnormal secretion of adipocytokines. Thus, this study was conducted to clarify the relationship between visceral fat area (VFA) precisely evaluated using abdominal computed tomography (CT) scanning and the number of metabolic risk factors in an elderly sample.

METHODS

Subjects

Subjects who voluntarily participated in geriatric health examination were recruited at a community clinic from September 1 to November 30, 2005. Two hundred seventy-two subjects aged 65 and older who had no impairments in

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DOI: 10.1111/j.1532-5415.2010.03018.x

0002-8614/10/$15.00

activities of daily living and consented to this study were selected.

Medical history and information on medications and smoking status were obtained from all subjects. Body weight, height, and waist circumference were measured, and BP was measured in the sitting position. Body mass index (BMI) was calculated (weight/height^2, kg/m^2). Venous blood samples were collected in the early morning after a 12-hour fast.

People with a history of cancer or gastrointestinal tract surgery; under treatment for endocrine disease or heart failure; taking pioglitazone, metformin, insulin, alpha-blockers, beta-blockers, beta-stimulators, or hormone therapy (including glucocorticoids); and with serum albumin of 3.0 g/dL or lower, serum creatinine greater than 1.5 mg/dL, or blood hemoglobin of 10.0 g/dL or lower were excluded because such factors as abnormal fat metabolism and insulin resistance might have affected them, leaving 218 subjects to be enrolled in this study.

The ethics committee of Abe Clinic approved this study, and written informed consent was obtained from all subjects.

VFA Measurement

VFA was obtained from a cross-sectional image at the umbilical level in the supine position using CT scanning (X Vision Scanner, Toshiba Medical Systems, Tokyo, Japan) and calculated using commercially available software (Fat Scan, N2 System, Osaka, Japan).

Definition of Metabolic Risk Factors

Components of the metabolic syndrome except waist circumference were defined using the criteria of the International Diabetes Federation (IDF): systolic BP (SBP) of 130 mmHg, greater or diastolic BP (DBP) of 85 mmHg or greater, or treatment with antihypertensive drug; fasting serum triglyceride level of 150 mg/dL or greater or treatment with fibrate; serum HDL-C level less than 40 mg/dL in men and less than 50 mg/dL in women; fasting plasma glucose of 100 mg/dL or greater or treatment with an antidiabetic drug.[15]

Homeostasis Model Assessment of Insulin Resistance and Serum Adiponectin Level

Homeostasis model assessment of insulin resistance (HOMA-IR), calculated as fasting insulin level (μIU/mL) × early morning fasting blood glucose level (mg/dL)/405, was evaluated to determine degree of insulin resistance.[16,17] Subjects with diabetes mellitus were excluded from HOMA-IR calculation because of a lack of reliability of their data.

Serum level of adiponectin was measured using an enzyme-linked immunosorbent assay (Human Adiponectin ELISA Kit, Otsuka, Tokyo, Japan).

Statistical Analysis

The subjects were divided into four groups according to individual calculated VFA values in men and women. High BP, high triglycerides, low HDL-C, and high blood glucose were used as metabolic risk factors. The number of metabolic risk factors was calculated as their sum (0–4). Data were expressed as means ± standard deviations or standard errors. The statistical significance of differences was assessed using unpaired *t*-tests for two groups and analysis of variance for three or more groups, followed by the Fisher protected least significant difference test to compare each group. Multiple regression analysis was performed to determine independent factors for the number of metabolic risk factors. The correlation of VFA with HOMA-IR or serum adiponectin level was analyzed using the Pearson correlation coefficient.

$P<.05$ was considered significant. Statistical analysis was performed using Stat View software (version 5.0, SAS Institute, Inc., Cary, NC).

RESULTS

Clinical characteristics of the subjects are depicted in Table 1. Mean VFA in men was significantly higher than in women, although BMI (kg/m^2) was comparable. The prevalence of subjects with high BP was 79.4% in men and 78.7% in women, including 46.8% in men and 43.2% in

Table 1. Clinical Characteristics of Study Population

Characteristic	Men (n = 63)	Women (n = 155)
Age, mean ± SD (range)	74.5 ± 7.1 (65–93)	75.3 ± 6.7 (65–92)
Body mass index, kg/m^2, mean ± SD (range)	22.9 ± 2.8 (15.4–29.4)	22.5 ± 3.3 (15.9–33.4)
Waist circumference, cm, mean ± SD (range)	86.6 ± 8.3 (63.0–104.3)	83.7 ± 11.0 (54.0–111.0)
Visceral fat area, cm^2, mean ± SD (range)	134.8 ± 53.0 (33.2–258.3)	91.2 ± 44.8* (17.5–240.5)
Components of metabolic syndrome, n (%)†		
High blood pressure	50 (79.4)	122 (78.7)
High serum triglycerides	8 (12.7)	15 (9.7)
Low HDL-C	9 (14.3)	33 (21.3)
High blood glucose	21 (33.3)	42 (27.1)
Smoking status, n (%)		
Current	14 (22.6)	8 (5.2)
Former	24 (38.7)	4 (2.6)
Never	24 (38.7)	143 (92.6)
Past history, n (%)		
Cerebral infarction	5 (8.1)	5 (3.2)
Ischemic heart disease	1 (1.6)	6 (3.9)
Medications, n (%)		
Antihypertensive drugs	29 (46.8)	67 (43.2)
Fibrates	0 (0.0)	3 (1.9)
Statins	7 (11.3)	38 (24.5)
Antidiabetic drugs	4 (6.5)	2 (1.3)

* $P<.001$ vs men.

