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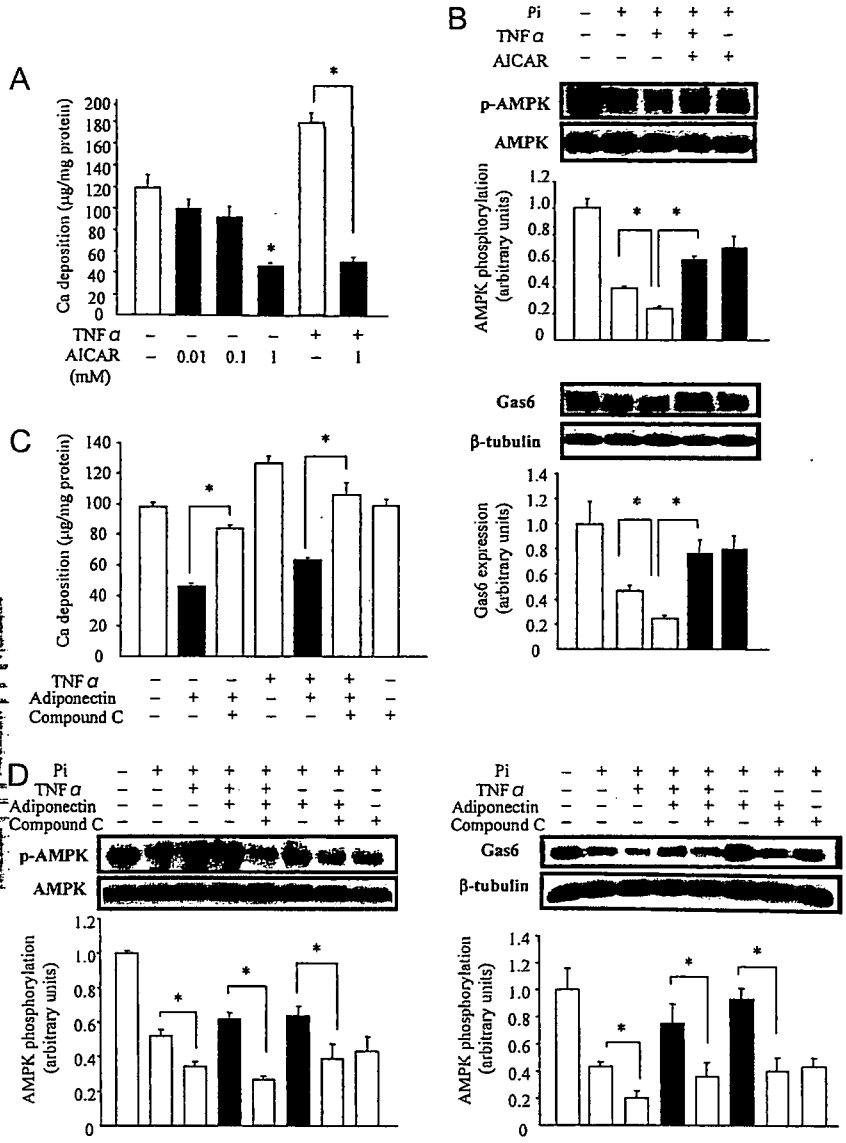

FIG. 5. AMPK plays an important role in Pi-induced calcification. HASMC were treated with or without AICAR (1 mM), a pharmacological activator of AMPK and TNFα (20 ng/ml) in calcification medium for 6 d. A and B, Ca deposition (n = 6) (A) was measured, and immunoblotting with antibodies to p-AMPK, AMPK, Gas6, and β-tubulin (B) was performed (n = 3). HASMC were cultured with or without compound C (1 μM), a chemical inhibitor of AMPK, adiponectin (300 ng/ml), and TNFα (20 ng/ml) in calcification medium for 6 d. C and D, Ca deposition (C) was evaluated (n = 6) and immunoblotting with antibodies to p-AMPK, AMPK, Gas6, and β-tubulin (D) was performed (n = 3). All values are presented as mean ± SE. * $P < 0.05$ by Bonferroni test. Each experiment was performed in triplicate for each condition.

AMPK plays a critical role in VSMC calcification and is regulated by adiponectin and TNFα

It has been reported that AMPK is a central signaling molecule in adiponectin's action (19, 20). We investigated whether AMPK is involved in the effect of adiponectin on Pi-induced calcification. First, we examined the activity of AMPK during calcification. Immunoblot analysis showed that phosphorylated AMPK was markedly down-regulated in the presence of Pi for 10 d, whereas the expression of total AMPK was not changed (Fig. 4A). TNFα further inhibited its phosphorylation in the presence of Pi, without changing total AMPK (Fig. 4B). In the case of adiponectin, AMPK phosphorylation was remarkably stimulated in a time-dependent manner (Fig. 4C). As shown in Fig. 4D, adiponectin further restored AMPK phosphorylation that was inhibited by Pi and TNFα in a calcification-promoting condition.

To clarify the causal relationship between AMPK and calcification, we tried to activate AMPK by treatment with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) (25). In HASMC, AICAR significantly inhibited Ca deposition in a concentration-dependent manner (Fig. 5A). In addition, TNFα-stimulated Ca deposition was also blunted by AICAR. Interestingly, AICAR restored Gas6 expression down-regulated by Pi and TNFα (Fig. 5B). Next, to investigate whether the effect of adiponectin is dependent on AMPK, we tried to block AMPK using compound C, a chemical inhibitor of AMPK. As shown in Fig. 5C, compound C clearly abrogated the inhibitory effect of adiponectin both on Pi- and TNFα-induced calcification. The increase in Gas6 expression as well as AMPK phosphorylation by adiponectin was also abolished by compound C (Fig. 5D). These results suggest that AMPK regulates Gas6 expression, followed by regulation of Ca deposition in HASMC.

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Transcription activity of Gas6 is regulated by adiponectin and TNFα via AMPK

To investigate whether Gas6 expression is transcriptionally regulated by adiponectin, TNFα, and AMPK, a promoter study was undertaken. Reporter assay using the −1.9-kb Gas6-luciferase DNA construct revealed that adiponectin completely reversed the down-regulation of Gas6 transcription activity by TNFα. Furthermore, compound C abrogated the effect of adiponectin on Gas6 transcription activity, indicating that adiponectin and TNFα regulate Gas6 expression at the transcription level via AMPK activity (Fig. 6). F6

Discussion

The present study showed that adiponectin has a protective effect against Pi-induced calcification and, furthermore, has an antagonistic effect on TNFα-augmented calcification. Based on our previous finding that Pi-induced calcification is dependent on apoptotic cell death in HASMC, we examined the role of adiponectin and TNFα in Pi-induced apoptosis. As expected, we found that adiponectin had an inhibitory effect and TNFα had a stimulatory effect on Pi-induced apoptosis. This study also demonstrated the regulation of Gas6 expression by TNFα and adiponectin, a suppressive effect and a promoting effect, respectively, at the transcriptional level. Akt, a critical downstream effecter of Gas6, was activated by adiponectin, whereas TNFα had an opposite action on its phosphorylation. Given that adiponectin and TNFα did not affect Gas6 expression and Akt phosphorylation in the absence of Pi (data not shown), the effects of adiponectin and TNFα on these molecules may dependent on Pi-induced responses. These results suggest that Gas6 is the target of adiponectin and TNFα in regulating Pi-induced apoptosis, accompanied by modulation of the Akt-dependent survival pathway.

As reported previously (6), Pi-induced VSMC calcification is associated with both phenotypic transition to osteoblastic cells via sodium-dependent phosphate cotransporter and apoptotic cell death. In our preliminary experiments, the expression of osteopontin, an osteoblastic marker, was not affected by TNFα and adiponectin (data not shown). Although this result suggests little influence of TNFα and adiponectin on osteoblastic differentiation of VSMC, extensive and systematic investigation including other markers of osteoblastic differentiation is needed to conclude this issue.

Multiple lines of clinical evidence show that adiponectin has protective actions on the cardiovascular system (26, 27). Circulating levels of adiponectin in humans are as high as 500–30,000 μg/ml (28). Therefore, the concentration of adiponectin (300 ng/ml) used in this study are within physiological levels. Especially, consistent with our findings, adiponectin has been implicated in apoptosis of cardiovascular cells (19, 23, 29). Adiponectin inhibits apoptosis in cardiac myocytes and fibroblasts that are exposed to hypoxia-reoxygenation stress (19). In endothelial cells, adiponectin has been reported to inhibit serum starvation-induced apoptosis (23). *In vivo* experiments have also shown that adiponectin-deficient mice develop larger myocardial infarcts due to increased myocardial cell apoptosis and TNFα expression (17). Taking these observations together with our results, the antiapoptotic actions of adiponectin contribute to the inhibition of VSMC calcification.

Most effects of adiponectin have been attributed to the activation of AMPK, which affects many aspects of cellular metabolism including glucose uptake (30, 31), glucose utilization (32), and fatty acid oxidation (33, 34). Recently, AMPK activation in VSMC has been suggested as a target to prevent or treat vascular disease (35, 36). AICAR-induced AMPK activation inhibited angiotensin II-stimulated VSMC proliferation, and administration of AICAR prevented neointimal formation in a rat balloon injury model (35). AMPK activation in VSMC elicited cell cycle arrest at the G1 phase and inhibited cell proliferation via p53 up-regulation (36). Furthermore, in the heart, the inhibitory effects of adiponectin on ischemic injury-induced apoptosis have been shown to be dependent on AMPK activation (19). The results of *in vitro* studies also revealed that AMPK signaling is essential for the antiapoptotic activities of adiponectin on endothelial cells (23). These observations are consistent with the finding of the present study that AMPK activated by adiponectin stimulated Gas6 expression to restore the survival pathway, leading to the suppression of calcification.

