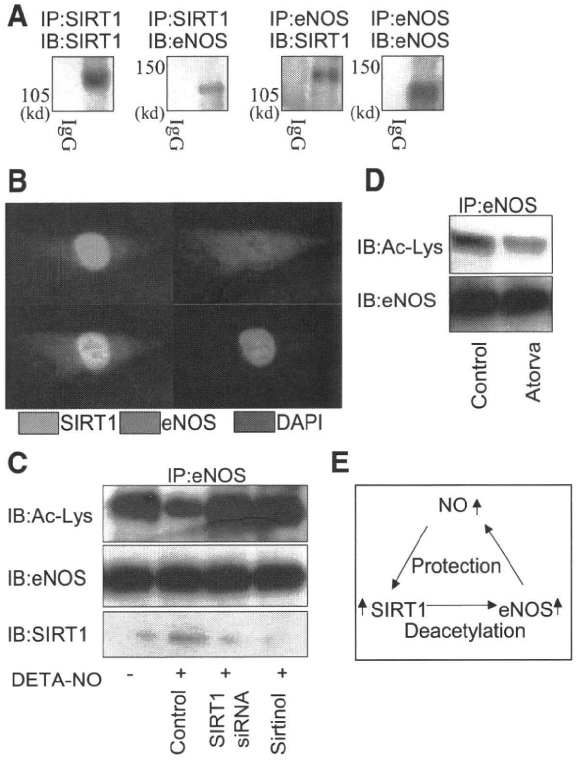

Figure 4. A, Coimmunoprecipitation of eNOS and SIRT1 in HUVEC. SIRT1 and eNOS were overexpressed, and whole-cell lysates were immunoprecipitated (IP) with anti-SIRT1 or anti-eNOS antibodies. Immunoprecipitates were immunoblotted (IB) with anti-SIRT1 and anti-eNOS antibodies. B, Double immunofluorescence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

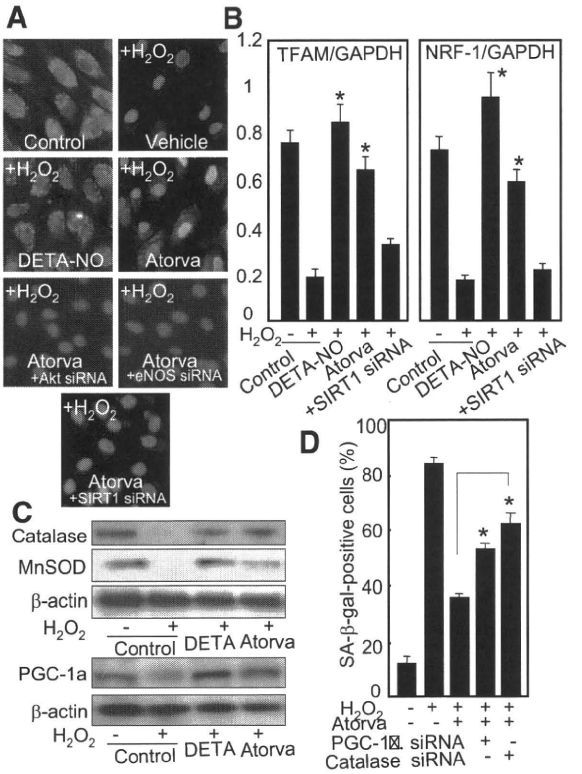

Figure 5. A, MitoTracker Red fluorescence was evaluated in atorvastatin (100 nmol/L)-treated cells at 10 days after addition of H_2O_2. Inhibition of SIRT1, eNOS, and Akt by siRNA abrogated the effect of atorvastatin. B, mRNA levels of TFAM and NRF-1 were quantified by real-time polymerase chain reaction.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

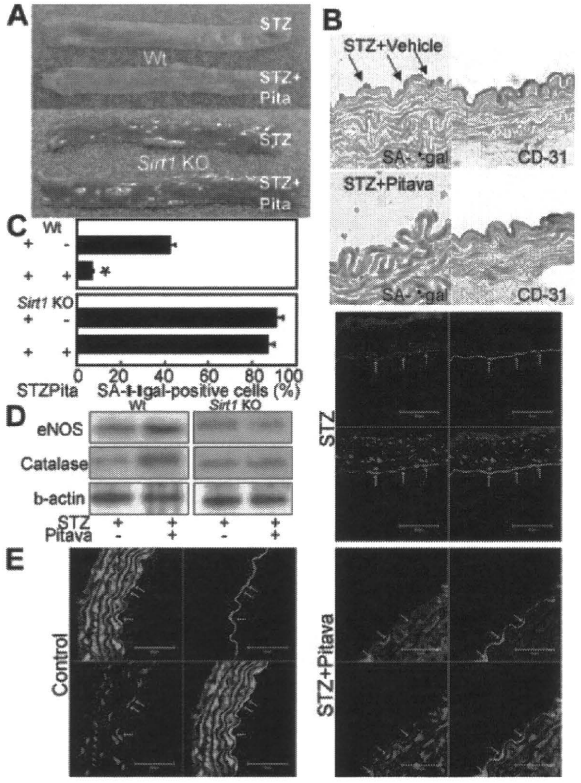

Figure 6. A, SA-βgal staining of thoracic aorta from C57/BL6 wild-type mice or *Sirt1*-heterozygous knockout mice receiving pitavastatin (3 mg/kg per day) at 7 days after a single intraperitoneal injection of STZ (60 mg/kg). B and C, Number of SA-βgal-stained cells in pitavastatin-treated thoracic aorta.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

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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_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.