† Components of the metabolic syndrome were diagnosed according to the definition of the International Diabetes Federation: high blood pressure = systolic blood pressure ≥130 mmHg, diastolic blood pressure ≥85 mmHg, or treatment with antihypertensive drug; high serum triglycerides = fasting serum triglyceride level ≥150 mg/dL or treatment with fibrates; low high-density lipoprotein cholesterol (HDL-C) = serum HDL-C level <40 mg/dL in men and <50 mg/dL in women; high blood glucose = fasting plasma glucose ≥100 mg/dL or treatment with antidiabetic drugs.

SD = standard deviation.

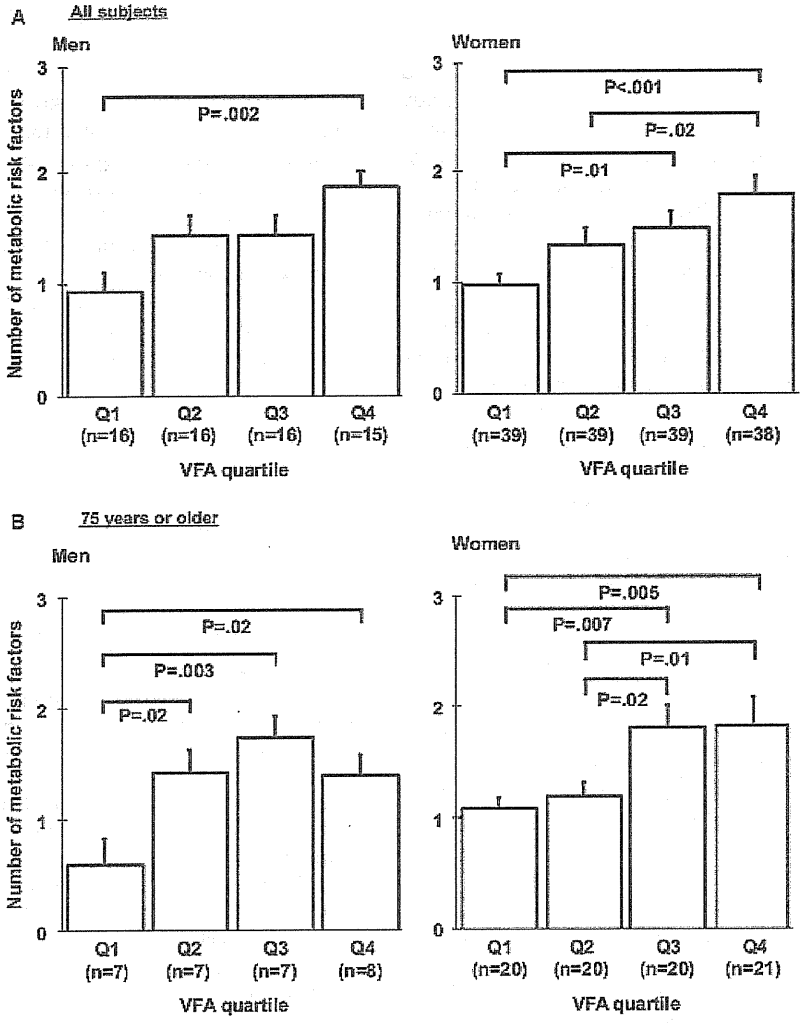

Figure 1. Number of metabolic risk factors according to quartile (Q) of visceral fat area (VFA) in all subjects (A) and subjects aged 75 and older (B). Metabolic risk factors include high blood pressure, high serum triglycerides, low serum high-density lipoprotein cholesterol, and high blood glucose. Data are expressed as means ± standard errors.

women receiving antihypertensive treatment. The prevalence of subjects who had never smoked was markedly higher in women (92.6%) than in men (38.7%).

Figure 1A shows the relationship between VFA and number of metabolic risk factors. The number of risk factors was greater with larger VFA values in men and women. This positive relationship was also observed in subjects aged 75 and older, especially in women (Figure 1B).

Next, multiple regression analysis was performed to detect independent factors for number of metabolic risk factors, using age, VFA, and BMI as independent variables. In men, VFA and in women, VFA and age were positively correlated with number of risk factors (Table 2). BMI was not correlated with number of metabolic risk factors in men or women. Moreover, when waist circumference was added in this multiple regression analysis, VFA was significantly correlated with number of metabolic risk factors in men and women ($P = .02$; data not shown). Waist circumference was not correlated with number of metabolic risk factors in men or women ($P = .85$ in men, $P = .08$ in women; data not shown).