In the present study, we further demonstrated that adiponectin significantly augmented the transcriptional activity of Gas6 that was decreased by TNFα. Indeed, suppression of AMPK by compound C clearly abrogated this beneficial effect of adiponectin. This result suggests that AMPK participates in the transcriptional regulation of Gas6 by adiponectin and TNFα. Several studies support that AMPK regulates the expression of particular genes at the transcriptional level (37–39). For example, AMPK activation by AICAR enhanced activation protein 1-mediated proopiomelanocortin promoter activities, which were completely abolished by compound C (37). AMPK has been shown to mediate the transcription signal that leads to the repression of phosphoenolpyruvate carboxykinase expression, a key enzyme of gluconeogenesis, through phosphorylation of AQ: D

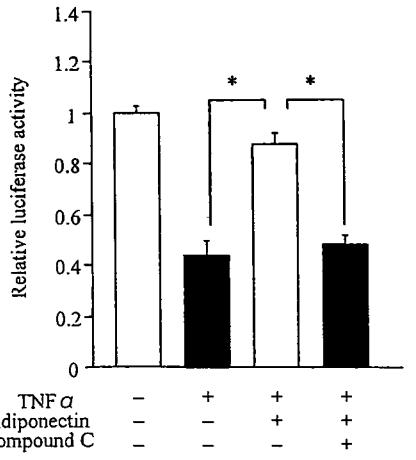

FIG. 6. Effect of adiponectin and TNFα on Gas6 promoter activity. HASMC were transfected with the Gas6 promoter-luciferase construct using lipofectamine 2000. Twenty-four hours after transfection, adiponectin (300 ng/ml), compound C (1 μM), and TNFα (20 ng/ml) were added. Cells were incubated for an additional 44 h. Luciferase activity was normalized to that of vehicle-treated cells. All values are presented as mean ± SE (n = 4). *, $P < 0.05$ by Bonferroni test. Each experiment was performed in triplicate for each condition.

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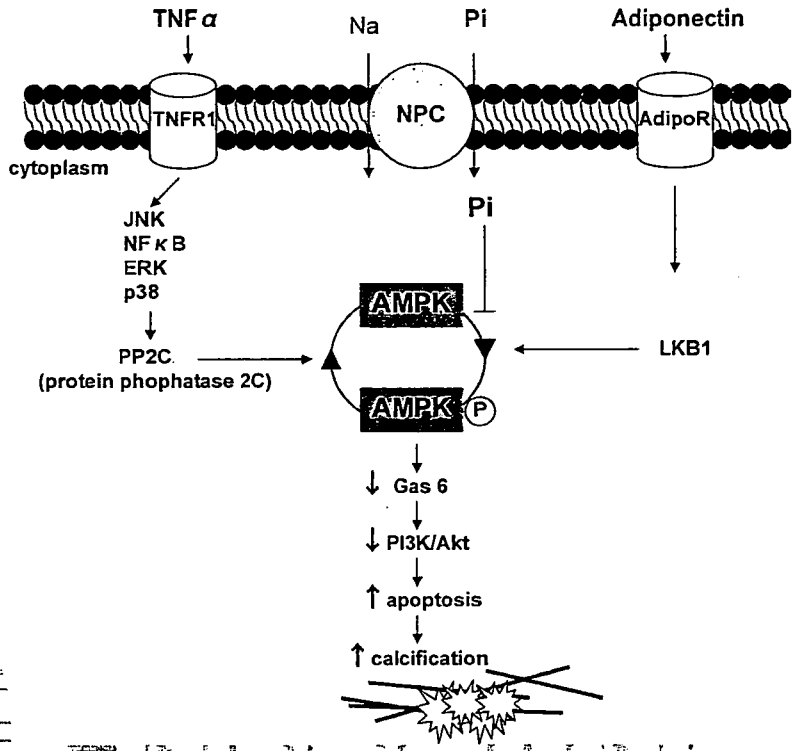
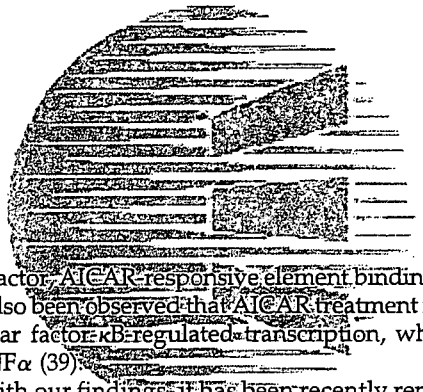


FIG. 7. Scheme of the effect of TNFα and adiponectin on Pi-induced calcification. In HASMC, exogenous Pi is internalized by sodium-dependent phosphate cotransporter (NPC, such as Pit-1) and inhibits AMPK phosphorylation, followed by down-regulation of the Gas6-mediated survival pathway. This pathway stimulates apoptosis, leading to subsequent development of calcification. TNFα directly suppresses AMPK activation by promoting PP2C activation via TNF receptor-1 (TNFR1). On the other hand, adiponectin activates LKB1-AMPK pathway via adiponectin receptors (AdipoR). AMPK activation modulated by TNFα and adiponectin contributes to the regulation of Pi-induced calcification.



a transcription factor-AICAR-responsive element binding protein (38). It has also been observed that AICAR treatment is able AQ: E to reduce nuclear factor-κB-regulated transcription, which is activated by TNFα (39).

Consistent with our findings, it has been recently reported that TNFα directly suppresses AMPK activation by promoting protein phosphatase 2C (PP2C) activity via TNF receptor-1 (40). PP2C has been proposed as one of modulators of the covalent regulation of AMPK (41). Increased PP2C levels account for the reduced AMPK activity and phosphorylation AQ: F after TNFα treatment (40). On the other hand, LKB1 is the well-known, principal upstream kinase of AMPK (42, 43) that is regulated by adiponectin (44). AMPK activation by adiponectin is considered to be mediated by the cell surface receptors adiponectin receptors 1 and 2 (45). Another adiponectin receptor, T-cadherin, has recently been identified (46). In preliminary experiments, we found that all of the three adiponectin receptors were endogenously expressed in HASMC, and Pi did not affect their expression (data not shown). Taking these observations together, we hypothesized the mechanism of regulation by adiponectin and TNFα F7 on Pi-induced vascular calcification (Fig. 7). However, further intensive investigations are required to elucidate the role of each player in VSMC calcification.

In summary, adiponectin inhibited VSMC calcification and antagonized the stimulatory effect of TNFα. This action was caused by preventing apoptosis via AMPK activation, followed by restoration of the Gas6-mediated survival pathway. AMPK regulated Gas6 expression at the transcriptional level. AMPK activation regulated by adiponectin and TNFα in vascular calcification might be a key to the management of cardiovascular disease.

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B—Please confirm or correct postal codes added to the affiliations line.

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D—Please confirm or correct expansion of AP1.

E—Please confirm or correct expansion of NF-κB.

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Original article

Sirt1 modulates premature senescence-like phenotype in human endothelial cells

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Abstract

Yeast Sir2 plays critical roles in gene silencing, stress resistance and longevity. Mammalian Sirt1 NAD^+-dependent protein deacetylase, the closest homolog of Sir2, regulates cell cycle, cellular senescence, apoptosis and metabolism, by functional interactions with a number of biological molecules such as p53. To investigate a role of Sirt1 in endothelial dysfunction and premature senescence, we examined the effects of Sirt1 inhibition in human umbilical vein endothelial cells (HUVEC). Sirt1 inhibition by sirtinol, which is a 2-hydroxy-1-napthaldehyde derivative, or siRNA for Sirt1-induced premature senescence-like phenotype, as judged by increased senescence-associated β-galactosidase (SA-β-gal) activity, sustained growth arrest and enlarged and flattened cell morphology at 10 days after the treatment. Sixty-four percent of sirtinol (60 μmol/L)-treated HUVEC was SA-β-gal-positive, whereas only 17% of vehicle-treated cells were positive. Sirt1 inhibition by sirtinol or Sirt1 siRNA increased PAI-1 expression and decreased both protein expression and activity of eNOS. Treatment with sirtinol or Sirt1 siRNA increased acetylation of p53, while p53 expression was unaltered. Impaired epidermal growth factor-induced activation of mitogen-activated protein kinases was associated with Sirt1 inhibition-induced senescence-like growth arrest. Conversely, overexpression of Sirt1 prevented hydrogen peroxide-induced SA-β-gal activity, morphological changes and deranged expression of PAI-1 and eNOS. These results showed that Sirt1 inhibition increased p53 acetylation and induced premature senescence-like phenotype in parallel with increased PAI-1 and decreased eNOS expression. Our data suggest that Sirt1 may exert protective effects against endothelial dysfunction by preventing stress-induced premature senescence and deranged expression of PAI-1 and eNOS.

Keywords: Sirt1; Sirtinol; Premature senescence; Endothelial dysfunction; Oxidative stress

1. Introduction

Replicative senescence is the limited ability of mammalian cells to divide when cultured in vitro [1]. Cessation of cell division after extended propagation in weeks – or months – culture results from the attrition of telomeres and is termed replicative senescence. In contrast to replicative senescence, stressors such as oxidative stress elicit irreversible growth arrest within just a few days, which is referred to as stress-induced premature senescence (SIPS) [2,3]. Although cellular phenotypes of replicative senescence and SIPS are quite similar or indistinguishable, unlike replicative senescence, SIPS can be induced independent of telomere attrition. In either type of cellular senescence, it is accompanied by a specific set of changes in cell function, morphology and gene expression [4,5]. Well-established biomarkers of cellular senescence include staining for senescence-associated β-galactosidase (SA-β-gal) at pH 6.0 as opposed to endogenous lysosomal enzyme detected at pH 4.0 in normal cells.

Recently, premature senescence of endothelial cells has been proposed to be involved in endothelial dysfunction and atherogenesis [6]. Increased plasminogen activator inhibitor-1 (PAI-1) expression and reduced endothelial nitric oxide synthase (eNOS) activity, which play key roles in endothelial dysfunction and atherogenesis [7], are associated with premature senescence of vascular endothelial cells. Indeed, previous studies have shown that senescence of endothelial cells leads to endothelial

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dysfunction and may contribute to the progression of atherosclerosis [8,9]. Human aortae with atherosclerotic lesion exhibited premature senescence-like phenotype, including SA-β-gal activity and increased expression of PAI-1 [10], in association with decreased production of nitric oxide (NO) in endothelial cells [6]. In addition to the role as a prothrombotic factor, the induction of PAI-1 is part of cellular senescence program and has been used as an indicator of cellular senescence in various cell types, including endothelial cells [11,12]. Moreover, NO production [13] and eNOS expression [14,15] are markedly attenuated in senescent endothelial cells. However, the molecular mechanisms underlying premature senescence and endothelial dysfunction remain to be clarified.