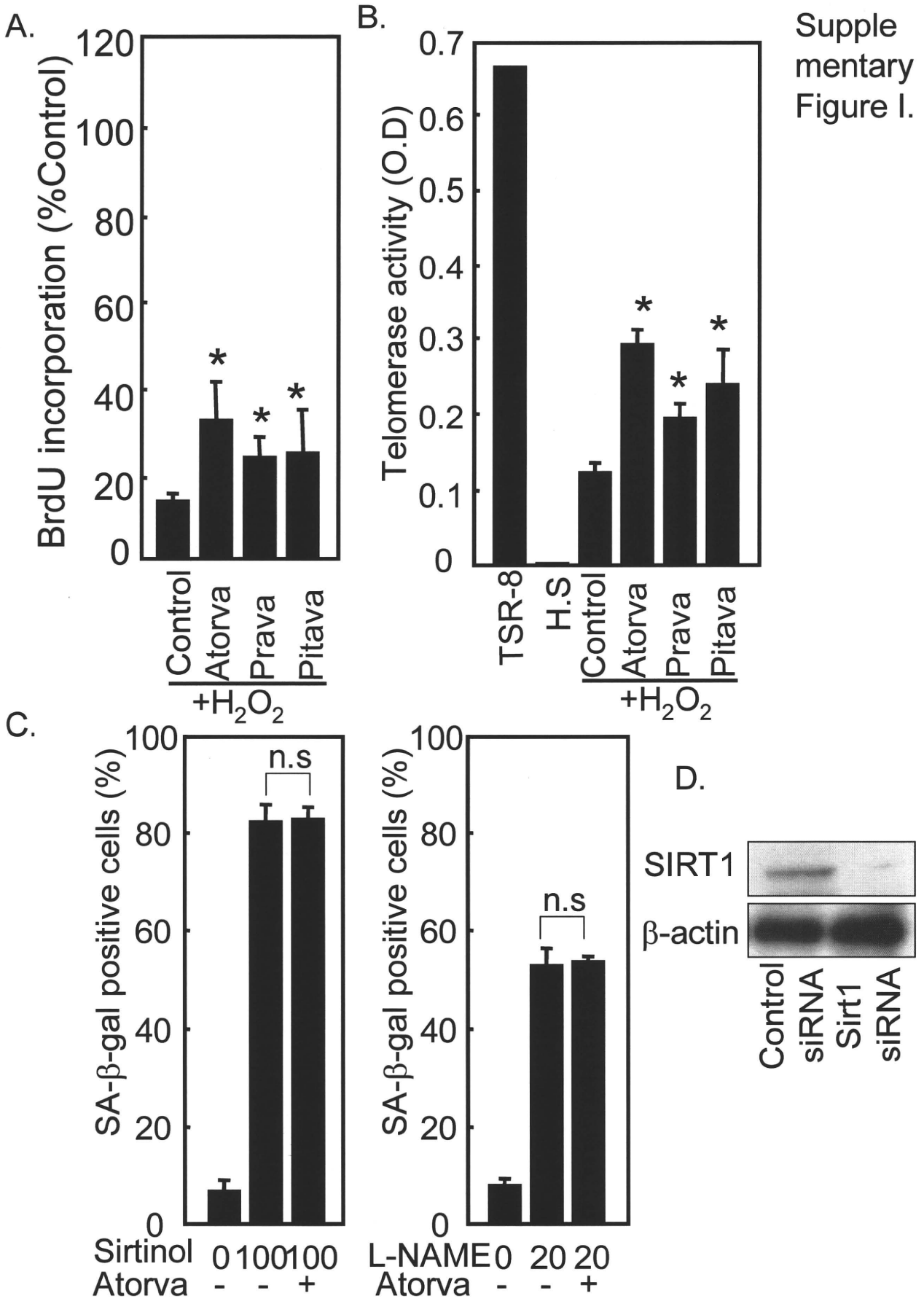
Supplementary Figure II. Overexpression of SIRT1 (10 μg) and eNOS (10 μg) inhibited oxidative-stress induced senescence-like phenotype in HEK293 cells. Expression of SIRT1 and eNOS were detected by western blotting analysis (**A**). For detection of a senescence-like phenotype, senescent morphological 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.

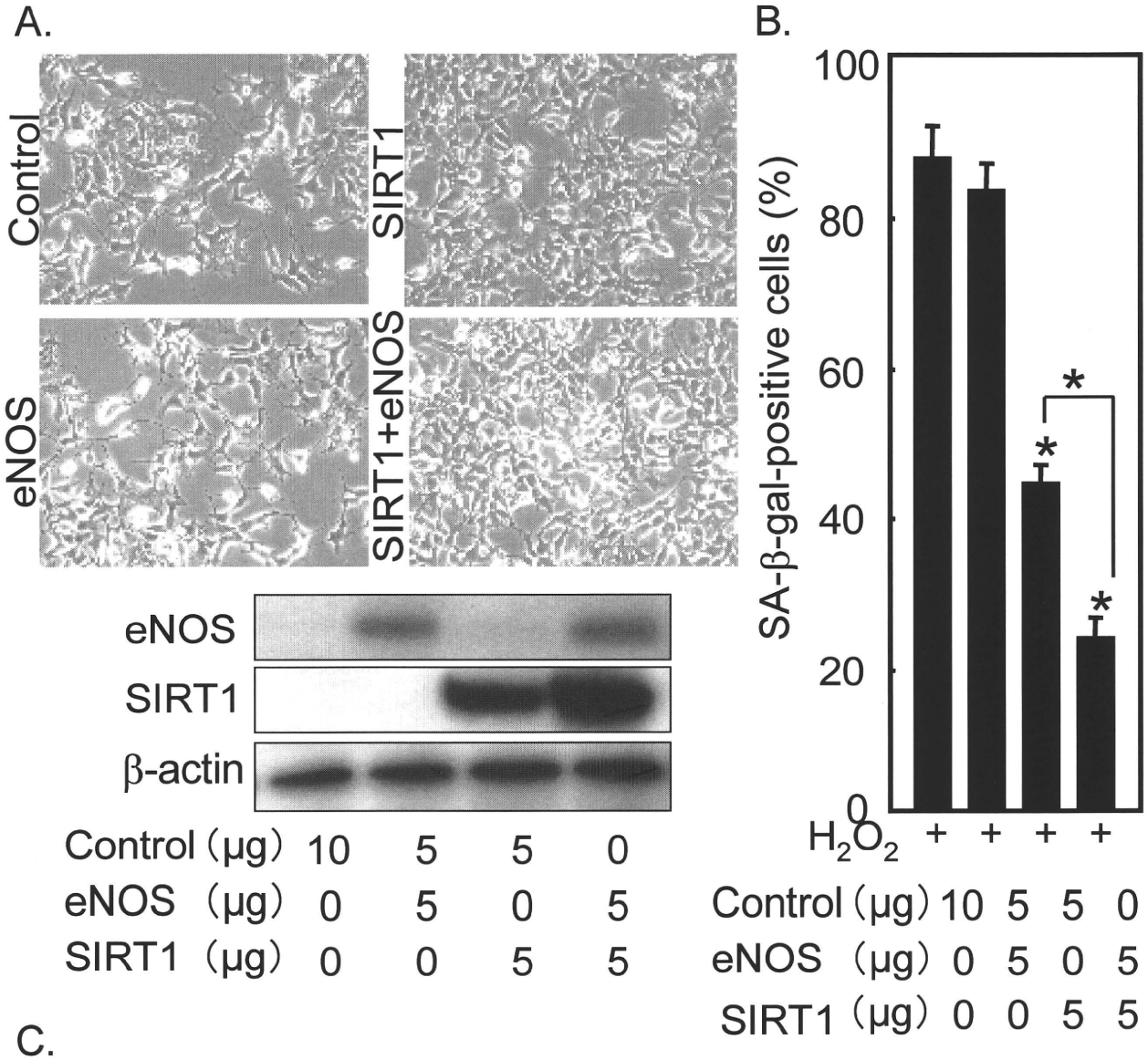
Supplementary Figure III. A. Treatment with resveratrol (10, 30, 100 μmol/L) increased SIRT1 and eNOS expression. **B.** Treatment with resveratrol (res) (100 μmol/L) increased eNOS activity ($^{*}p<0.05$, N=3). **C.** Knockdown of PGC-1α and catalase reversed the inhibitory effect on senescence of resveratrol (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.