Table 2. Multiple Regression Analysis with Number of Metabolic Risk Factors

Independent Variable	Coefficient (Standard Error)	Standardized Coefficient	*P*-Value
Men*			
Age	0.012 (0.014)	0.10	.39
VFA	0.006 (0.002)	0.39	.01
BMI	0.055 (0.047)	0.18	.25
Women†			
Age	0.027 (0.011)	0.19	.01
VFA	0.007 (0.002)	0.33	.001
BMI	0.010 (0.028)	0.04	.72

*Correlation coefficient (R) = 0.515, coefficient of determination (R^2) = 0.265, $P < .001$.

†$R = 0.393$, $R^2 = 0.154$, $P < .001$.

VFA = visceral fat area; BMI = body mass index.

Metabolic risk factors indicate components of the metabolic syndrome except abdominal obesity according to the definition of the International Diabetes Federation.

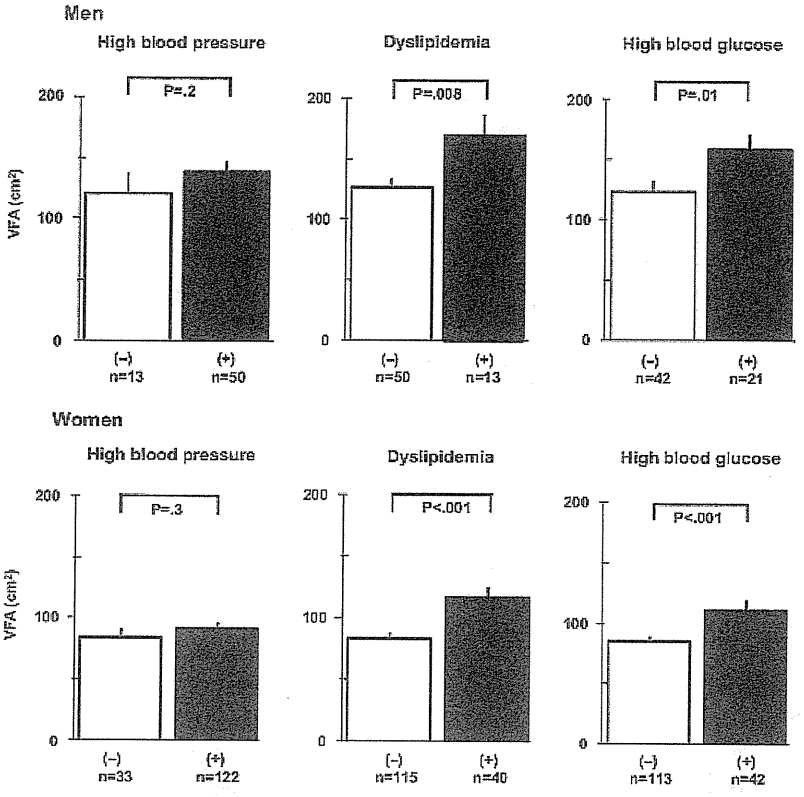

Figure 2. Visceral fat area (VFA) in the absence (–) and presence (+) of each metabolic risk factor. Dyslipidemia includes high triglycerides, low high-density lipoprotein cholesterol, or both. Data are expressed as means ± standard errors.

The relationship between each metabolic risk factor and VFA in elderly subjects was examined. As shown in Figure 2, men and women with dyslipidemia (high triglycerides, low HDL-C, or both) had a significantly greater mean VFA than those without dyslipidemia. Similar results were observed in subjects with and without high blood glucose, although there was no significant difference in VFA between subjects with and without high BP. Changing the cutoff values to 140/90 mmHg from 130/85 mmHg in this analysis made no difference in the results ($P = .25$ in men, $P = .41$ in women; data not shown). A simple regression analysis between VFA and SBP or DBP in subjects not receiving antihypertensive treatment showed no correlation (SBP: $P = .51$ in men, $P = .72$ in women; DBP: $P = .81$ in men, $P = .11$ in women; data not shown).

Finally, a significant negative correlation was observed between VFA and serum adiponectin and a positive correlation between VFA and HOMA-IR in men and women (Figure 3).

DISCUSSION

VFA is associated with metabolic abnormalities, as previously shown in studies of middle-aged populations.[7–9] This association was still observed after adjustment for age and BMI, suggesting that visceral fat accumulation might be a strong risk factor for the metabolic syndrome even in older adults. This association was observed even in subjects aged 75 and older, and VFA was correlated with components of the metabolic syndrome even in subjects who on average had a normal BMI.

