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Umegaki H.	Pathophysiology of cognitive dysfunction in older people with type 2 diabetes: vascular changes or neurodegeneration?	Age and Ageing	39(1)	8-10	2010

IV. 研究成果の刊行物・別刷

Dose-Dependent Modulatory Effects of Insulin on Glucose-Induced Endothelial Senescence In Vitro and In Vivo: a Relationship between Telomeres and Nitric Oxide

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ABSTRACT

The elderly are prone to postprandial hyperglycemia that increases their cardiovascular risk. Although insulin therapy is necessary to treat diabetes, high plasma concentrations of insulin may cause the development of atherosclerosis and accelerate endothelial senescence. We assumed that high glucose causes stress-induced premature senescence and replicative senescence and examined the regulatory role of insulin in endothelial senescence and functions under different glucose conditions. Exposure of human endothelial cells to high glucose (22 mM) for 3 days increased senescence-associated-β-galactosidase activity, a senescence marker, and decreased telomerase activity, a replicative senescence marker. Physiological concentrations of insulin preserved telomere length and delayed endothelial senescence under high-glucose conditions. The effect of insulin under high-glucose conditions was associated with reduced reactive oxygen species and increased nitric oxide (NO). Small interfering RNA targeting endothelial NO synthase reduced the antisenescence effects of insulin. Physiological concentrations of insulin also reversed high glucose-induced increases in p53 and vascular cell adhesion molecule-1 and decreases in senescence marker protein-30. On the other hand, when insulin was given at any concentrations under normal glucose or at high concentrations under high glucose, its ability to promote cellular senescence was unrelated to endothelial NO. Finally, streptozotocin-induced diabetes showed more senescent cells in the aortic endothelium of aged rats compared with age-matched control and insulin-treated animals. Conclusively, the regulatory effects of insulin on endothelial senescence were modulated by the glucose environment. These data may help explain insulin's complicated roles in atherosclerosis in the elderly.

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Introduction

Diabetes mellitus is a common and serious metabolic disease worldwide. It affects 240 million people, and those numbers are still increasing. Diabetic patients have a ~2.5- to 4-fold increased risk of cardiovascular events, and their life spans can be shortened by as many as 10 years (Fox et al.,

2004). In the elderly, before diabetes is diagnosed, postprandial hyperglycemia is common because of the delay in insulin secretion to food intake, and their cardiovascular risk increases (Rodriguez et al., 1996).

Diabetes mellitus and aging are closely associated with atherosclerosis, an inflammatory disease characterized by endothelial dysfunction and oxidative stress, such as reactive oxygen species (ROS), and leads to the destruction of nitric oxide (NO) (Hayashi et al., 1991; Ignarro and Napoli, 2004). Insulin is necessary to treat diabetes; however, elevated insulin levels might be associated with cardiovascular events (Murcia et al., 2004; Muniyappa et al., 2007). Insulin can

ABBREVIATIONS: ROS, reactive oxygen species; NO, nitric oxide; NOS, NO synthase; eNOS, endothelial NOS; IGF, insulin-like growth factor; PI3-K, phosphatidylinositol 3-kinase; L-Arg, L-arginine; L-NAME, N^G-nitro-L-arginine methyl ester; AICAR, 5′-aminoimidazole-4-carboxamide ribonucleoside; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; HUVEC, human umbilical venous endothelial cell; HAEC, human aortic endothelial cell; SA-β-gal, senescence-associated-β-galactosidase; NOx, nitrite and nitrate; siRNA, small interfering RNA; VCAM-1, vascular cell adhesion molecule-1; STZ, streptozotocin; SMP30, senescence marker protein-30; CM-H$_2$DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester; VE, vascular endothelial; NG, normal glucose; HG, high glucose; EHG, extremely high glucose.

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progress atherosclerosis through the migration and proliferation of smooth muscle cells (Stout, 1990). Therefore, insulin is a double-edged sword in the treatment of diabetics; it reduces oxidative stress and glucose toxicity, but it contributes to the atherogenic process.

Insulin and insulin-like growth factor-1 (IGF-1) signaling promotes aging in *Caenorhabditis elegans* and mice through the activation of phosphatidylinositol 3-kinase (PI3-K) and FOXO/DAF16 pathways (Miyauchi et al., 2004). Recent clinical trials, such as Action to Control Cardiovascular Risk in Diabetes, warrant strict glucose control in the diabetic elderly because of the possible increased risk of cardiovascular diseases. However, the contribution of insulin is unclear. The detrimental effects of insulin may be evident in the elderly, suggesting an important, but unclear, role of insulin signaling in both atherosclerosis and aging (Action to Control Cardiovascular Risk in Diabetes Study Group et al., 2008).