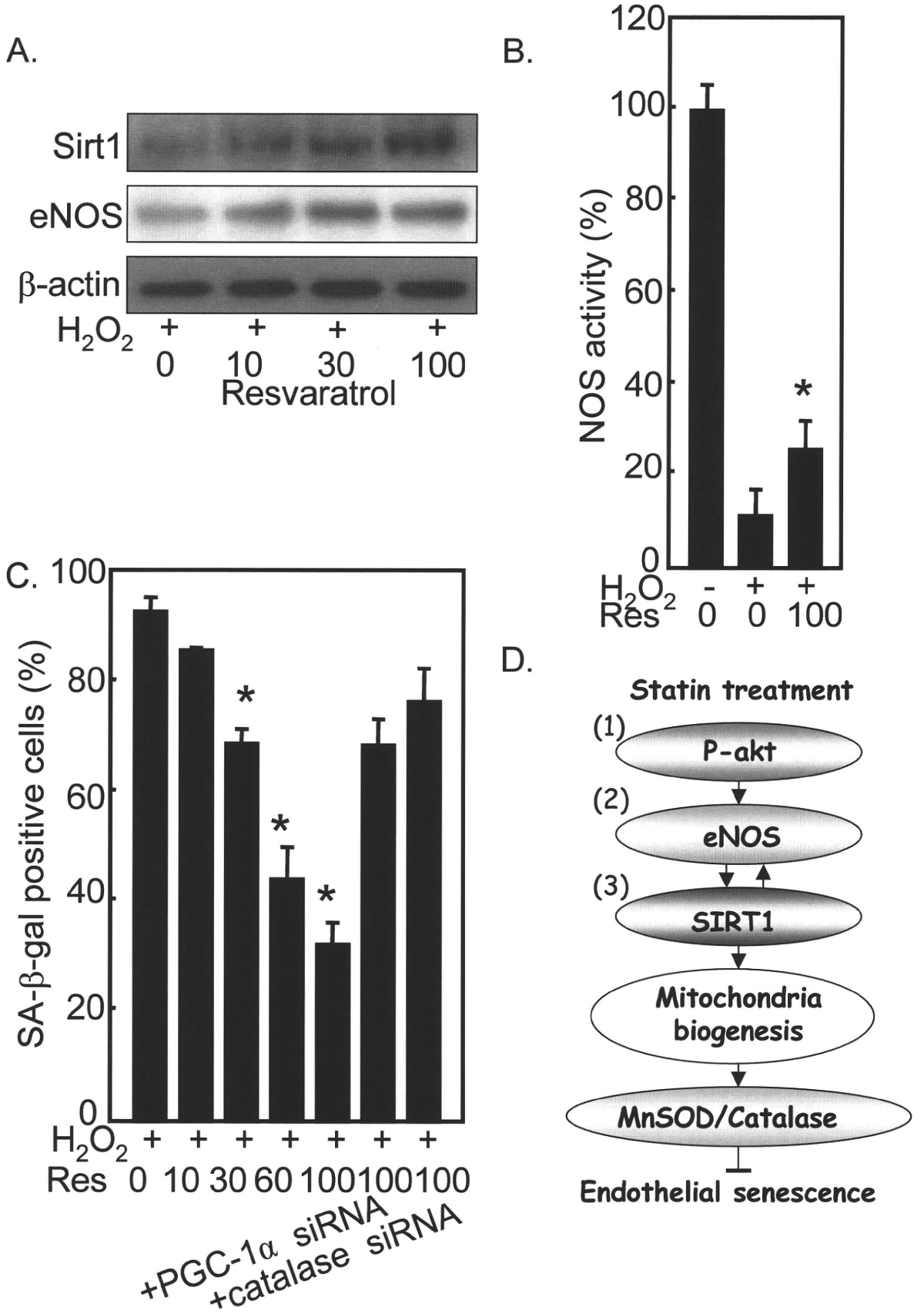
Supplementary Figure I.



Supplementary Figure II.

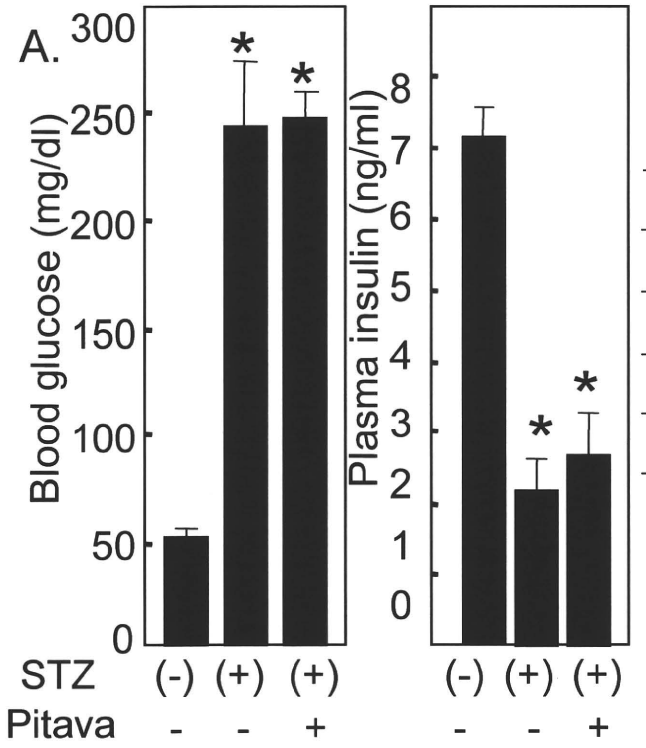


Supplementary Figure III.



Supplementary Figure IV.

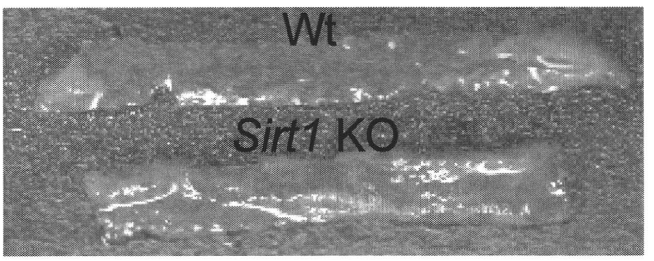
A.



B.

Mouse Group	STZ+Vehicle	STZ+Pitava
BW (g)	29.9±2.1	28.7±2.2
sBP (mmHg)	146.3±9.5	147.2±6.3
dBP (mmHg)	61.4±6.4	64.8±4.9
PR (/min)	463.1±35.5	475.5±42.6

C.



STZ (-)

Hypertension Research (2010) 33, 587–591

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ORIGINAL ARTICLE

Association of low testosterone with metabolic syndrome and its components in middle-aged Japanese men

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Epidemiological studies have shown that low testosterone is associated with metabolic syndrome (MetS) in Caucasian men. We investigated whether testosterone level is related to the prevalence of MetS in middle-aged Japanese men. A cross-sectional survey was conducted in 194 men aged 30–64 years (49 ± 9). Blood sampling was performed in the morning after a 12-h fast, and the relationship between plasma hormone and MetS was analyzed. Low total testosterone was associated with MetS according to the Japanese criteria (HRs of 2.02 by quartile of testosterone; 95% CI=1.43–2.87) and the International Diabetes Federation criteria (HRs of 1.68 by quartile of testosterone; 95% CI=1.25–2.25). Age-adjusted regression analyses revealed that testosterone was significantly related to the MetS parameters of obesity (β=−0.365 and −0.343 for waist circumference and body mass index, respectively), hypertension (β=−0.278 and −0.157 for systolic and diastolic blood pressure, respectively), dyslipidemia (β=−0.242 and 0.228 for triglycerides and high-density lipoprotein cholesterol, respectively), insulin resistance (β=−0.253 and −0.333 for fasting plasma glucose and homeostasis model assessment of insulin resistance, respectively) and adiponectin (β=0.216). Inclusion of waist circumference into the model largely weakened the association of testosterone with other metabolic risk factors. In contrast, high estradiol was associated with MetS and its parameters, mostly attributing to the positive correlation between estradiol and obesity. Dehydroepiandrosterone sulfate was not associated with MetS or its parameters. These results suggest that low testosterone is associated with MetS and its parameters in middle-aged Japanese men. The association between estradiol and MetS needs further investigation.
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Keywords: androgen; estrogen; insulin resistance; obesity; sex hormone

INTRODUCTION

There is growing awareness that metabolic syndrome (MetS) is one of the most important threats to public health because of its association with type 2 diabetes mellitus, cardiovascular disease and mortality.[1–3] In men, it is well established that endogenous androgens decline with advancing age,[4] and low testosterone levels have been associated with insulin resistance,[5] type 2 diabetes,[6,7] hypertension[8] and increased cardiovascular and all-cause mortality.[9,10] Moreover, men with low testosterone are likely to have more components of MetS in cross-sectional studies,[11–13] and longitudinal studies show that lower total testosterone predicts higher frequency of MetS.[14,15] These data were mostly from studies with Caucasian men in western countries. Regarding Japanese men, one study showed that testosterone was positively correlated with plasma adiponectin.[16] However, there are no reports showing a relationship between testosterone and MetS or its components in Japanese men.