Nevertheless, in multiple regression analysis, BMI was not correlated with number of metabolic risk factors in men or women. These results suggest that, for the evaluation of metabolic abnormalities in older adults, VFA is more useful than BMI because BMI in older adults might reflect not only visceral fat mass, but also lower muscle mass and intercellular fluid associated with aging. Thus, because of a reduction of muscle mass with aging, studies that use only BMI would underestimate the health effect of body fatness. Moreover, even if waist circumference was added in this multiple regression analysis, VFA was significantly correlated with number of metabolic risk factors in men and women, but waist circumference was not, suggesting that VFA rather than waist circumference may strongly predict metabolic abnormalities. Data from the Diabetes Prevention Program Research Group showed that visceral adipose tissue predicted the development of type 2 diabetes mellitus better than BMI or waist circumference, but analyses were not limited to older adults (only 20% were ≥60).[18] Thus, it would be important to assess the value of VFA prospectively in predicting the worsening of metabolic risk factors and age-related diseases (e.g., diabetes mellitus and cardiovascular disease).

A strength of this study is the precise assessment of visceral fat according to CT scanning instead of the generally used waist circumference for assessment of abdominal obesity. In many clinical studies, large waist circumference, representing visceral fat accumulation, has been reported to

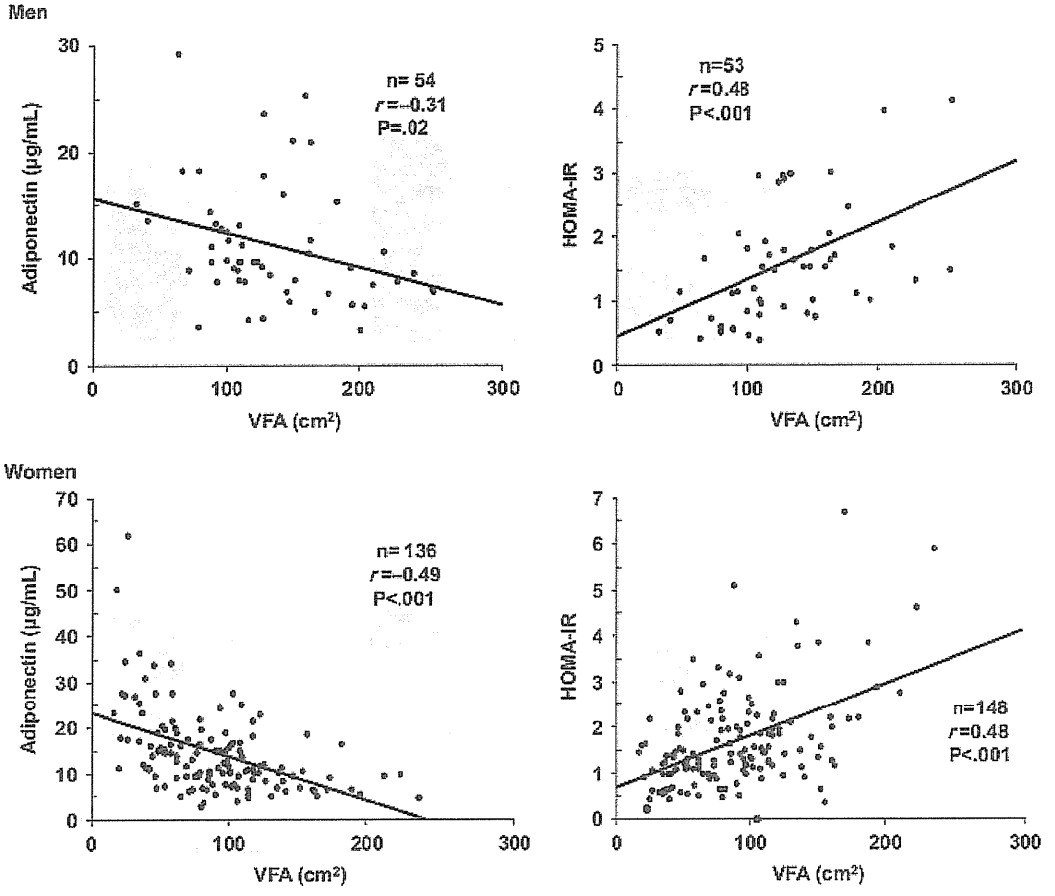

Figure 3. Correlation between visceral fat area (VFA) and serum adiponectin in all subjects and homeostasis model assessment of insulin resistance (HOMA-IR) in older men and women without diabetes mellitus. There was a significant negative correlation between VFA and serum adiponectin and a positive correlation between VFA and HOMA-IR in men and women. r = correlation coefficient.

be associated with greater cardiovascular disease and mortality.[19–21] As the mechanism of this association, it has been proposed that visceral fat accumulation is associated with metabolic abnormalities through insulin resistance and abnormal secretion of adipocytokines.[22,23] This study confirmed that visceral fat accumulation was negatively correlated with serum adiponectin level and positively correlated with insulin resistance as estimated by HOMA-IR in older adults. These findings suggest that older adults with visceral fat accumulation might tend to show metabolic abnormalities through decreased secretion of adiponectin and exacerbation of insulin resistance, similar to middle-aged adults with abdominal obesity.