Sir2 (silent information regulator-2), an NAD^+-dependent histone deacetylase, is highly conserved in organisms ranging from archaea to humans [16,17]. In 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 [18–20]. Mammalian Sirt1 NAD^+-dependent protein deacetylase, the closest homolog of Sir2, regulates cell cycle, premature senescence, apoptosis and metabolism by interacting with a number of molecules, including p53, PML, Foxo, Ku70 and PPAR-γ [21–26]. A previous study has shown that Sirt1 antagonizes p53-mediated premature senescence in mouse embryo fibroblasts [25]. Recently, we have demonstrated that Sirt1 inhibition induces premature senescence-like growth arrest in human cancer cells [28].

In the present study, to test the hypothesis that Sirt1 plays a role in endothelial premature senescence and dysfunction in vitro, we evaluated the effects of inhibition and overexpression of Sirt1. Here, we demonstrate in human vascular endothelial cells that inhibition of Sirt1-induced premature senescence-like phenotype in parallel with acetylation of p53, and that Sirt1 overexpression reverted premature senescence induced by hydrogen peroxide.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from CAMBREX (Walkersville, MD) and maintained in endothelial growth medium (EGM-2, EGM-2 singleQuots, CAMBREX). Population doubling levels (PDL) were calculated as described previously [29], and all experiments were performed at PDL of 8–9, unless otherwise stated.

2.2. Sirt1 inhibition and overexpression

Exponentially proliferating cells were washed three times with growth medium and exposed for 24 h to the indicated concentrations of sirtinol (Calbiochem, San Diego, CA), a cell permeable 2-hydroxy-1-napthaldehyde derivative and a chemical inhibitor of NAD^+-dependent protein deacetylases of sirtuins. Trichostatin A (TSA) was purchased from Sigma (St. Louise, MO). After exposure, the cells were washed three times with inhibitor-free medium and cultured for up to additional 9 days. Proliferating cells were transfected with 200 pmol/L siRNA for Sirt1 (GAT GAA GTT GAC CTC CTC A [22] and TGA AGT GCC TCA GAT ATT A [28]), Sirt2 (sc-40988, Santa Cruz Biotechnology, Inc., CA), or control siRNA (Darmacom, Chicago, IL) using silMPORTER (Upstate, Charlottesville, VA). Sirt1 was overexpressed by transfection with pIRES-Sirt1, which was kindly provided by Dr. R.A. Weinberg [26,27], using jetPEI-HUVEC (Polyplustransfection, Illkirch, France) according to the manufacturer's instruction. Three days after the transfection, HUVEC were treated with 100 μmol/L hydrogen peroxide (Sigma) for 1 h and then washed three times with medium and were cultured for up to 10 days. Inhibition of p53 was performed by transfection of 4 μmol/L non-targeted oligonucleotide (5′-GGAGCCAGGGGGGAGGG-3′) or p53 anti-sense oligonucleotide (5′-CCCTGCTCCCCCTGGCTCC-3′).

2.3. Senescence-associated β-galactosidase (SA-β-gal) Staining

At 10 days after the treatment, the proportion of SA-β-gal-positive cells was determined as described by Dimri et al. [30].

2.4. BrdU incorporation assay

BrdU incorporation was analyzed using a commercially available kit (Roche, Indianapolis).

2.5. NOS activity assay

NOS activity of endothelial cells was determined using a NOS assay kit (Calbiochem) according to the manufacturer's instructions.

2.6. Immunoblotting

Cells were lysed on ice for 1 h in buffer (50 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, 1%NP-40, 0.1% SDS, 1 mmol/L dithiothreitol, 1 mmol/L sodium vanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin and 10 mmol/L sodium fluoride). Equal amounts of protein were separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking, the filters were incubated with the following antibodies; anti-p53, anti-acetyl-histone H3 (Lys14), anti-phosho-p44/42 MAPK (Thr202/Tyr204), anti-p44/42 MAPK, anti-phospho-p38 MAPK (Thr180/Tyr182), anti-p38, anti-phospho-JNK (Thr183/Tyr185), anti-JNK (Cell Signaling, Danvers, MA), anti-PAI-1 (Molecular Innovations, Southfield, MI), anti-eNOS (BD Transduction Laboratories, San Jose, CA), anti-Sirt1 (rabbit monoclonal IgG, recognizes human and mouse Sirt1), anti-acetyl-p53 (Lys373/382 and Lys320), anti-acetyl-histone H4 (Lys16) (Upstate), anti-Sirt2 (Santa Cruz) and anti-β-actin (Sigma). After washing and incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Amersham, Piscataway, NJ) for 1 h, the antigen–antibody complexes were visualized by an enhanced chemiluminescence system (Amersham).

2.7. Data analysis

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

3. Results

3.1. Inhibition of Sirt1 induces premature senescence-like phenotype in HUVEC

To investigate whether Sirt1 modulates stress-induced premature senescence-like phenotype in HUVEC, we first examined the effect of Sirt1 inhibition. Sirt1 was inhibited by sirtinol, a specific chemical inhibitor of sirtuins, and Sirt1 siRNA. Sirtinol increased the acetylation of histone H3 (Lys14) and H4 (Lys16), endogenous substrates for Sirt1 (Fig. 1A), as expected. However, the protein expression of Sirt1 was not affected. Knockdown of Sirt1 with siRNA was confirmed by Western blotting (Fig. 1D). Treatment with sirtinol or Sirt1 siRNA induced sustained senescence-like growth arrest for up to 10 days (Fig. 1B). BrdU incorporation was also inhibited by sirtinol or Sirt1 siRNA at 10 days after treatment (Fig. 1C), indicating that the premature senescence-like phenotype and decreased cell number of HUVEC were accompanied by the attenuated DNA synthesis. We examined the effects of sirtinol or Sirt1 siRNA treatment on SA-β-gal activity, a characteristic feature of senescence-related growth arrest. Sirtinol or Sirt1 siRNA significantly increased SA-β-gal activity compared with untreated cells or control siRNA at 10 days after treatment; 64% of sirtinol (60 μmol/L)-treated HUVEC were SA-β-gal-positive, whereas only 17% of vehicle-treated cells were positive (Figs. 2A and B). Sirtinol or Sirt1 siRNA increased SA-β-gal activity in a time-dependent manner (data not shown). These cells also exhibited senescence-like morphological changes, that is, enlarged and flattened shapes (Fig. 2A). Next, because sirtinol can inhibit other NAD^+-dependent protein deacetylases of sirtuin family, especially Sirt2 [32], we examined the effect of Sirt2 inhibition by Sirt2 siRNA on senescence-like phenotype at 10 days after treatment. Sirt2 siRNA successfully eliminated the protein expression of Sirt2 (Fig. 1D) but did not affect the rate of senescence-associated β-galactosidase-positive cells (Fig. 2B).

3.2. Senescent-like phenotype resulting from Sirt1 inhibition is mediated by p53

p53 plays a pivotal role in cellular senescence [11]. Previous studies have shown that Sirt1 inhibition promotes stress-induced apoptosis by enhancing p53 acetylation [21,26], while ectopically expressed Sirt1 blocks p53-mediated premature senescence in mouse embryo fibroblasts [25]. Therefore, we examined whether inhibition of Sirt1 affects p53 in HUVECs. Consistent with pervious studies [21,25,26], Sirt1 inhibition by sirtinol or Sirt1 siRNA increased the acetylation of p53 after 3 days and the expression of p53 after 5 days (Figs. 3A and C). We also treated HUVEC with TSA, an inhibitor of classes I and II HDACs. Acetylation of p53 was increased at Lys373/382 and Lys320 in HUVECs treated with sirtinol but

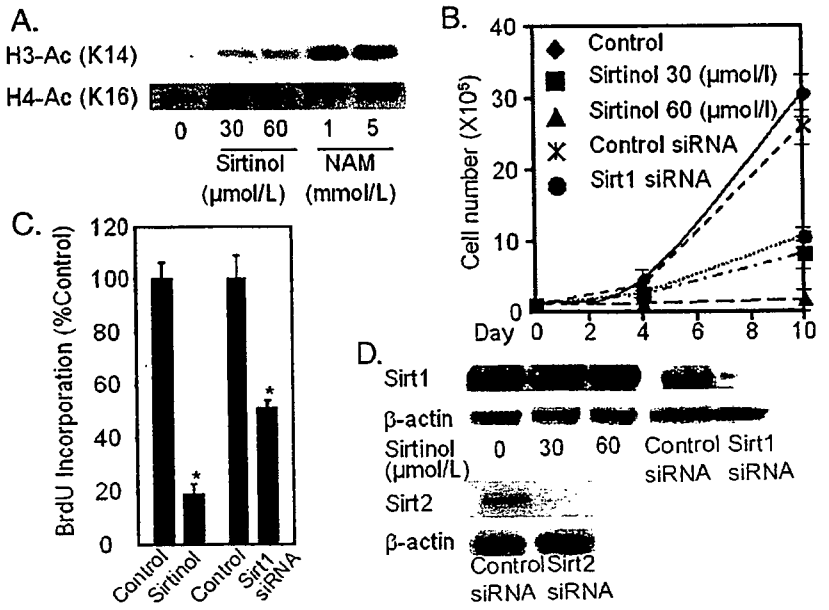

Fig. 1. Effects of Sirt1 inhibition on Sirt1 expression, acetylation of histone H3, H4 and cell growth in HUVEC. (A) Western blot analysis for acetylated histone H3 at lysine 14 (K14) and histone H4 at lysine 16 (K16) at 24 h after treatment with sirtinol (30 and 60 μmol/L) or nicotinamide (1 and 5 mmol/L), a physiological Sirt1 inhibitor. (B) The effects of sirtinol or Sirt1 siRNA on cell growth for 10 days. (C) BrdU incorporation analysis at 10 days after treatment with sirtinol or Sirt1 siRNA. (D) Western blot analysis for Sirt1 expression at 24 h after treatment with sirtinol or at 72 h after transfection of Sirt1 siRNA. Western blot analysis for Sirt2 expression at 3 days after transfection of Sirt2 siRNA. β-Actin served as a loading control.