Recently, we reported that low testosterone is an independent determinant of endothelial dysfunction in middle-aged men[17] and is a predictor of cardiovascular events in men with coronary risk factors,[18] suggesting a link between testosterone and cardiovascular pathology. Given these findings, this study investigated the relationship of endogenous testosterone with MetS in middle-aged Japanese men.

METHODS

Subjects

Enrollment screening included consecutive, apparently healthy male subjects aged 30–64 years who underwent medical examinations at either our department or at two clinics located in Tokyo. After exclusion of subjects who met the exclusion criteria,. 194 subjects (104 from our department and 90 from the clinics) were enrolled. Exclusion criteria included history of cardiovascular disease (stroke, coronary heart disease, congestive heart failure and peripheral arterial disease), malignancy or overt endocrine disease or use of steroid hormones, because these conditions may influence plasma sex hormones and/or the components of MetS. Other exclusion criteria were diabetic subjects

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on insulin injection or hypoglycemic agent drugs or with hemoglobin A1c >8%, and subjects on β-blockers[19] or fibrates. History, physical examination and laboratory tests were performed for all subjects. Of the included subjects, 23% (n=44) were taking anti-hypertensive drugs (angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, calcium channel blockers and diuretics), and 22% were taking statins. Each subject gave written, informed consent before study enrollment. The study protocol was approved by the ethics committee of the Graduate School of Medicine at the University of Tokyo.

Assays of metabolic risk factors and plasma hormones

Clinical information was collected at baseline when each patient attended the initial medical examination. Blood sampling and measurement of height, weight, waist circumference and blood pressure were performed in the morning after a 12-h overnight fast. Blood pressure was measured at least twice using an automated, digital electrosphygmomanometer (Omron Healthcare, Kyoto, Japan) on the non-dominant arm in a sitting position, and the average was used for statistical analysis.

Serum total cholesterol and triglyceride were measured enzymatically, and serum high-density lipoprotein (HDL) cholesterol was measured by the heparin-$Ca^{2+}Ni^{2+}$ precipitation method. Low-density lipoprotein cholesterol was determined using the Friedewald formula or the direct, liquid, selective detergent method when triglycerides were >400 mg per 100 ml. Plasma glucose was assayed by the glucose oxidase method, and hemoglobin A1c was measured by high-performance liquid chromatography. Plasma total testosterone, dehydroepiandrosterone sulfate and estradiol were determined using sensitive radioimmunoassays. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as fasting insulin ($\mu IU\,ml^{-1}$)×fasting plasma glucose (mg per 100 ml)/405. Patients with a fasting plasma glucose >140 mg per 100 ml were excluded from the HOMA-IR calculation because of a lack of data reliability. Serum adiponectin was measured using an enzyme-linked immunosorbent assay (Human Adiponectin ELISA kit, Otsuka Pharmaceutical, Tokyo, Japan). These assays were performed by a commercial laboratory (SRL, Tokyo, Japan). The intra-assay coefficients of variation for the measurements were <5%.

Definition of MetS

We applied both the Japanese criteria[20] and the International Diabetes Federation (IDF) criteria for Japanese ethnicity[21] for the diagnosis of MetS. In the Japanese criteria, MetS was diagnosed when waist circumference ⩾85 cm and two or more of the following three components were present: (1) HDL cholesterol <40 mg per 100 ml and/or triglyceride ⩾150 mg per 100 ml; (2) systolic blood pressure ⩾130 mm Hg and/or diastolic blood pressure ⩾85 mm Hg and (3) fasting plasma glucose ⩾110 mg per 100 ml. Subjects taking anti-hypertensive medications were considered hypertensive for statistical purposes.

In the IDF criteria for Japanese ethnicity, MetS was diagnosed when waist circumference ⩾85 cm and two or more of the following four components were present: (1) HDL cholesterol <40 mg per 100 ml; (2) triglyceride ⩾150 mg per 100 ml; (3) systolic blood pressure ⩾130 mm Hg and/or diastolic blood pressure ⩾85 mm Hg and (4) fasting plasma glucose ⩾100 mg per 100 ml. Subjects taking anti-hypertensive medications were considered hypertensive for statistical purposes.

Data analysis

Values are expressed as the mean ± s.d. in the text unless otherwise stated. Pearson's simple correlation coefficients were calculated between plasma hormones and the number of MetS components. Differences between the quartile groups of sex hormones were analyzed using one-factor ANOVA followed by the Newman–Keuls' test. Logistic regression analysis was performed to determine the association of sex hormones with the diagnosis of MetS. Furthermore, multiple regression analysis was performed to determine the association between sex hormones and metabolic risk factors for MetS. A value of $P<0.05$ was considered statistically significant. The data were analyzed using SPSS (Version 17.0, SPSS, Chicago, IL, USA).

RESULTS

Sex hormones and MetS criteria

Characteristics of the study subjects are shown in Table 1. Twenty-three and 32% of the subjects were diagnosed with MetS according to the Japanese criteria and the IDF criteria, respectively. The prevalence is comparable with that reported in middle-aged Japanese men.[22,23]

As plasma total testosterone was negatively correlated with the number of MetS components (Figure 1a), the association of testosterone with MetS was analyzed by quartile of testosterone. As shown in Figure 2a, lower testosterone was associated with a step-wise increase in the number of MetS components. Age-adjusted logistic regression analysis revealed that the hazard ratios for MetS diagnosis by quartile decline of testosterone were 2.02 (95% CI=1.43–2.87) and 1.68 (95% CI=1.25–2.25) according to the Japanese criteria and the IDF criteria, respectively.

Interestingly, plasma estradiol was positively correlated with the number of MetS components (R=0.285, $P<0.001$); therefore, the association with MetS was also analyzed by quartile of estradiol. As shown in Figure 2b, higher estradiol was associated with a step-wise increase in the number of MetS components. Age-adjusted logistic regression analysis revealed that the hazard ratios for MetS diagnosis by quartile increment of estradiol were 1.48 (95% CI=1.06–2.06) and 1.63 (95% CI=1.20–2.21) according to the Japanese criteria and the IDF criteria, respectively. Dehydroepiandrosterone sulfate was not associated with MetS components or diagnosis (data not shown).