No association was observed between high BP and VFA. Although the high rate (nearly 80%) of high BP may have affected this result, an additional analysis of this study showed no association between VFA and high BP using a modified cutoff value (140/90 mmHg). Moreover, the simple regression analysis showed no correlation between VFA and SBP or DBP in subjects not receiving antihypertensive treatment. These results suggest that factors other than visceral fat accumulation, such as sclerosis of blood vessels and enhancement of salt sensitivity, both of which are associated with aging, might affect BP in older adults. To the contrary, impaired energy metabolism (e.g., high blood glucose and dyslipidemia) was closely associated with visceral fat accumulation.

It has been reported that weight-reduction therapy using diet, exercise, or both is efficacious in terms of improvement of insulin resistance and dyslipidemia even in older adults.[24,25] Thus, taking together the results of this study and these reports, it appears that the beneficial effects of weight-reduction therapy for older adults even with normal BMI might result from a reduction of visceral fat mass and subsequent improvement in energy metabolism. However, severe dietary therapy for weight reduction is difficult to achieve in elderly patients and has potential risks of causing micronutrient deficiencies,[26–28] generalized weakness, and loss of lean body mass.

There are some limitations of this study. First, because of exclusion criteria, the results of this study might not be generalizable to the general elderly population,. Second, this study did not determine the effects of other body parameters such as subcutaneous fat and nonfat mass on metabolic abnormalities. Third, with the cross-sectional design, causal relationships cannot be established between VFA and metabolic risk factors. Finally, it remains to be determined whether metabolic syndrome in older adults

contributes to cardiovascular events or mortality.[29,30] Confirmation by a large prospective study with precise assessment, such as CT scanning, will be needed to determine whether visceral fat accumulation in older adults directly contributes to cardiovascular events or mortality.

In conclusion, this study suggests that visceral fat accumulation is associated with metabolic risk factor clustering even in older adults with normal BMI. These results provide important insight into the management of metabolic abnormalities in older adults.

ACKNOWLEDGMENTS

Conflict of Interest: This study was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (18890055 and 20249041) and Mitsui Sumitomo Insurance Welfare Foundation.

The authors do not have any conflict of interest with a company whose products or services are directly related to the subject matter of the manuscript.

Author Contributions: Study concept and design: Kazushi Nomura, Masato Eto, Masahiro Akishita, Yasuyoshi Ouchi. Acquisition of subjects and data: Kazushi Nomura, Taro Kojima, Tetsuro Nakamura, Masahiro Akishita. Analysis and interpretation of data: Kazushi Nomura, Masato Eto, Sumito Ogawa, Katsuya Iijima, Atsushi Araki, Masahiro Akishita, Yasuyoshi Ouchi. Preparation of manuscript: Kazushi Nomura, Masato Eto.

Sponsor's Role: The funding institutes that supported this research did not participate in the study design, methods, subject recruitment, data collection, analysis, or preparation of the manuscript.

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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 and Yasuyoshi Ouchi

Arterioscler Thromb Vasc Biol 2010;30;2205-2211; originally published online Aug 12, 2010;
DOI: 10.1161/ATVBAHA.110.210500
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 1079-5642. Online ISSN: 1524-4636

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 (H_2O_2), 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]

Received on: May 6, 2010; final version accepted on: July 23, 2010.

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Arterioscler Thromb Vasc Biol **is available at http://atvb.ahajournals.org** **DOI: 10.1161/ATVBAHA.110.210500**

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, geranylgeranylpyrophosphate (GGPP), farnesylpyrophosphate (FPP), Y27632, and LY294002 were purchased from Sigma (St. Louis, Mo). (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)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,[16] 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^G-nitro-L-arginine methyl ester hydrochloride (L-NAME, Sigma) diluted in medium. Proliferating cells were transfected with 200 pmol/L short interfering RNA (siRNA) for SIRT1 (GAT GAA GTT GAC CTC CTC A[17] and TGA AGT GCC TCA GAT ATT A) using silMPORTER (Upstate Cell Signaling Solutions). siRNAs for Akt, eNOS, PGC-1α, and catalase were purchased from Santa Cruz Biotechnology Inc. The pIRES-SIRT1 plasmid was provided by Dr M. Takata and Dr R.A. Weinberg,[18] and pcDNA3-eNOS plasmid was provided by Dr T Hayashi.[19] SIRT1 and eNOS were overexpressed by transfection using Lipofectamine LTX and PLUS reagents (Invitrogen) for HEK293 cells and jetPEI-HUVEC (Polyplustransfection, Illkirch, France) for HUVEC 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 H_2O_2 diluted in EGM-2. After treatment, HUVEC were trypsinized, reseeded at a density of 1×10^5 cells per 60-mm dish, and cultured with EGM-2 containing the above compounds for 10 days. The proportion of SA-βgal-positive cells was determined as described by Dimri et al.[20]

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-mangenese superoxide dismutase (MnSOD), anti-PGC-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 fluorescent quantitative detection system (Bioflux Co, Tokyo, Japan). The following primers were used: NRF-1, forward, 5′-GATGGCACTGTCTCACTTATCC-3′, reverse, 5′-CTGATGCTTGCGTCGTCT-3′; TFAM, forward, 5′-CATCTGTCTTGGCAAGTTGTCC-3′, reverse, 5′-CCACTCCG-CCCTATAAGCATC-3′; GAPDH, forward, 5′-ACCACAGTCCAT-GCCATCAC-3′, reverse, 5′-TCCACCACCCTGTTGCTGTA-3′.