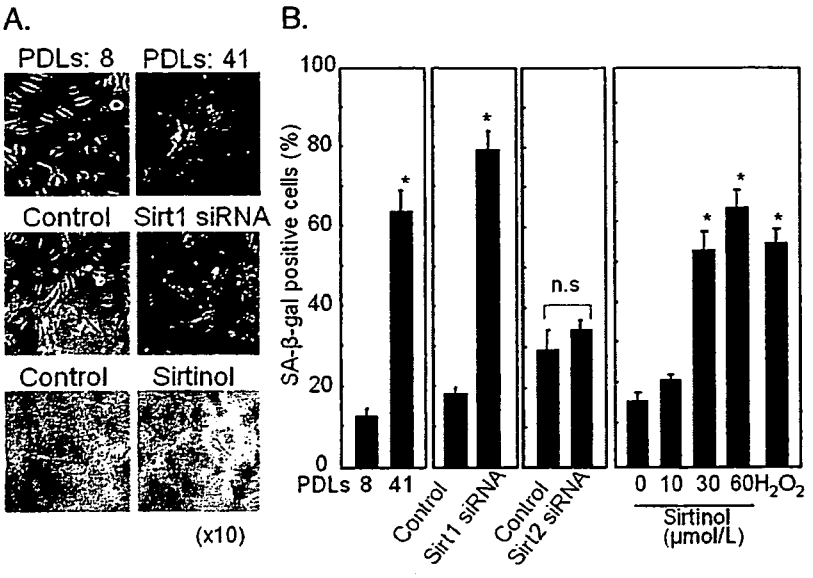

Fig. 2. Effects of Sirt1 inhibition on senescence-associated β-galactosidase. (A) Photomicrographs of HUVEC stained for SA-β-gal. Population doubling levels (PDLs) for the young and senescent cells are indicated, and sirtinol (60 μmol/L)-, Sirt1 siRNA-treated cells were at PDL 8. (B) SA-β-gal-positive cells at 10 days after sirtinol or Sirt1 or Sirt2 siRNA treatment. HUVEC treated with hydrogen peroxide (H_2O_2, 100 μmol/L) for 1 h and the cells at PDL 8 served as positive controls for SA-β-gal staining. $n=3$ for each group. *$p<0.05$ vs. control.

not with TSA, suggesting that endogenous Sirt1 plays an important role in deacetylation of p53 in HUVECs (Fig. 3D). We further examined whether the effects of Sirt1 inhibition were mediated by p53. We found that blockade of p53 by antisense oligonucleotides reversed the effects of Sirt1 inhibition on senescence associated β-galactosidase activity (Fig. 3E) and senescence-like morphological changes (data not shown) at 10 days after Sirt1 inhibition.

Next, we examined the activation status of mitogen-activated protein kinases (MAPKs) in response to epidermal growth factor (EGF). When untreated with sirtinol or Sirt1 siRNA, upon exposure to EGF, robust phosphorylation (activation) of extracellular-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK was observed in HUVEC. By contrast, in sirtinol- or Sirt1 siRNA-treated cells, EGF-stimulated phosphorylation of ERK, JNK and p38 MAPK was markedly attenuated (Fig. 3F). The protein expression of ERK, JNK and p38 MAPK did not differ between sirtinol- or Sirt1 siRNA-treated and untreated cells.

3.3. Sirt1 inhibition promotes endothelial dysfunction

To investigate further the role of Sirt1 in premature senescence and endothelial function, we examined PAI-1 expression, eNOS expression and NOS activity in HUVEC. Sirt1 inhibition by sirtinol or Sirt1 siRNA increased PAI-1 expression by three-fold and decreased eNOS expression and NOS activity (Figs. 4A, B and D). These changes were similar to those observed in HUVEC that underwent hydrogen peroxide-induced premature senescence and also replicative senescence at 41 PDL (Figs. 4A, C and D). The protein expression of PAI-1 and eNOS were increased and decreased, respectively, at 3 days after the addition of sirtinol or Sirt1 siRNA and further increased and reduced at 10 days after sirtinol or Sirt1 siRNA treatment (Fig. 5A). Likewise, the activity of NOS was reduced at 3 and 10 days after sirtinol or Sirt1 siRNA addition in a time-dependent manner (Fig. 5B). These results indicate that Sirt1 inhibition-induced premature senescence-like phenotype was associated with endothelial dysfunction.

To investigate the relationship between p53 and these protein expressions, we inhibited p53 by anti-sense oligonucleotide for p53. Anti-sense p53 reduced the expression of p53 and prevented induction of PAI-1 expression by Sirt1 inhibition, as compared with non-targeting oligonucleotide, although reduced eNOS expression was not reverted (Fig. 5C).

3.4. Overexpression of Sirt1 blocks oxidative stress-induced premature senescence

Next, we examined whether Sirt1 overexpression can protect HUVEC from oxidative stress-induced premature senescence. As shown previously [3,31], hydrogen peroxide induced premature senescence (Fig. 2B). Overexpression of Sirt1 significantly reduced the number of cells positive for SA-β-gal and inhibited senescence-associated morphological changes (Figs. 6A and B). Moreover, overexpression of Sirt1 reversed the changes in eNOS and PAI-1 expression (Fig. 6C) and blocked hydrogen peroxide-induced growth arrest (Fig. 6D). By contrast, when untreated with hydrogen peroxide, over-expression of Sirt1 did not alter cell growth compared with empty vector. In HUVEC transfected with Sirt1, Sirt1 protein expression was increased up to 5 days after treatment with hydrogen peroxide, and then started declining and returned to

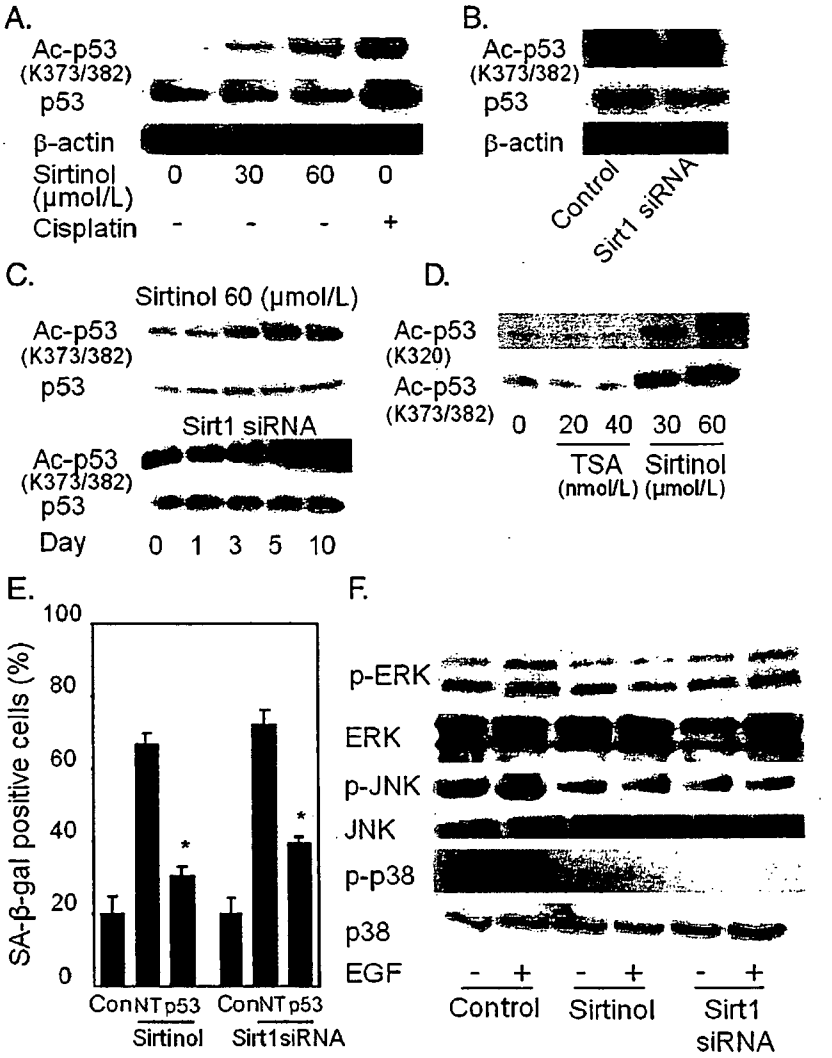

Fig. 3. Effects of Sirt1 inhibition on p53 and EGF-stimulated phosphorylation of MAPKs in HUVEC. (A, B) Acetylation of p53 (Ac-p53) at lysine *373/382* (*K373/382*) and total protein level of p53 were evaluated by Western blotting analysis at 24 h after treatment with sirtinol (A) and at 72 h after transfection with Sirt1 siRNA (B). Treatment with cisplatin (100 μmol/L) for 4 h served as a positive control. β-Actin served as a loading control. (C) Acetylation and total protein levels of p53 for 10 days after treatment with sirtinol or Sirt1 siRNA. (D) Acetylation of p53 at lysine 372/383 and lysine 320 (K372/383 and K320) was evaluated by Western blot analysis at 24 h after treatment with sirtinol (30 and 60 μmol/L) or Trichostatin A (TSA, 20 and 40 nmol/L). (E) Effect of transfection of non-targeted oligonucleotide (NT, 4.0 μmol/L, 5′-GGAGCCAGGGGGGAGGG-3′) or p53 anti-sense (p53, 4 μmol/L, 5′-CCCTGCTCCCCCTGGCTCC-3′) on SA-β-gal activity in sirtinol- or Sirt1 siRNA-treated cells. $n=3$ for each group. *$p<0.05$ vs. NT control. (F) EGF-stimulated phosphorylation of MAPKs in sirtinol- or Sirt1 siRNA-treated cells. At 10 days after treatment with sirtinol or Sirt1 siRNA, following overnight serum starvation, the cells were exposed to EGF (50 ng/ml) for 20 min.

normal level at 10 days after hydrogen peroxide treatment (Fig. 6E).