Table 1 Characteristics of study subjects (N=194)

Age (years)	49 ± 9	[30–64]
Body mass index ($kg\,m^{-2}$)	25.2 ± 4.0	[17.3–41.9]
Waist circumference (cm)	87 ± 10	[69–125]
Hip circumference (cm)	96 ± 7	[80–125]
Waist/hip ratio	0.94 ± 0.06	[0.78–1.09]
Systolic blood pressure (mm Hg)	126 ± 14	[95–183]
Diastolic blood pressure (mm Hg)	79 ± 11	[50–128]
Triglycerides (mg per 100 ml)	162 ± 135	[32–880]
HDL cholesterol (mg per 100 ml)	54 ± 16	[26–110]
Free fatty acids ($mEq\,l^{-1}$)	0.53 ± 0.28	[0.08–2.08]
LDL cholesterol (mg per 100 ml)	128 ± 29	[54–213]
Fasting plasma glucose (mg per 100 ml)	98 ± 13	[76–158]
Hemoglobin A1c (%)	5.2 ± 0.6	[4.0–8.0]
Insulin ($\mu U\,ml^{-1}$)	6.7 ± 4.0	[1.0–21.2]
HOMA-IR	1.64 ± 1.04	[0.21–5.50]
Total testosterone ($nmol\,l^{-1}$)	19.1 ± 6.2	[4.6–38.2]
DHEA-S ($\mu mol\,l^{-1}$)	5.89 ± 2.37	[1.12–12.0]
Estradiol ($pmol\,l^{-1}$)	92.5 ± 43.7	[18.4–216.6]
Metabolic syndrome (MetS) and its components		
MetS (Japanese criteria), n (%)		44 (23)
MetS (IDF criteria), n (%)		62 (32)
Waist circumference ⩾85 cm, n (%)		110 (56)
High blood pressure, n (%)		89 (46)
HDL cholesterol <40 mg per 100 ml, n (%)		34 (18)
Triglycerides ⩾150 mg per 100 ml, n (%)		79 (41)
Fasting plasma glucose ⩾110 mg per 100 ml, n (%)		23 (12)
Fasting plasma glucose ⩾100 mg per 100 ml, n (%)		73 (38)

Abbreviations: DHEA-S, dehydroepiandrosterone sulfate; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; IDF, International Diabetes Federation; LDL, low-density lipoprotein.
Values are expressed as the mean ± s.d. (range). High blood pressure was defined if subjects showed systolic blood pressure ⩾130 mm Hg and/or diastolic blood pressure ⩾85 mm Hg, or were taking antihypertensive medications.

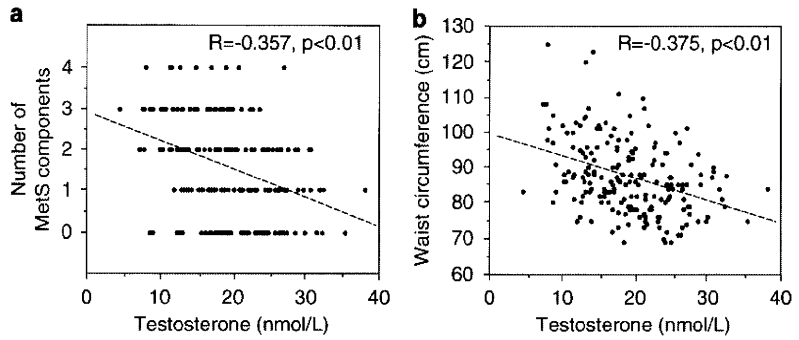

Figure 1 Scattergrams and regression lines (dotted lines) showing the correlation between testosterone and the number of metabolic syndrome (MetS) components (**a**) or waist circumference (**b**).

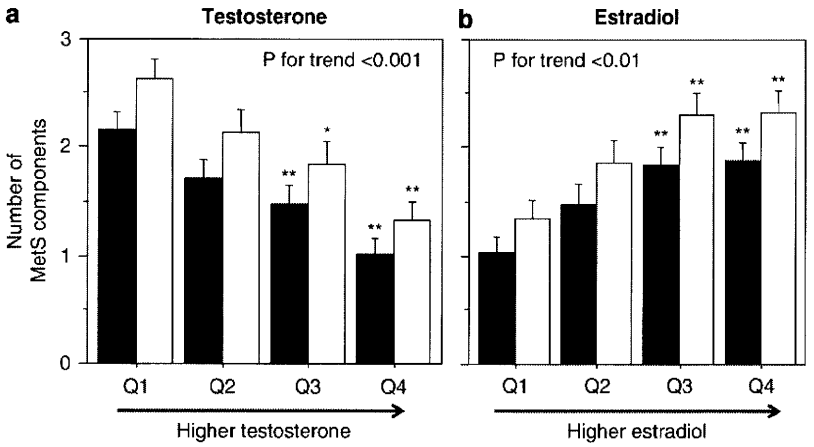

Figure 2 Number of metabolic syndrome (MetS) components according to quartiles of plasma testosterone (**a**) and estradiol (**b**). MetS components were defined according to the Japanese criteria (closed bars) and the IDF criteria for Japanese ethnicity (open bars). Values are expressed as the mean ± s.e.m. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* Q1. Cut offs of the quartiles were 14.1, 18.7 and 23.4 nmol l^{-1} (405, 540 and 674 ng per 100 ml) for testosterone, and 55, 101 and 125 pmol l^{-1} (15.0, 27.5 and 34.0 pg ml^{-1}) for estradiol.

Sex hormones and metabolic risk factors

The associations of plasma sex hormones with each of the metabolic risk factors were analyzed. As shown in Table 2, the unadjusted model shows that testosterone was significantly related to parameters of MetS except for diastolic blood pressure. Testosterone was not related to low-density lipoprotein cholesterol, but this parameter is not included in the definitions of MetS used here. Adjustment for age did not considerably influence the results of the regression analysis, but the association between testosterone and diastolic blood pressure became significant after adjustment for age. In contrast, inclusion of waist circumference into the model weakened the association of testosterone with metabolic risk factors. As a result, systolic blood pressure, triglycerides, fasting plasma glucose and HOMA-IR were significantly related to testosterone. The significant association for diastolic blood pressure, HDL cholesterol, free fatty acids, hemoglobin A1c, insulin and adiponectin were attenuated after adjustment for age and waist circumference. Adjustment for body mass index or waist/hip ratio instead of waist circumference showed similar results (data not shown).

As shown in Table 3, estradiol showed weaker association than testosterone with parameters of MetS, but was significantly related to body mass index, waist circumference, systolic blood pressure, HDL

Table 2 Multiple regression analysis determining the impact of plasma testosterone on metabolic risk factors

	Unadjusted	*Age adjusted*	*Age+waist adjusted*
Body mass index	−0.376*	−0.343*	ND
Waist circumference	−0.378*	−0.365*	ND
Waist/hip ratio	−0.353*	−0.384*	ND
Systolic blood pressure	−0.230**	−0.278*	−0.169***
Diastolic blood pressure	−0.114	−0.157***	−0.098
Triglycerides	−0.247*	−0.242*	−0.182***
HDL cholesterol	0.252*	0.228**	0.065
Free fatty acids	−0.208**	−0.209**	−0.137
LDL cholesterol	−0.054	−0.056	−0.020
Fasting plasma glucose	−0.231**	−0.253**	−0.228**
Hemoglobin A1c	−0.166***	−0.220**	−0.137
Insulin	−0.331*	−0.307*	−0.129
HOMA-IR	−0.349*	−0.333*	−0.159***
Adiponectin	0.222**	0.216**	0.046

Abbreviations: HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein; ND, not determined.
Regression coefficients with plasma testosterone as an independent variable and each of risk factors as a dependent variable are shown. Age and/or waist circumference were included in multiple regression models as indicated. $^{*}P<0.001$, $^{**}P<0.01$, $^{***}P<0.05$.