Animal Experiments

The animal experiments were approved by our institutional review board. Twelve-week-old specific pathogen free (SPF) male wild-type B57/BL6 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.[21] 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 H_2O_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.

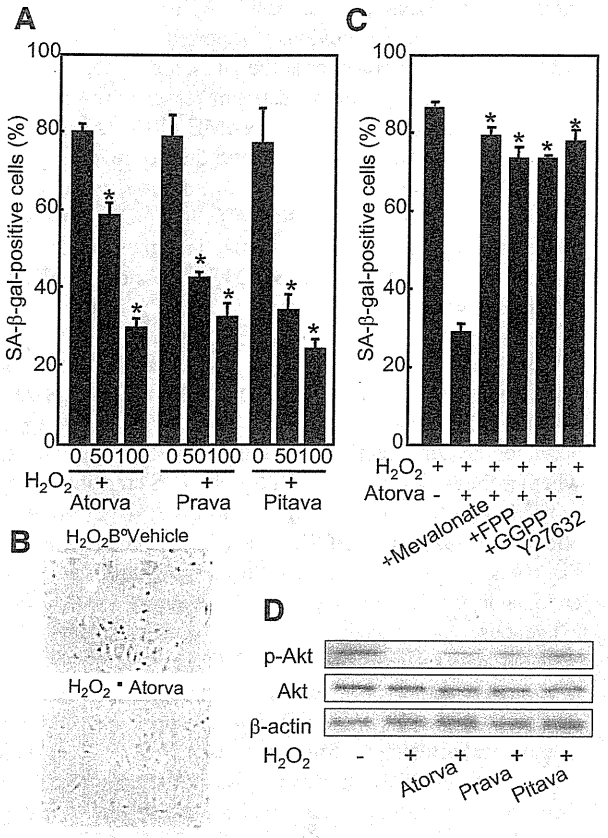

Figure 1. A and B, Atorvastatin (atorva), pravastatin (prava), and pitavastatin (pitava) (50 and 100 nmol/L) inhibited H_2O_2 (100 μmol/L)-induced endothelial senescence as judged by SA-βgal staining (A) (*$P<0.05$, n=3) and morphological changes (B) at 10 days after addition of H_2O_2. C, Atorvastatin (100 nmol/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-βgal staining (*$P<0.05$, n=3). D, Treatment with atorvastatin, pravastatin, or pitavastatin (100 nmol/L) increased phosphorylation of Akt at Ser473. p-Akt indicates phospho-Akt.

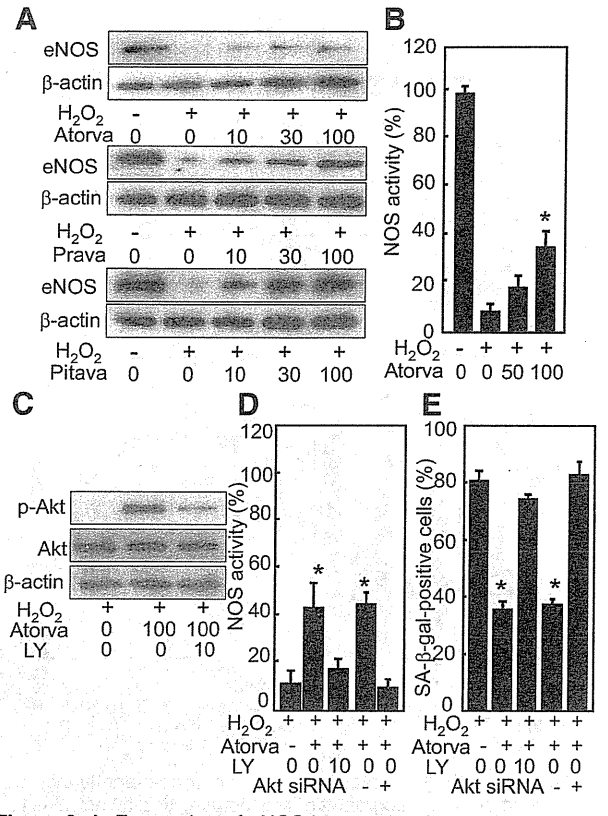

Figure 2. A, Expression of eNOS in atorvastatin, pravastatin, and pitavastatin-treated (10, 30, or 100 nmol/L) cells. B, NOS activity was measured after treatment with atorvastatin (atorva) (*$P<0.05$, n=3). C, Treatment with LY294002 (LY) (10 μmol/L) for 6 hours inhibited phosphorylation of Akt at Ser473. 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 atorvatatin (100 nmol/L) as judged by SA-βgal staining (*$P<0.05$, n=3).

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,[22] 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.