4. Discussion

We found that Sirt1 inhibition by a specific chemical inhibitor of sirtuins, sirtinol or gene knockdown by Sirt1 siRNA induced premature senescence-like phenotype, as judged by representative markers for premature senescence, SA-β-gal activity, enlarged and flattened cell morphology, sustained growth arrest, increased PAI-1 expression and suppressed activation of MAPKs in response to a growth factor, EGF. Another chemical inhibitor of sirtuins, splitomicin, also elicited similar effects in HUVEC (data not shown). Sirt1 inhibition-induced premature senescence-like growth arrest was associated with acetylation of p53. These results are consistent with previous studies by others and us showing that: (1) over-expression of Sirt1 antagonizes premature senescence through deacetylation of p53 in mouse fibroblasts [25]; and (2) sirtinol induces premature senescence-like phenotype in human cancer cells [28]. Sirtinol is a cell-permeable 2-hydroxy-1-napthaldehyde derivative that acts as a specific and direct inhibitor of all NAD^+-dependent protein deacetylases of sirtuin family. Therefore, our results brought by sirtinol might involve the possible influence of other sirtuins, especially Sirt2 [32]. In this regard, we also examined the role of Sirt2 in premature senescence-like

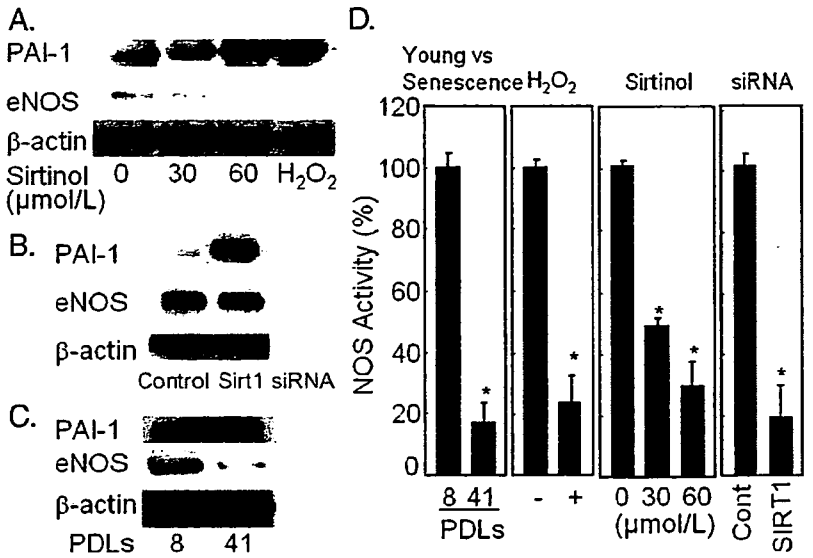

Fig. 4. Effects of Sirt1 inhibition on PAI-1 and eNOS expression in HUVEC. (A, B) PAI-1 and eNOS expressions were evaluated by Western blotting at 10 days after treatment with sirtinol (A) or Sirt1 siRNA (B). Treatment with hydrogen peroxide (H_2O_2, 100 μmol/L) for 1 h served as a positive control for the phenotype of premature senescence. (C) The expression of PAI-1 and eNOS in young (PDL 8) and senescent (PDL 41) HUVEC. (D) The effects of sirtinol or Sirt1 siRNA treatment on NOS activity. HUVEC at PDL 8 and the cells treated with hydrogen peroxide served as positive controls. $n=3$ for each group. $^*p<0.05$ vs. control.

phenotype in HUVEC. Little influence of gene knockdown by Sirt2 siRNA suggests that Sirt2 does not play a major role in our model of premature senescence, even though Sirt2 may be a target enzyme of sirtinol. However, there are 7 mammalian sirtuins and several might be involved in human endothelial senescence. Further studies are needed to address this point.

Moreover, overexpression of Sirt1 prevented premature senescence induced by hydrogen peroxide in HUVEC. This finding is in accord with a previous report that overexpression of Sirt1 enhanced stress resistance against hydrogen peroxide through deacetylation of p53 in human mesangial cells [33]. Taken together, these findings suggest that Sirt1 may play an important role in stress resistance and the modulation of stress-induced premature senescence (SIPS), at least in part, through deacetylation of p53.

Sirtinol- or Sirt1 siRNA-induced premature senescence was accompanied by attenuated response to EGF in terms of activation of MAPKs signaling. A hallmark feature of senescent

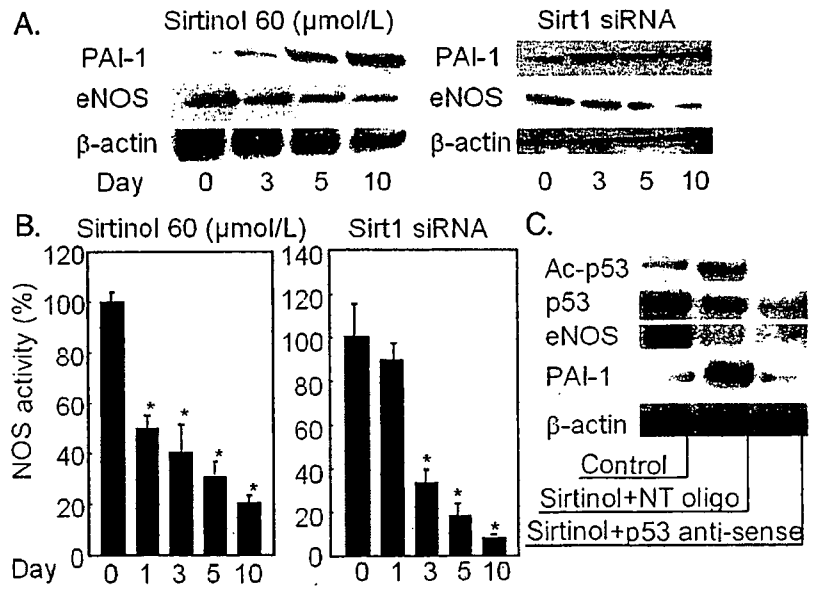

Fig. 5. Time-dependent alterations in PAI-1 and eNOS expression by inhibition of Sirt1 in HUVEC. (A, B) PAI-1 and eNOS expression (A) and NOS activity (B) were evaluated after treatment with sirtinol or Sirt1 siRNA. (C) The effect of transfection of non-targeted oligonucleotide (NT oligo) and p53 antisense (4.0 μmol/L) on PAI-1 and eNOS expression in sirtinol-treated cells. $^*p<0.05$ vs. control.

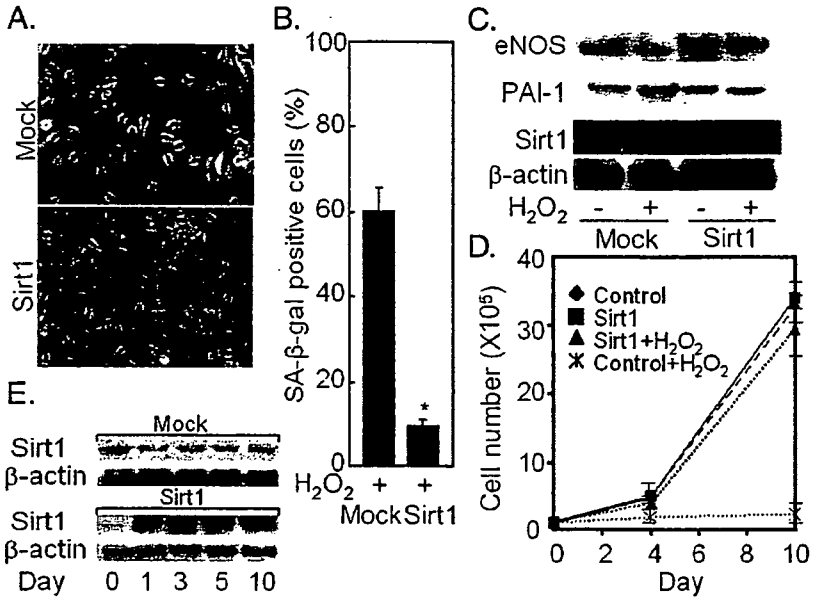

Fig. 6. Effects of ectopically expressed Sirt1 on hydrogen peroxide-induced premature senescence in HUVEC. (A–C) The effects of Sirt1 overexpression on cell morphology (A), SA-β-gal staining (B) and the expression of PAI-1 and eNOS (C) in HUVEC treated with and without hydrogen peroxide (H_2O_2, 100 μmol/L) for 1 h. The effects of Sirt1 overexpression on SA-β-gal-positive cells in hydrogen peroxide-treated HUVEC. *$p<0.05$ vs. Mock. (D) The effects of Sirt1 overexpression on cell growth. $n=3$ for each group. (E) Sirt1 protein expression in HUVEC transfected with empty vector and pIRES-Sirt1 during the 10-day culture.

cells is unresponsiveness to cell growth stimuli in induction of c-*fos* as well as cell proliferation. Our results are in agreement with previous studies that growth factor-stimulated activation of the ERK pathway is impaired in senescent human diploid fibroblasts [34].