Table 3 Multiple regression analysis determining the impact of plasma estradiol on metabolic risk factors

	Unadjusted	*Age adjusted*	*Age+waist adjusted*
Body mass index	0.279*	0.260*	ND
Waist circumference	0.346*	0.338*	ND
Waist/hip ratio	0.102	0.082	ND
Systolic blood pressure	0.133	0.158**	0.042
Diastolic blood pressure	0.036	0.058	−0.002
Triglycerides	0.105	0.094	−0.012
HDL cholesterol	−0.207***	−0.193***	−0.040
Free fatty acids	0.087	0.091	0.049
LDL cholesterol	−0.056	−0.056	−0.094
Fasting plasma glucose	0.130	0.141	0.095
Hemoglobin A1c	0.040	0.067	−0.030
Insulin	0.240***	0.228***	0.038
HOMA-IR	0.250***	0.243***	0.060
Adiponectin	−0.267*	−0.262*	−0.114

Abbreviations: HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein; ND, not determined.
Regression coefficients with plasma estradiol as an independent variable and each of risk factors as a dependent variable are shown. Age and/or waist circumference were included in multiple regression models as indicated. *$P<0.001$, **$P<0.05$, ***$P<0.01$.

cholesterol, insulin, HOMA-IR and adiponectin after adjustment for age. Further adjustment for waist circumference, body mass index or waist/hip ratio (Table 3 and data not shown) eliminated the significant associations between estradiol and these metabolic parameters. Dehydroepiandrosterone sulfate was not significantly related to parameters of MetS in unadjusted or adjusted analyses (data not shown).

DISCUSSION

In this study, cross-sectional analysis of 194 middle-aged Japanese men showed that low testosterone is positively related to MetS, MetS components and additional metabolic risk factors. Adjustment for obesity parameters such as waist circumference, body mass index and waist/hip ratio greatly diminished the association, but low testosterone retained weak associations with some metabolic risk factors including systolic blood pressure, triglycerides, fasting plasma glucose and HOMA-IR. Taken together, results in this statistical model suggest that abdominal obesity is an important contributor to the association between low testosterone and MetS, but additional factors may also impact testosterone. To our knowledge, this is the first report showing the significant association between low testosterone and MetS in Japanese men.

Several mechanisms have been suggested for the causal relationship between low testosterone and abdominal obesity. Activation of the lipoprotein lipase and lipolysis[24] may explain the effect of testosterone on adipose tissue. Many studies including a medium-sized, randomized controlled trial[25] and a meta-analysis[26] showed the inverse effect of testosterone on adiposity. Conversely, it has been reported that men with MetS are prone to hypogonadism.[27] This finding might be due to elevated leptin levels that interfere with gonadotropin-stimulated androgen production[28] and to increased aromatase activity in adipose tissue that leads to higher circulating estradiol and suppression of testosterone production by negative feedback.[29] These findings suggest a bi-directional causal relationship between low testosterone and obesity.

After adjustment for waist circumference, testosterone was weakly but significantly related to some metabolic risk factors including systolic blood pressure, triglycerides, fasting plasma glucose and HOMA-IR, which is consistent with earlier reports.[5,6,8,12] Testosterone is likely to be involved in the pathogenesis of MetS, irrespective of obesity. For example, testosterone increases the hepatic production of apolipoprotein A-1 and consequently increases HDL cholesterol,[30] improves insulin sensitivity and increases muscle strength.[31] There was no significant correlation between age and testosterone ($R=0.114$, $P=0.12$). This result may be because the cohort was limited to middle-aged men (30–64 years old) . However, age was included in the multivariate analyses in this study, because it is well established that testosterone declines with age.[4]

The positive association found between testosterone and adiponectin is in agreement with earlier reports.[16,32,33] However, the direct action of testosterone on adiponectin production/secretion might be different from these findings, because testosterone decreases adiponectin secretion in mice and in adipocytes.[34,35] Accordingly, abdominal obesity may underlie the positive correlation between testosterone and adiponectin in men.

In this study, estradiol was associated positively with MetS and its components, consistent with an earlier report.[12] This relationship may be independent of testosterone because estradiol was not correlated with testosterone by simple regression analysis ($R=-0.019$, $P=0.80$), and the inclusion of both testosterone and estradiol into the multiple regression model as covariates did not influence the association of each other with MetS parameters (data not shown). The relationship between estradiol and MetS might be attributed to increased aromatase activity and subsequent elevation of circulating estradiol in obese subjects.[29] Increased estradiol may subsequently suppress pituitary function,[29] and lead to a further decrease in testosterone. Comprehensive assessment of sex hormone, gonadotropin and components of MetS reveal a causal relationship. Unfortunately, we could not measure gonadotropin because of limited plasma. Further investigation is needed to address the mechanistic and pathophysiological interactions between sex hormones and MetS.

There are some limitations to our study. First, the cross-sectional design does not clarify the causal relationship between sex hormones and MetS. As there may be bi-directional causalities as mentioned above, longitudinal follow-up studies and hormone replacement studies should be performed in Japanese populations. Second, active forms of testosterone such as bioavailable and calculated free testosterone were not measured. A direct assay of bioavailable testosterone or of sex hormone-binding globulin (required for free testosterone calculation) was not available for the study. Third, the potential influence of medications on the measured parameters cannot be denied, although the exclusion of subjects on statins ($n=40$) or anti-hypertensive drugs ($n=44$) did not seriously affect the association of testosterone with waist circumference (statins, $R=-0.304$, $P<0.01$; anti-hypertensives, $R=-0.337$, $P<0.01$) and the number of MetS components (statins, $R=-0.274$, $P<0.01$; anti-hypertensives, $R=-0.278$, $P<0.01$). Fourth, because the sample size ($n=194$) is relatively small, the finding needs to be confirmed in a larger cohort.

In summary, this study suggests that low testosterone is associated with MetS and its parameters in middle-aged Japanese men. We also found a positive but weaker association between estradiol and MetS. These associations were largely attenuated by adjustment for waist circumference. Our results reinforce the need to address the causal relationship and pathophysiological interactions between sex hormones and MetS.

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Review

SIRT1/eNOS Axis as a Potential Target against Vascular Senescence, Dysfunction and Atherosclerosis

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Sir2 (silent information regulator-2), an NAD$^+$-dependent histone deacetylase, is highly conserved in organisms ranging from archaea to humans. Yeast Sir2 is responsible for silencing at repeated DNA sequences in mating-type loci, telomeres and rDNA, and plays critical roles in DNA repair, stress resistance and longevity.

The phenomenon of human aging is known to be a critical cardiovascular risk factor. Senescence of endothelial cells has been proposed to be involved in vascular dysfunction and atherogenesis. Recent studies have demonstrated that mammalian *Sirt1* NAD$^+$-dependent protein deacetylase, the closest homologue of Sir2, regulates vascular angiogenesis, homeostasis and senescence. This review focuses on SIRT1 as a potential therapeutic target against atherosclerosis.

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Key words; SIRT1, Vascular senescence, Dysfunction, Atherosclerosis

Introduction

Recent studies demonstrate that cellular senescence is involved in various pathological conditions, such as atherosclerosis. In this review, we discuss the potential protective effect of SIRT1 on vascular endothelial cells.