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 H_2O_2, 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 in atorvastatin-treated endothelial cells. Treatment with LY294002 inhibited phosphorylation of Akt at Ser473 and abrogated the eNOS activation (Figure 2C and 2D). Treatment with Akt siRNA also abrogated the eNOS activation (Figure 2D). After treatment with Ly294002 or Akt siRNA, many senescent cells were observed in the presence of statins (Figure 2E). These results indicate that the antisenescent property of statins and increased eNOS activation are attributable to a direct stimulation of the Akt pathway.

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

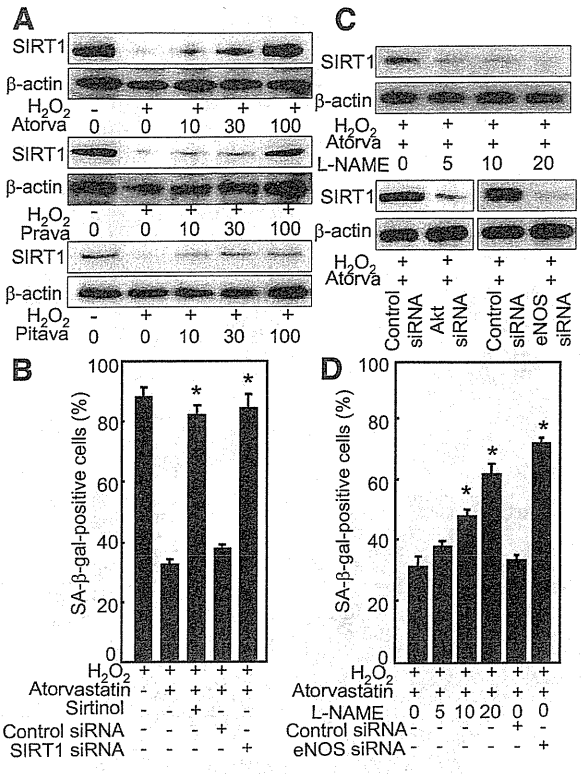

Figure 3. A, SIRT1 expression was dose-dependently increased by treatment with atorvastatin, pravastatin, or pitavastatin (10, 30, 100 nmol/L). B, Inhibition of SIRT1 by sirtinol or SIRT1 siRNA reversed the atorvastatin (100 nmol/L)-induced reduction of SA-βgal-positive cells (*$P<0.05$, n=3). C and 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.[25] 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 H_2O_2 (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,[26] 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 H_2O_2 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-

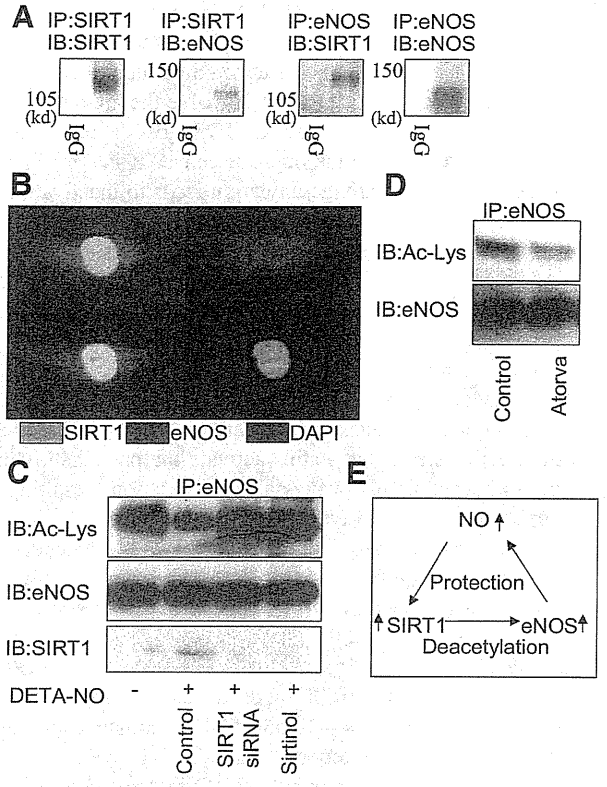

Figure 4. A, Coimmunoprecipitation of eNOS and SIRT1 in HUVEC. SIRT1 and eNOS were overexpressed, and whole-cell lysates were immunoprecipitated (IP) with anti-SIRT1 or anti-eNOS antibodies. Immunoprecipitates were immunoblotted (IB) with anti-SIRT1 and anti-eNOS antibodies. B, Double immunofluorescence 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.