Cellular senescence could contribute to aging processes, such as atherosclerosis (Minamino and Komuro, 2007). Senescent endothelial cells are found in human atherosclerotic lesions but not in nonatherosclerotic lesions (Hayashi et al., 2007), which suggests that cellular senescence contributes to atherogenesis. However, the role of diabetes is not fully understood.

Senescence ensuing from cell replication is termed "replicative senescence," which implicates an intrinsic mechanism responsible for the life span of somatic cells (Hayashi et al., 2008). Mitosis-related telomere shortening is critical. A decrease in telomerase activity precedes telomere shortening (Bosnar et al., 1998). The senescence response is elicited by many stressful stimuli, such as DNA damage (McLaren et al., 2004) and ROS (Parrinello et al., 2003). Human cells exposed to these stressors display features of "stress-induced premature senescence" within several hours or a few days that are probably related to telomerase disorganization rather than telomere shortening per se (Yokoi et al., 2006; Minamino and Komuro, 2007).

Hyperglycemia generates oxidative stress that pushes normal endothelial cells to premature senescence (Hayashi et al., 2006; Yokoi et al., 2006). Hyperglycemia is observed ordinarily not only in diabetic individuals but also in the elderly, who display impaired glucose tolerance. This study aimed to delineate the regulatory role of insulin in endothelial senescence on cardiovascular risks. We hypothesized that insulin may act differently on endothelial senescence in a manner that can be affected by glucose concentrations and endothelial NO.

Materials and Methods

Materials. D-glucose, D-mannitol, L-arginine [L-Arg; a substrate of NO synthase (NOS)], N^G-nitro-L-arginine methyl ester (L-NAME; an NOS inhibitor), and insulin were purchased from Sigma-Aldrich (St. Louis, MO). Apocynin (an NADPH oxidase inhibitor), 5'-aminoimidazole-4-carboxamide ribonucleoside (AICAR; an AMP-activated protein kinase agonist), and LY294002 (2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; a PI3-K inhibitor) were purchased from Calbiochem (San Diego, CA).

Cell Culture. We used two types of endothelial cells. Human umbilical venous endothelial cells (HUVECs) and human aortic endothelial cells (HAECs) were purchased from Lonza Walkersville Inc. (Walkersville, MD) and cultured in endothelial cell growth medium-2 until the start of the experiment. The cells were cultured in

modified endothelial cell growth medium-2 that lacked IGF-1 but contained 2% fetal bovine serum during the experimental term. It contained only less than 10^{-12} M insulin, which was considered to have no affect on our outcome. According to our previous study (Hayashi et al., 2006), five- to seven-passage subconfluent cells were used in the experiments. Cells were harvested at subconfluence and seeded into six-well plates.

Research Design. The effects of various concentrations of insulin were examined in HUVECs or HAECs cultured under normal glucose (5.5 mM; the same as human plasma) or high glucose (22 or 31 mM) for 72 h to 28 days. Mannitol was used to rule out the effect of osmotic pressure. Senescence-associated-β-galactosidase (SA-β-gal), telomerase activities, ROS generation, endothelial NOS (eNOS) expression, and NOx (nitrite and nitrate) were assessed. To elucidate the possible mechanisms of the effects of insulin, L-Arg, L-NAME, apocynin, AICAR, LY294002, and small interfering RNA (siRNA) targeted to eNOS were treated during the same term as insulin.

Pulmonary Microvascular Leakage. SA-β-gal activity was measured by flow cytometry as described previously (Kurz et al., 2000). After the experiment, HUVECs were incubated with C_{12}FDG (fluorogenic substrate 5-dodecanoyl-aminofluorescein di-β-D-galactopyranoside; 33 mM) at 37°C for 30 min. Cells were trypsinized and analyzed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ). Cytochemical staining for SA-β-gal was performed at pH 6 using the senescence detection kit (Bio Vision Research Products, Mountain View, CA) (Canela et al., 2007).

Human Telomerase Activity Assay. Telomerase activity was measured using the TeloTAGGG Telomerase PCR ELISA^{PLUS} kit (Roche Diagnostics, Mannheim, Germany) (Hayashi et al., 2006). This assay is based on the telomere repeat application protocol (trap) assay. Protein concentrations were determined using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA).

Human Telomere Length Assay. Telomere length was measured by fluorescence in situ hybridization using flow cytometry (Canela et al., 2007).