Sirtuins

During the last decade, aging research has progressed through the use of lower organism models, such as the budding yeast *Saccharomyces cerevisiae* and the nematode *Caenorhabditis elegans*. In *S. cerevisiae*, the Sir2 (silent information regulator-2) family of genes governs budding exhaustion and replicative life span[1, 2]; deletions of Sir2 shorten life span and an extra copy of this gene increases life span. In addition to promoting longevity, the activity of Sir2 is enhanced by caloric restriction (CR), which extends life span in diverse species. Sir2 has been identified as an NAD$^+$-dependent histone deacetylase and is responsible for maintenance of chromatin silencing and genome stability[3]. Sir2 genes are conserved during evolution, and seven homologs of sirtuins (*Sirt1-7*) have been cloned in mammals. Mammalian sirtuins have diverse cellular localizations, modify multiple substrates, and affect cellular functions. SIRT1 is localized in the cytoplasm and nucleus, and SIRT6 and SIRT7 are localized in the nucleus. SIRT3, SIRT4 and SIRT5 reside in the mitochondria and SIRT2 is localized in the cytoplasm. SIRT1, SIRT2, SIRT3 and SIRT5 are NAD$^+$-dependent deacetylases, whereas SIRT4 and SIRT6 are primarily mono-ADP-ribosyl transferases.

SIRT1, the closest homologue of Sir2, targets a wide range of transcriptional regulators, including p53, PML (promyelocytic leukemia protein), FoxO (forkhead box O), NF-κB (nuclear factor κB) and PPAR-γ (peroxisome proliferators-activated receptor-γ)[4-8]. Like yeast Sir2, SIRT1 regulates the cell cycle, senescence, apoptosis and metabolism, and might act as a longevity factor in mammals.

Endothelial Senescence Induces Vascular Dysfunction and Atherosclerosis

The phenomenon of human aging is known to

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be a critical cardiovascular risk factor. Minamino *et al.* proposed that the senescence of endothelial cells is involved in endothelial dysfunction and atherogenesis[9]. Histological study of human atherosclerotic lesions has demonstrated the existence of vascular cells that exhibit the morphological features of senescence[10].

Moreover, it has been reported that angiogenesis becomes impaired with advancing age[11] and that aging reduces the antithrombogenic properties of the endothelium[12]. These senescent changes of vascular structure and function have been suggested to result in the increased risk of atherosclerotic cardiovascular disease in the elderly.

According to the free-radical theory, reactive oxygen species (ROS) may be potential candidates responsible for vascular dysfunction and atherosclerosis[13], and upon the production of high levels of ROS, the redox balance is disturbed and cells shift into a state of oxidative stress, which subsequently leads to endothelial dysfunction and senescence with shortening of telomeres[14]. Endothelial NO synthase (eNOS) activity is reduced in human senescent endothelial cells, accompanied by a reduction of nitric oxide (NO) production. Endothelial-derived NO regulates vascular relaxation and has athero-protective effects[15]. Intriguingly, endothelial NO can protect against a state of oxidative stress, and activation of eNOS and subsequent production of NO delay endothelial cellular senescence[16, 17].

SIRT1 Plays a Critical Role in Endothelial Homeostasis

SIRT1 likely plays a critical role in endothelial homeostasis by regulating endothelial nitric oxide synthase (eNOS). A recent study showed that levels of cGMP and eNOS are elevated in tissues of calorie-restricted mice, and production of NO by CR increases SIRT1 expression. The induction of SIRT1 expression is blunted in eNOS-deficient mice, and eNOS has been implicated in regulation of the expression of SIRT1[18].

It has been reported that SIRT1 promotes endothelial-dependent vasodilation by targeting eNOS for deacetylation, leading to enhanced NO production[19]. Intriguingly, SIRT1 has been shown to directly bind to eNOS, which is deacetylated at lysines 496 and 506 in the calmodulin-binding domain and posttranscriptionally leads to activation of eNOS. Inhibition of SIRT1 by a deacetylase-defective mutant SIRT1 decreases NO bioavailability and inhibits endothelium-dependent vasorelaxation. Consistent with these results, we showed that SIRT1 inhibition by sirtinol or RNAi-mediated knock down induced a premature senescent phenotype in human endothelial cells. Conversely, overexpression of SIRT1 prevented oxidative stress-induced endothelial senescence[20]. Intriguingly, a micro RNA (miR-217) was recently identified and miR-217 induced endothelial senescence through direct inhibition of SIRT1[21].

In addition to endothelial protection, SIRT1 regulates the angiogenic activity of endothelial cells. It has been reported by Potente *et al.* that SIRT1 deacetylase activity plays a critical role in the angiogenesis of endothelial cells[22]. Knockdown of SIRT1, but not SIRT2-7, was uniquely associated with loss of sprouting angiogenesis *in vitro*. Moreover, *Sirt1* mutant mice, which have genetic deletion of SIRT1 activity in the endothelium postnatally, have impaired formation of new vessels in response to angiogenic signals such as ischemic stress.

Cilostazol Inhibits Oxidative Stress-Induced Premature Senescence via Up-Regulation of SIRT1 in Human Endothelial Cells

A PDE3 inhibitor, cilostazol, is used as a vasodilating anti-platelet drug for treating intermittent claudication, and in preclinical studies was shown to have a protective effect on endothelial cells by increasing eNOS activity[23]. Cilostazol increases intracellular cAMP content accordingly and activates protein kinase A (PKA) and PI3K/Akt signaling[24]. We found that treatment with cilostazol inhibited the senescent phenotype. Cilostazol increased eNOS activity, expression of eNOS and the phosphorylation of eNOS at Ser^{1177} in parallel with the phosphrylation of Akt at Ser^{473}. These results suggest that the protective effect against a senescent phenotype may be attributable to an increase in NO via eNOS activation by cilostazol[25].

To explore the mechanism by which cilostazol prevents endothelial senescence, we considered that an increase in NO production could promote the longevity gene, SIRT1. We found that cilostazol significantly increased SIRT1 mRNA and protein in a concentration-dependent manner. In contrast, SIRT1 inhibition abrogated the effect of cilostazol on specific senescent changes. Although NO is known to be involved in reducing oxidative stress and the progression of atherosclerosis, we suggest that the NO-mediated prevention of senescence is attributable to SIRT1 function (**Fig. 1**). These findings implicate the eNOS-NO-SIRT1 axis as one of the fundamental determinants of endothelial senescence, and the role of SIRT1 as a driver of cellular stress resistance and longevity is note-

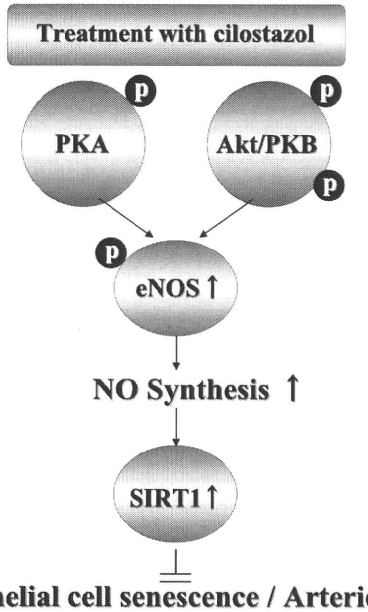

Fig. 1. Signaling pathway of inhibition of endothelial senescence, dysfunction and atherosclerosis by cilostazol treatment.

worthy in the context of its expression profile (**Fig. 2**).