Endothelial dysfunction has been implicated in the development of cardiovascular diseases, including hypertension and atherosclerosis [35]. Nevertheless, molecular mechanisms responsible for endothelial dysfunction remain to be determined. Recently, SIPS of endothelial cells has been proposed as a contributor to endothelial dysfunction [36,37]. We found that Sirt1 inhibition increased PAI-1 expression and decreased eNOS expression and NOS activity in HUVEC, and that overexpression of Sirt1 reverted hydrogen oxide-induced alterations in these proteins. Collectively, these findings suggest that Sirt1 may protect from endothelial dysfunction by inhibiting premature senescence and thereby contribute to maintenance of vascular homeostasis. One can reasonably speculate therefore that Sirt1 may exert salutary actions against cardiovascular diseases (e.g., hypertension, atherosclerosis) by preventing SIPS-associated endothelial dysfunction. However, further studies will be required to clarify this point.

p53 is a major substrate for Sirt1, and that deacetylation of p53 regulates cell cycle, cellular senescence and stress resistance in various cell types, including cardiac myocytes [38]. Consistent with previous studies [21,25,26], Sirt1 inhibition by sirtinol or Sirt1 siRNA increased the acetylation of p53 after 3 days and the expression of p53 after 5 days. This result is consistent with the finding by van der Veer et al. [39] that nicotinamide phosphoribosyltransferase-induced and Sirt1-mediated p53 deacetylation was associated with the decreased expression of p53 in vascular smooth muscle cells. A recent study has revealed that PAI-1 is a direct downstream target gene of p53 and a major player in the induction of cellular senescence [40]. In agreement with this, we found that inhibition of p53 reversed SA-β-gal activity and PAI-1 expression that were induced by Sirt1 inhibition. These results indicate that the acetylation of p53 may play a role in Sirt1 inhibition-induced premature senescence-like phenotype in HUVEC.

Mitosis-related telomere shortening is the critical event for replicative senescence. However, telomere attrition is dispensable for SIPS, which can be induced in a few days. A previous study has shown that changes in telomere length or in telomerase activity were not detected when SIPS was induced by oxidative stress in human endothelial cells [41]. Moreover, a recent study has demonstrated that telomere extension by overexpression of human telomerase does not affect SIPS [2], although it rescues replicative senescence [42]. These data clearly indicate that in contrast to replicative senescence, telomere erosion does not have an important role in the induction of SIPS.

Previous studies have shown senescence-like phenotype in endothelial cells in atherosclerotic lesion in humans and rodents [6,37], as discussed above. Oxidative stress and other stressors have been implicated in the pathogenesis of endothelial dysfunction, hypertension and atherosclerosis [35]. In contrast, there is no direct in vivo evidence indicating excessive replication of endothelial cells, which in turn causes replicative senescence, in cardiovascular diseases. In aggregate, it is reasonably conceivable that SIPS rather than genuine replicative senescence may be a major contributor to senescence-like phenotype of endothelial cells in cardiovascular diseases. Therefore, we focused on the role of Sirt1 in SIPS-like phenotype in endothelial cells in the present study.

In summary, our data showed that Sirt1 inhibition increased p53 acetylation and induced SIPS-like phenotype in HUVEC in parallel with increased PAI-1 and decreased eNOS expression, and that Sirt1 overexpression reversed the SIPS induced by oxidative stress. These results suggest that Sirt1 may exert protective effects against endothelial dysfunction by preventing premature senescence and deranged expression of PAI-1 and eNOS in endothelial cells.

4.1. Perspectives

Prevention and reversal of endothelial dysfunction, a major pathogenic factor for cardiovascular diseases, has been an issue of intense investigation for a number of years. Recently, SIPS has been proposed as a contributor to endothelial dysfunction. Nonetheless, it remains largely unknown how stressors such as oxidative stress induces SIPS in endothelial cells. Our data suggest an important role for Sirt1 in the regulation of SIPS. Collectively, these findings highlight Sirt1 as a possible molecular target to prevent and/or treat cardiovascular diseases, including hypertension and atherosclerosis by protecting endothelial cells from SIPS-involved dysfunction. This possibility deserves further investigation.

Acknowledgments

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EFFECTS OF AGE AND SEX ON PLASMA ADRENOMEDULLIN LEVELS IN PATIENTS WITH OBSTRUCTIVE SLEEP APNEA SYNDROME

To the Editor: It has been recognized that obstructive sleep apnea syndrome (OSAS) is one of the risk factors of cardiovascular disorders, including hypertension, ischemic heart disease, and cerebrovascular diseases.[1–3] Although the incidence of sleep apnea increases with age, the pathological roles of OSAS have not been fully established. OSAS-induced hypoxic stress and oxidative stress increase circulating inflammatory mediators, including adhesion molecules, inflammatory cytokines, and high-sensitivity C-reactive protein, leading to hypertension and cardiovascular events.[4,5] The stress and its related inflammatory molecules are implicated in the production of adrenomedullin (AM), which is a potent endothelial-derived vasodilator.[6] Circulating AM levels are higher in adults with untreated OSAS than in adults without OSAS.[7] Because plasma AM is closely correlated with pulse wave velocity and atherosclerosis progression in middle-aged and elderly patients, higher levels of AM may be a surrogate marker for hypertension and the progression of atherosclerosis.[8] Furthermore, treatment with nasal continuous positive airway pressure (nCPAP) mostly reversed the higher AM levels in subjects with OSAS. Thus, OSAS treatment may prevent atherosclerosis and cardiovascular events, although circulating AM levels and therapeutic response to OSAS have not been examined in elderly people. Furthermore, female sex hormones increase AM-induced vasodilation by increasing the expression of AM2 receptor components in rats.[9] Sex may affect circulating AM levels in humans.

Age and sex differences in AM levels in patients with OSAS were examined. Eighty middle-aged (aged 40–60) and 80 elderly (aged 60) patients were compared with OSAS and 80 middle-aged and 80 elderly age- and body mass index (BMI)-matched subjects without OSAS. The patients had to fulfill the following criteria: absence of renal and renovascular hypertension, systolic blood pressure (BP) greater than 160 mmHg or diastolic BP greater than 95 mmHg, chronic renal and hepatic diseases, and diabetes mellitus. Patients who smoked or had systemic infections at the time of the study or within 4 weeks before the study were excluded. No patients were being treated with antihypertensive agents. These subjects were examined using polysomnography; subjects with an apnea-hypopnea index (AHI) less than five were controls, and those with an AHI of five or greater were determined to have OSAS. To assess OSAS-induced hypoxia quantitatively, the oxyhemoglobin desaturation index (ODI) was used in this study as previously described.[4] ODI was defined as $ODI = \Sigma(90 - \text{oxygen saturation})t$, where t is time of desaturation (hours).[4]

Circulating AM levels and sleep study variables were compared. Then 3 months of nCPAP treatment was performed in the patients. Peripheral blood was obtained from the subjects at 7:30 a.m. to 8:00 a.m. before and after the 3 months treatment with nCPAP. The AM was measured using a specific radioimmunoassay.

There were no significant differences in BMI between the patients and controls in each age group, whereas AHI in subjects OSAS was markedly greater than in controls (Table 1). There were no significant differences in BP or metabolic indices. The AHI values in elderly (50.1 ± 3.2 events/h) and middle-aged (51.6 ± 3.0) subjects with OSAS were considerably greater than in age-matched controls (3.8 ± 0.3 and 3.6 ± 0.4, respectively). There were significant differences in baseline ODI between patients with OSAS and controls, suggesting that the patients with OSAS were exposed to a

Table 1. Circulating Adrenomedullin (AM) Levels and Other Variables in Elderly and Middle-Aged Patients with Obstructive Sleep Apnea Syndrome (OSAS) and Controls

Characteristic	Middle-Aged Subjects with OSAS (n = 80)	Elderly Subjects with OSAS (n = 80)	Middle-Aged Controls (n = 80)	Elderly Controls (n = 80)
	Mean ± Standard Error			
Age	46.8 ± 2.2	65.8 ± 2.2	45.1 ± 2.2	64.8 ± 2.1
Body mass index	33.4 ± 0.9	32.1 ± 0.9	32.8 ± 1.1	31.5 ± 1.1
Systolic blood pressure, mmHg	138.1 ± 3.7	139.1 ± 4.7	135.6 ± 4.1	137.1 ± 3.7
Diastolic blood pressure, mmHg	82.1 ± 3.2	80.1 ± 3.2	78.9 ± 3.8	78.1 ± 2.8
Total cholesterol, mg/dL	202.9 ± 7.9	199.1 ± 6.9	202.9 ± 7.9	198.3 ± 10.8
High-density lipoprotein cholesterol, mg/dL	43.0 ± 2.2	41.0 ± 2.1	43.6 ± 2.3	42.2 ± 2.1
Triglyceride, mg/dL	144.1 ± 10.7	137.1 ± 12.7	140.1 ± 10.7	132.0 ± 10.9
Fasting plasma glucose, mg/dL	98.4 ± 1.3	94.6 ± 1.3	97.6 ± 1.4	90.3 ± 1.1
Hemoglobin A1c, %	5.7 ± 0.1	5.8 ± 0.1	5.6 ± 0.1	5.27 ± 0.1
Total sleep time, min	368.1 ± 20.3*	348.1 ± 20.3*	440.3 ± 20.9	414.3 ± 20.9
Apnea–hypopnea index, events/h	51.6 ± 3.0*	50.1 ± 3.2*	3.8 ± 0.3	3.6 ± 0.4
Lowest oxygen saturation, %	67.2 ± 2.1*	68.9 ± 3.0*	95.8 ± 0.5	94.1 ± 0.5
Oxyhemoglobin desaturation index	2.45 ± 0.32*	2.41 ± 0.36*	0.02 ± 0.01	0.02 ± 0.01
Arousal index,/h	42.4 ± 3.1*	40.2 ± 2.2*	8.3 ± 3.1	8.3 ± 3.1
AM level, pg/mL	49.1 ± 3.7	51.1 ± 4.1	24.8 ± 1.9	25.7 ± 2.1

Note: There were 40 men and 40 women in each group.

*$P < .001$ versus control group.

significantly greater degree of hypoxia than the control subjects. The magnitude of ODI was not different between middle-aged and elderly patients with OSAS.

Circulating AM levels in middle-aged and elderly patients with OSAS were significantly greater than in the age- and BMI-matched controls, although neither age nor sex affected them (Table 1). nCPAP treatment significantly decreased the higher levels of circulating AM in the patients irrespective of age and sex. After 3 months of treatment with nCPAP, AM levels in elderly patients (26.5 ± 2.4 pg/mL) were not different from those of middle-aged patients (24.7 ± 2.1 pg/mL).