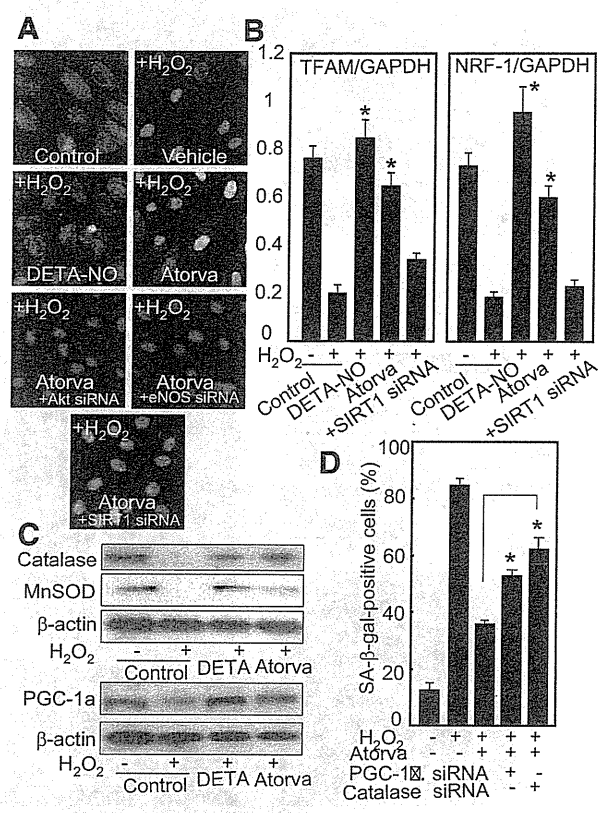

Figure 5. A, MitoTracker Red fluorescence was evaluated in atorvastatin (100 nmol/L)-treated cells at 10 days after addition of H_2O_2. Inhibition of SIRT1, eNOS, and Akt by siRNA abrogated the effect of atorvastatin. B, mRNA levels of TFAM and NRF-1 were quantified by real-time polymerase chain reaction. 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=3).

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 completely reversed this (Figure 5B). Concomitantly, the expression of MnSOD and catalase were also increased (Figure 5C). PGC-1α is the principal regulator of mitochondria biogenesis. Therefore, we examined the expression of PGC-1α. As expected, treatment with atorvastatin increased the expression of PGC-1α. To clarify the involvement of PGC-1α and catalase, PGC-1α and catalase siRNA was transfected in atorvastatin-treated cells. Knockdown of PGC-1α and catalase reversed the inhibitory effect of atorvastatin on senescence (Figure 5D). Moreover, to confirm involvement of SIRT1 activation, we treated cells with the SIRT1 direct activator resveratrol. Treatment with resveratrol increased SIRT1, eNOS expression, and eNOS activation (Supplemental Figure IIIA and IIIB). As shown in Supplemental Figure IIIC, activation of SIRT1 by resveratrol inhibited a senescent phenotype, and knockdown of PGC-1α and catalase abrogated it. These results indicated that the molecular mechanism of the antioxidative effect of statins was attributable to increased MnSOD/catalase expression through upregulation of SIRT1 (Supplemental Figure IIID).

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 IVA). 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

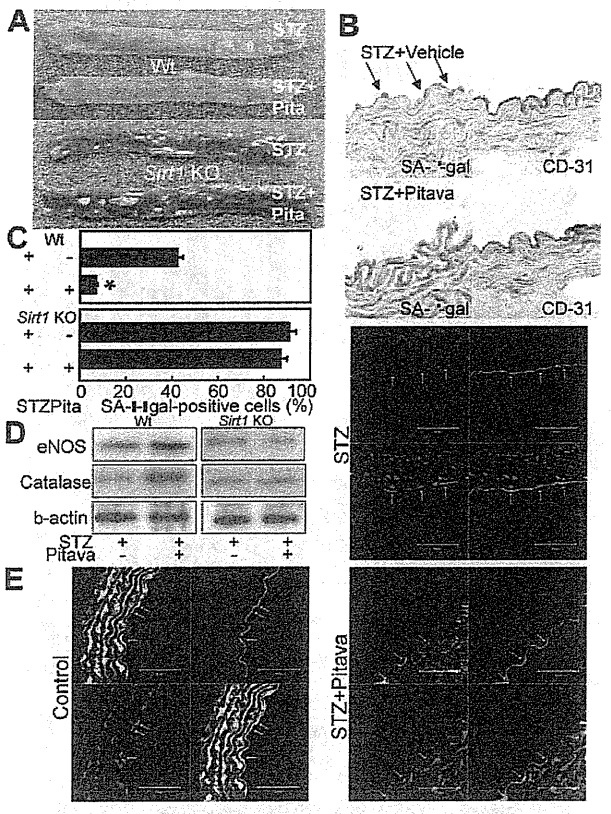

Figure 6. A, SA-βgal staining of thoracic aorta from C57/BL6 wild-type mice or *Sirt1*-heterozygous knockout mice receiving pitavastatin (3 mg/kg per day) at 7 days after a single intraperitoneal injection of STZ (60 mg/kg). B and C, Number of SA-βgal-stained cells in pitavastatin-treated thoracic aorta. 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 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.[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 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 H_2O_2 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.[30] 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.

Sources of Funding

This work was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sports of Japan (20249041, 18590801, and 18890056).

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_2O_2 (100 μmol/L)-induced endothelial senescence as judged by BrdU incorporation (A) and telomerase activity (B) at 10 days after addition of H_2O_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.