In addition to these results, we found that drugs utilized for drug-eluting stents (DES), including paclitaxel and limus family members (e.g. sirolimus, everolimus), inhibit the growth of endothelial cells and lead to endothelial senescence caused by delayed re-endothelialization[26]. We showed that the development of endothelial senescence induced by sirolimus and everolimus is SIRT1-dependent, whereas paclitaxel acts through a SIRT1-independent pathway. Because the effects of sirolimus and everolimus involve SIRT1 modulation, cilostazol reverses sirolimus- or everolimus-induced senescence. Our results could have the interesting clinical implication that triple anti-platelet therapy may have more beneficial effects on endothelial senescence than standard dual therapy with sirolimus- or everolimus-eluting stents.

Activation of SIRT1, a Potential Therapeutic Target against Atherosclerosis

CR extends life span in diverse species. A recent study by Colman *et al.* at Wisconsin National Primate Center (WNPRC) reported that calorie-restricted Rhesus macaques showed a lower incidence of age-related diseases, such as cancer, diabetes, and cardiovascular disease, and lower age-related mortality[27]. Resveratrol, a CR mimetic, is a polyphenolic activator

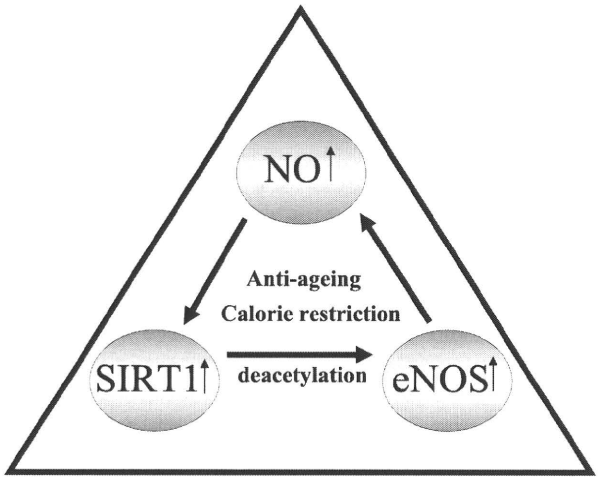

Fig. 2. Possible mechanisms of prevention of vascular senescence, dysfunction and atherosclerosis.

of SIRT1. Resveratrol increases mitochondrial biogenesis in endothelial cells via the activation of eNOS and SIRT1[28]. Resveratrol has also been shown to increase the expression of eNOS, and a combination of resveratrol with an HMG-CoA reductase inhibitor (statin) increased the activation of eNOS, resulting in increased functional recovery in a model of acute myocardial infarction[29]. Therefore, we suggest that increased NO bioavailability by other pharmaceutical products, such as statins, or agents with phytoestrogenic properties, such as resveratrol, may exert a protective effect against endothelial senescence via up-regulation of eNOS and SIRT1, and this possibility deserves further investigation. Our results and the findings by other laboratories indicate that micromolar levels of resveratrol are sufficient to exert vasculoprotective effects[26, 30]. Considering that each gram of fresh grape skin contains 50-100 μg resveratrol and it is found mainly in high-quality red wine at a concentration of 20 to 60 μmol/L as previously reported[31], it becomes apparent that effective concentrations are unlikely to be reached in plasma *in vivo*. However, resveratrol is a lipophilic substance, and exhibits higher bioavailability and slower clearance, and has been shown to accumulate in tissues such as the heart, liver, and kidney[32]. By daily consumption of grapes, berries, red wine or dietary supplements containing resveratrol, an effective concentration of resveratrol may be achievable *in vivo*. Recently, novel small molecule activators of Sirt1 (SRTs), even 1000-fold more potent than resveratol, have been identified (Sirtris Pharmaceuticals Inc., Boston, USA)[33]. SRTs induce many of the beneficial metabolic changes observed with CR/resveratrol treat-

ment[34]. We propose that using these chemical agents to activate SIRT1 may be a new attractive therapy for protection against vascular senescence, dysfunction and atherosclerosis.

Conclusions

SIRT1 is likely to play an important role in the prevention of human cardiovascular disease, including atherosclerosis. There is some evidence that SIRT1 interacts with the vascular eNOS/NO system. Just as the French paradox stands out as an excellent example of a reduced incidence of cardiovascular disease, activation of SIRT1 may have a beneficial effect on vascular senescence, dysfunction and atherosclerosis.

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Aortic arch calcification detectable on chest X-ray is a strong independent predictor of cardiovascular events beyond traditional risk factors

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ABSTRACT

Objectives: Arterial calcification makes the management of hemodynamics more difficult. Some reports have previously shown that simple assessment of aortic calcification using plain radiography is associated with cardiovascular (CV) events; however, these studies simply assessed whether aortic calcification was present or absent only, without considering its extent. Here, we evaluated validity of grading aortic arch calcification (AAC) to predict new CV events.

Methods and results: We retrospectively reviewed chest X-rays in 239 asymptomatic out-patients who underwent measurement of endothelial function at the 1994–2000 without past history of CV events. The extent of AAC was divided into four grades (0–3). Among these subjects, the follow-up of CV events in 209 patients was completed. At baseline, AAC grade was positively related to age, pulse pressure, diabetes and renal dysfunction. Impairment of endothelial function, as determined by flow-mediated dilation (FMD), was also correlated to increasing AAC grade. Fifty-seven CV events in total occurred during a mean follow-up period of 69 ± 45 months. With multivariate adjustment, Kaplan–Meier analysis showed that the incidence was significantly higher in patients with higher AAC grade (grades 2 and 3) than in those with grade 0 or 1 ($p < 0.01$, log-rank test). Two kinds of multivariate Cox-proportional hazards analyses showed the predictive values of AAC grade were significant (hazard ratio, 2.49; $p = 0.01$, 2.56; $p < 0.01$, respectively), and the predictive power was superior to that of renal dysfunction or FMD. In addition, the prediction was valuable even in patients without CKD.

Conclusions: AAC detectable on chest X-ray is a strong independent predictor of CV events beyond traditional risk factors including endothelial dysfunction. Risk stratification by assessment of AAC may provide important information for management of atherosclerotic disease.

1. Introduction

Atherosclerotic vascular damage manifests as two kinds of features, namely atherosis and sclerosis, leading to complications of several ischemic diseases [1,2]. These pathological conditions result in arterial wall thickening (localized morphological changes) and arterial stiffening (functional changes), respectively [3]. Especially, arterial wall stiffness resulting from calcification makes the management of hemodynamics more difficult in the elderly. Ectopic calcium deposition in the aorta and arteries contributes to inappropriate blood pressure (BP) conditions, such as variable BP, isolated systolic hypertension and orthostatic hypotension [3,4], and increases after-load, leading to left ventricular hypertrophy (LVH) [5]. In addition, loss of elastic recoil due to arterial calcification results in unstable hemodynamic consequences, finally leading to a decline in end-organ perfusion and subsequent cardiovascular (CV) events [3]. Eventually, organ damage leads to a decline in quality of life and long-term outcome.

Arterial calcification is anatomically separated into two types, medial and intimal calcification [6,7]. Medial calcification, which is frequently seen in the elderly [8], diabetes [9] and chronic renal failure [10], is observed as continuous linear deposits along the internal elastic lamina. On the other hand, intimal calcification, which is seen as patchy scattered deposits occurring within atherosclerotic plaques, is associated with plaque vulnerability [11]. In patients with advanced atherosclerosis, these calcified lesions result from overlapping pathological mechanisms. However, it is difficult to distinguish these calcific changes in the arterial wall solely by radiographic techniques without a pathologic approach.

Several imaging examinations have been employed to detect and quantify arterial calcification in routine clinical work. Recently, high-tech non-invasive examinations, such as electron beam-

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