These results indicated that plasma AM levels were higher in middle-aged and elderly patients with OSAS and could be deceased with nCPAP treatment, regardless of age and sex. The augmented increase in AM caused by severe nocturnal hypoxemia and oxidative stress due to OSA may overcome the age-dependent increase of AM levels in middle-aged and elderly patients with OSAS. Because AM is reported to induce cell surface expression of adhesion molecules, including E-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 (ICAM-1), on human endothelial cells, the higher level of AM is one of the mechanisms of higher levels of ICAM-1 in patients with OSAS.[10] The current study also indicates that treatment with nCPAP may be effective for the prevention of cardiovascular complications in elderly patients with OSAS.

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NATURAL THERAPIES—WHEN IGNORANCE IS NOT BLISS!!

To the Editor: A 72-year-old Caucasian man with history of hypertension, dyslipidemia, depression, gastroesophageal reflux disease, and mild obesity presented to the hospital with intractable singultus (hiccups) for approximately 9 days, associated with anorexia and epigastric pain. He had presented to his primary care provider the day before admission and was given prochlorperazine without relief. His daily medications were metoprolol, furosemide, fluvastatin, gemfibrozil, paroxetine, and omeprazole. A thorough history revealed that he had added 2 tablespoons daily of acetic acid (household vinegar) to his routine for the previous 2 weeks after reading an article in a health magazine that indicated that vinegar helps decrease food intake (by promoting satiety) and lowers cholesterol. Physical examination was unremarkable except for mild epigastric tenderness.

Computerized tomography of the abdomen with oral and intravenous contrast was suggestive of acute pancreatitis, and amylase and lipase levels were elevated, at 876 and 1,187 U/L, respectively. Other laboratory parameters were normal. Brain imaging and endoscopy were negative, and furosemide and paroxetine were discontinued on admission. The patient received nothing by mouth and was maintained with intravenous fluids for 2 days. He experienced complete resolution of the pancreatitis and the hiccups.

Vinegar has a long history in medicine, including use by the father of modern medicine, Hippocrates, as an agent to fight acute infections and chronic coughs. Some modern studies support antimicrobial effects with food preparation; others claim it is a nematocyst inhibitor and protective in stings by some jellyfish. Recently, vinegar has been identified as an antiglycemic in subjects with and without glucose intolerance. In addition, it has been shown to increase satiety in healthy patients.[1]

There have been various adverse effects of vinegar described in the literature. Some of these include corrosive

Original Article

Association of Plasma Dehydroepiandrosterone-Sulfate Levels with Endothelial Function in Postmenopausal Women with Coronary Risk Factors

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Age-related decline of plasma dehydroepiandrosterone-sulfate (DHEA-S) levels may be associated with the risk of cardiovascular disease in women. We investigated whether plasma DHEA-S levels are related to endothelial function in postmenopausal women with coronary risk factors. One hundred and fifteen postmenopausal women (mean age±SD: 57±5 years; range: 48–65 years) who underwent measurement of flow-mediated vasodilation (FMD) of the brachial artery using ultrasonography were enrolled. Plasma hormone levels were determined in the morning after a 14-h fast, and the relationship between hormone levels and FMD was analyzed. DHEA-S was significantly correlated with %FMD (r=0.392, p<0.001), while estradiol, total testosterone and cortisol were not. %FMD in the highest quartile of DHEA-S was 1.8-fold higher than that in the lowest quartile (5.3±1.3 *vs.* 2.9±2.0 [means±SD], p<0.01). Multiple regression analysis revealed that DHEA-S was related to %FMD independent of age, body mass index, hypertension, hyperlipidemia, diabetes mellitus and smoking (β=0.344, p<0.01), and was itself independent of age, body mass index, systolic blood pressure, total cholesterol, high-density lipoprotein (HDL) cholesterol, fasting plasma glucose and smoking (β=0.291, p<0.05). In conclusions, plasma DHEA-S levels were weakly but significantly related to endothelial function in postmenopausal women independent of other coronary risk factors, suggesting a protective effect of DHEA on the endothelium. (*Hypertens Res* 2008; 31: 69–74)

Key Words: **endothelium, vasodilation, risk factor, man, nitric oxide**

Introduction

Plasma levels of dehydroepiandrosterone-sulfate (DHEA-S), the most abundant circulating steroid that is secreted from the adrenal cortex, decline with advancing age in men and women (*1*, *2*). The age-related decrease in DHEA-S concentrations has often been associated with the pathological processes of aging, such as osteoporosis, depression and dementia (*3*, *4*). A number of studies have investigated the link between DHEA-S and cardiovascular disease, though with inconsistent results (*5–7*). Among them, Haffner *et al.* have shown that low DHEA-S levels predicted ischemic heart disease mortality in diabetic women (*8*). The association of low DHEA-S levels with carotid artery atherosclerosis (*9*), obesity (*10*) and decreased diurnal blood pressure variability (*11*) also suggests a vasoprotective role of DHEA(-S) in women. Furthermore, experimental studies showing endothe-

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Table 1. Characteristics of Study Subjects (N=115)

Age, years	57±5	[48–65]
Body mass index, kg/m²	23.3±4.0	[17.6–35.0]
Risk factors		
Hypertension, *n* (%)	35 (30)	
Hyperlipidemia, *n* (%)	71 (62)	
Diabetes mellitus, *n* (%)	20 (17)	
Current smoker, *n* (%)	17 (15)	
Hemodynamic and vascular measurements		
Systolic blood pressure, mmHg	123±17	[93–170]
Diastolic blood pressure, mmHg	70±10	[52–100]
%FMD	4.8±2.4	[0.0–12.7]
%NTG	14.0±4.5	[4.1–22.5]
Carotid IMT, mm	0.92±0.22	[0.46–1.45]
Blood chemistry and hormones		
Total cholesterol, mmol/L	5.72±1.01	[3.73–8.96]
HDL cholesterol, mmol/L	1.66±0.42	[0.77–2.87]
Triglycerides, mmol/L	1.18±0.65	[0.36–3.49]
Fasting plasma glucose, mmol/L	5.42±1.22	[4.00–10.43]
Hemoglobin A1c, %	5.5±1.0	[4.3–9.6]
Estradiol, pmol/L	35±26	[18–160]
Testosterone, nmol/L	0.85±0.43	[0.21–2.01]
DHEA-S, μmol/L	2.28±1.07	[0.37–5.02]
Cortisol, nmol/L	316±121	[110–728]

Values except risk factors are expressed as mean±SD [range]. %FMD, percent flow-mediated dilation of brachial artery; %NTG, percent nitroglycerin-induced dilation of brachial artery; IMT, intima-media thickness of common carotid artery; HDL, high-density lipoprotein; DHEA-S, dehydroepiandrosterone-sulfate.

lium-dependent (*12*) and -independent (*13*) vasodilating effects of DHEA(-S) led us to hypothesize that postmenopausal women with low plasma DHEA-S levels would have impaired vasomotor function.

To test this hypothesis, we conducted a cross-sectional survey of 115 postmenopausal women by examining flow-mediated dilation (FMD) of the brachial artery and plasma sex hormones, and showed that low DHEA-S levels were associated with endothelial dysfunction.

Methods

Subjects

One hundred and fifteen postmenopausal women who underwent examination of vasomotor function of the brachial artery and intima-media thickness (IMT) of the carotid artery in our department were enrolled. The subjects were referred to our department to check their cardiovascular disease or risks. All of them were in chronic stable condition. A history was taken, and physical examination and laboratory tests were performed in all subjects. Subjects with a history of cardiovascular disease, including stroke, coronary heart disease, congestive heart failure and peripheral arterial disease, malignancy, overt endocrine disease or administration of steroid hormones, were excluded. The postmenopausal status of each subject was confirmed by the fact that at least 12 months had passed since her last menses and by the measurement of follicular stimulating hormone and estradiol. The characteristics of the study subjects are shown in Table 1.

Seventy-three percent of the subjects had one or more classical coronary risk factors, such as hypertension, hyperlipidemia, diabetes mellitus or current smoking. Hypertension, hyperlipidemia and diabetes mellitus were considered to be present based on the published diagnostic criteria (*14–16*) or if the subjects were taking any medications for these diseases. Eighty-six percent of the hypertensive subjects were treated: 75% with calcium antagonists, 19% with angiotensin-converting enzyme inhibitors, 10% with diuretics and 7% with β blockers. Eighty-six percent of the hyperlipidemic subjects were treated with statins, and 75% of the diabetic subjects were treated with oral hypoglycemic agents. None of the study subjects were taking nitrates. Each subject gave written informed consent before enrollment in this study. The study protocol was approved by the ethics committee of the Graduate School of Medicine, the University of Tokyo.

Vascular Measurement

Vasomotor function of the brachial artery was evaluated using an ultrasound machine according to the method described previously (*17*). Briefly, endothelium-dependent flow-mediated vasodilation (%FMD) was measured as the maximal percent change of the vessel diameter after reactive hyperemia. The subjects were examined in the morning after a 14-h overnight fast with no medication. Subsequently, endothelium-independent nitroglycerin-induced vasodilation (%NTG) was measured as the maximal percent change of the vessel diameter after sublingual administration of nitroglycerin spray (0.3 mg; Toa Eiyo Co., Tokyo). Carotid IMT was evaluated using an ultrasound machine as described previously (*17*).

Plasma Hormones

Blood sampling was performed in the morning of the vascular measurement after a 14-h overnight fast, and plasma was stored at −80°C until assay. Plasma estradiol, testosterone (total testosterone), DHEA-S and cortisol concentrations were determined using sensitive radioimmunoassays by a commercial laboratory (SRL Inc., Tokyo, Japan). The intra-assay coefficients of variation for these measurements were less than 5%.

Because plasma was deep-frozen for 3–7 years, we checked the change in titers using the stored samples, which had been measured at sampling 5–7 years before. The Pearson's correlation coefficients between the two measurements were 0.965

Table 2. Pearson's Correlation Coefficients between Age, Vascular Measurements and Plasma Hormones

	Age	%FMD	Carotid IMT
Estradiol	−0.108	0.041	−0.136
Testosterone	0.061	0.019	−0.088
DHEA-S	−0.198*	0.392†	−0.187*
Cortisol	0.074	0.140	0.064

%FMD, percent flow-mediated dilation of brachial artery; IMT, intima-media thickness of common carotid artery. †$p<0.001$, *$p<0.05$.

Table 3. Age-Adjusted Regression Coefficients between Vascular Measurements and Plasma Hormones

	%FMD	Carotid IMT
Estradiol	0.023	−0.098
Testosterone	0.029	−0.117
DHEA-S	0.366†	−0.146
Cortisol	0.156	−0.062

%FMD, percent flow-mediated dilation of brachial artery; IMT, intima-media thickness of common carotid artery. Standardized regression coefficients by multiple regression analyses with %FMD or carotid IMT as a dependent variable and age and each of the hormones as independent variables are shown. †$p<0.01$.

for estradiol ($n=34$), 0976 for testosterone ($n=20$), 0.991 for DHEA-S ($n=15$) and 0.937 for cortisol ($n=16$), indicating that there was no significant change in plasma titers in our frozen samples.

Data Analysis

Pearson's simple correlation coefficients between age, vascular measurements and plasma hormones were determined. Standardized regression coefficients from multiple regression analysis of vascular measurements in relation to age, coronary risk factors and plasma hormones were determined. Differences between the groups were analyzed using one-factor ANOVA, followed by Newman-Keuls' test. A value of $p<0.05$ was considered statistically significant. Statistical analyses were performed using the SPSS ver. 11.0 software.

Results

Changes in Plasma Hormones and Vascular Measurements According to Age and Coronary Risk Factors

Plasma levels of DHEA-S declined with age, while those of estradiol, testosterone and cortisol did not significantly change according to age (Table 2). %FMD decreased slightly but not significantly ($r=-0.166$, $p=0.08$), while carotid IMT increased significantly ($r=0.337$, $p<0.01$) with advancing age.

The subjects with hypertension, hyperlipidemia or diabetes showed impaired %FMD compared to those without these diseases (hypertension, 4.0±2.4 *vs.* 5.1±2.2; hyperlipidemia, 4.3±2.3 *vs.* 5.6±2.2; diabetes mellitus, 4.0±2.8 *vs.* 5.2±2.2; $p<0.05$). %FMD in the patients taking antihypertensive agents, statins or hypoglycemic agents was comparable to or smaller than that in the patients without medical agents (hypertension, 4.0±2.5 *vs.* 4.3±2.3, n.s.; hyperlipidemia, 4.1±2.2 *vs.* 5.1±2.4, n.s.; diabetes, 3.5±2.7 *vs.* 6.5±1.5, $p<0.05$), suggesting that the favorable effects of medical treatment on endothelial function, if present, might have been lost in patients with a long history of coronary risk factors. In contrast, none of the plasma hormones were significantly changed according to coronary risk factors or medications (data not shown).

Relationship between Plasma Hormones and Vascular Measurements

First, simple correlation coefficients between plasma hormones and vascular measurements were determined. As shown in Table 2, %FMD was positively correlated with DHEA-S, whereas carotid IMT was negatively correlated with DHEA-S. There was no significant correlation between vascular measurements and other steroid hormones.

Next, age-adjusted regression coefficients were determined, because age was correlated with some of the hormones and vascular measurements, as mentioned above. The results showed that none of the hormones were significantly related to carotid IMT (Table 3). In contrast, DHEA-S was significantly related to %FMD, independent of age. Furthermore, the age-adjusted regression coefficients between DHEA-S and %FMD were 0.374 in the subjects with no coronary risk factor or medication ($p<0.05$, $n=31$) and 0.399 in those with coronary risk factors ($p<0.05$, $n=84$), most of whom were taking some medications.

Finally, multiple regression analyses were performed to exclude the influence of coronary risk factors on the relationship between hormones and %FMD. As shown in Table 4, DHEA-S was related to %FMD, independent of age, body mass index, hypertension, hyperlipidemia, diabetes mellitus and current smoking (Model 1), and were independent of age, body mass index, systolic blood pressure, total cholesterol, high-density lipoprotein (HDL) cholesterol, fasting plasma glucose and current smoking (Model 2). Furthermore, the relationship between DHEA-S and %FMD was significant after addition of carotid IMT to the model (Model 3), suggesting that the relationship was not attributable to the effect of DHEA(-S) on the development of subclinical atherosclerosis. Also, the statistical result was unchanged after addition of nitroglycerin-induced dilation of the brachial artery to the model (Model 4), indicating that DHEA-S is related to endothelial function independent of arterial compliance. Estradiol,

Table 4. Regression Coefficients between %FMD and Plasma Hormones Adjusted for Coronary Risk Factors

	Model 1	Model 2	Model 3	Model 4
Estradiol	0.065	0.042	0.025	0.094
Testosterone	0.036	0.052	0.028	0.148
DHEA-S	0.344‡	0.291*	0.263*	0.250*
Cortisol	0.150	0.127	0.099	0.110

Standardized regression coefficients by multiple regression analyses with %FMD as a dependent variable and coronary risk factors (covariates used in each analysis are listed below) and each of the hormones as independent variables are shown. ‡$p<0.01$, *$p<0.05$. Model 1: age, body mass index, hypertension, hyperlipidemia, diabetes mellitus, and current smoking. Model 2: age, body mass index, systolic blood pressure, total cholesterol, high-density lipoprotein cholesterol, fasting plasma glucose and current smoking. Model 3: Model 2 plus carotid intima-media thickness. Model 4: Model 2 plus percent nitroglycerin-induced dilation of brachial artery. %FMD, percent flow-mediated dilation of brachial artery.

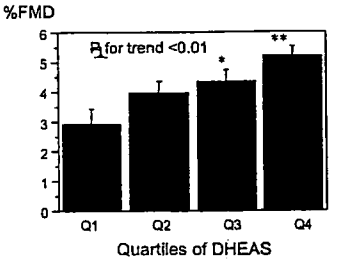

Fig. 1. *Percent flow-mediated dilation of the brachial artery (%FMD) according to quartiles of serum dehydroepiandrosterone-sulfate (DHEA-S). Values are expressed as the means±SEM. *$p<0.05$, **$p<0.01$ vs. Q1.*

testosterone and cortisol were not significantly related to %FMD in similar multivariate analyses (Table 4). As shown in Fig. 1, %FMD showed a stepwise increment according to quartiles of DHEA-S, and %FMD in the highest quartile of free testosterone was 1.8-fold higher than that in the lowest quartile (5.3±1.3 *vs.* 2.9±2.0, $p<0.01$).

All the variables except hormones were mandatorily incorporated into the multiple regression models shown in Table 4, because they are classical coronary risk factors. In forward and backward stepwise models including age, body mass index, hypertension, hyperlipidemia, diabetes mellitus, smoking and the four steroid hormones, DHEA-S, hypertension and hyperlipidemia (β=0.390, −0.224 and −0.295, respectively) were selected as significant variables. Next, in forward and backward stepwise models including age, body mass index, systolic blood pressure, total cholesterol, HDL cholesterol, fasting plasma glucose, smoking and the four steroid hormones, only DHEA-S and glucose were selected as significant variables (β=0.330 and −0.325, respectively). Taken together, these results show that DHEA-S was consistently related to %FMD in the multivariate analyses.

Discussion

In this cross-sectional study, plasma DHEA-S levels were positively correlated with %FMD, a surrogate marker of clinical atherosclerosis that reflects endothelial function (*18*, *19*). Adjustment for potential confounders such as age, coronary risk factors and nitroglycerin-induced dilation of the brachial artery had little influence on the results. These results suggest that endogenous DHEA(-S) plays a vasoprotective role in postmenopausal women.

The majority of DHEA exists as the sulfated form (DHEA-S) in circulation, and its concentration is reported to be 100- to 500-fold higher than that of testosterone and 1,000- to 10,000-fold higher than that of estradiol (*4*), suggesting an important physiologic role of DHEA(-S). Plasma DHEA-S levels do not drop during the menopause transition, but decline with aging (*1*, *2*, *20*). A recent study has shown a positive correlation between DHEA-S levels and the duration of reactive hyperemia, another less specific marker of endothelial function compared to FMD, in young women (26±6 years old, n=27) with polycystic ovary syndrome (*21*). In postmenopausal women, however, a correlation between DHEA-S levels and endothelial vasomotor function has not yet been reported. On the other hand, two studies have reported the effects of DHEA supplementation on FMD in postmenopausal women (*12*, *22*). Williams *et al.* (*12*) showed a significant increase in FMD after the 12-week administration of DHEA (50 mg/day) in healthy postmenopausal women, while Silvestri *et al.* (*22*) observed no effect using the same dose of DHEA for 4 weeks in postmenopausal women with increased cardiovascular risk. In middle-aged men, it has been reported that low-dose DHEA supplementation (25 mg/day) for 12 weeks improved FMD [23]. Experimental studies have also demonstrated the effects of DHEA on endothelial proliferation (*12*) and endothelial nitric oxide synthase (*26*), further supporting the notion that DHEA(-S) has a protective effect on the endothelium.

At this moment, intrinsic receptors for DHEA(-S) have not been identified. Thus, DHEA(-S) may exert its activity after conversion to androgens or estrogens *via* androgen or estrogen receptors, although in previous reports neither an androgen receptor antagonist nor an estrogen receptor antagonist blocked some of the *in vitro* effects of DHEA on endothelial cells (*12*, [24]. Testosterone supplementation in men [25,26] and estrogen replacement in postmenopausal women (*27*, *28*) improves endothelial vasomotor function. Activation of endothelial nitric oxide synthase and stimulation of nitric oxide production *via* androgen and estrogen receptors (*29*) might be attributable to the effect of DHEA(-S) on FMD. For