Western Blot Analysis. Immunoblotting was performed as described in our previous reports (Fukatsu et al., 2007; Miyazaki-Akita et al., 2007). Samples of cell homogenate (5–10 μg) were subjected to electrophoresis on polyacrylamide gels, and proteins were transferred to polyvinylidene difluoride filter membranes. The membrane was blotted with the indicated antibodies and processed via chemiluminescence. We note that the actual immunoblot data were obtained from exactly the same samples under exactly the same conditions.

Flow Cytometric Analysis of ROS Generation. Intracellular oxidant generation was detected with the fluorescent probe, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) (Invitrogen, Carlsbad, CA) (Chandra et al., 2003). Cells were incubated with CM-H_2DCFDA (10 mM) at 37°C for 30 min, and flow cytometry was performed.

Immunofluorescence and Confocal Analysis. Cultured endothelial cells were fixed with a 4% formalin solution and exposed to the fluorescent antibody overnight either with an anti-vascular cell adhesion molecule-1 (VCAM-1) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or an anti-VE-cadherin antibody (Alexis Biochemicals, San Diego, CA). Endothelial cells were treated with an ROS detection reagent (CM-H_2DCFDA; Invitrogen). The nucleus was counterstained with Hoechst 33258 (Nacalai Tesque, Kyoto, Japan). Images were observed using a Leica (Wetzlar, Germany) TCS-SP5 confocal system.

Transfection of eNOS siRNAs. siRNAs targeting human eNOS were developed in our laboratory (Miyazaki-Akita et al., 2007). Nonsilencing control siRNA (QIAGEN, Tokyo, Japan) was used as a negative control. A control with scrambled siRNA was also used as a control. The following sequences were used: 5'-CGAGGAGACUUCCGAAUCUUU-3' (sense) and 5'-PAGAUUCGGAAGUCUCCUCGUU-3' (antisense) for eNOS siRNA; 5'-UUCUUCGAACGUGUCACGUdTdT-3' (sense) and 5'-ACGUGACACGUUCGGAGAAdTdT-3' (antisense) for control siRNA. siRNA (1 nM)

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was transfected using Lipofectamine RNAiMAX (Invitrogen). After incubation for 72 h, the down-regulation of eNOS expression was confirmed by Western blotting and NOx levels.

Generation of Streptozotocin Diabetic Animal Model. We generated young (8 weeks old) and aged (82 weeks old) diabetic rats (Sprague-Dawley rats) using streptozotocin (STZ) (60 mg/kg i.p.). The control group was injected with the buffer solution alone. After we confirmed that plasma glucose levels were higher than 350 mg/dl, diabetic rats were randomly divided into two groups. The STZ-insulin group received insulin (4 IU/day s.c.), and the STZ group received saline alone. Plasma glucose levels and body weights were measured daily. After treatment for 7 days, the rats were sacrificed for measurements of SA-β-gal activity and other aging-related proteins.

Statistical Analysis. The data are presented as the mean ± S.E. Statistical analysis was performed using one- or two-way analysis of variance followed by Fisher's protected least-significant-difference test. A P value less than 0.05 was considered significant.

Results

Cellular Senescence Assessed by SA-β-Gal Activity. Both HUVECs and HAECs were examined to verify the similarity of the endothelial senescence responses to various stimuli in different types of endothelial cells. Glucose increased SA-β-gal activity in a concentration-dependent (Fig. 1, A and B) and time-dependent manner. Under normal glucose, all concentrations of insulin increased SA-β-gal activity in HUVECs and HAECs (Fig. 1, A and B). However, insulin at 10^{-10} M, a physiological concentration, prevented the increase in SA-β-gal activity that was induced by high-glucose conditions (Figs. 1 and 2A). However, treatment with supraphysiological concentrations of insulin (10^{-7} to 10^{-6} M) enhanced the high-glucose (22 mM)-induced increase in SA-β-gal activity (Figs. 1A and 2A), although insulin at 10^{-6} M did not cause further increase in SA-β-gal activity beyond that of extremely high glucose (31 mM) alone (Fig. 1A). To rule out an osmotic effect, we added 25 mM mannitol to 5.5 mM glucose and

9.5 mM mannitol to 21 mM glucose. Mannitol was without effect on cellular senescence (data not shown).

Replicative and Stress-Induced Senescence. Telomerase activity decreased significantly after 3 days of exposure to high glucose in HUVECs, and subsequently, telomere length was significantly shortened by 4 weeks, which indicated replicative senescence (Fig. 2, B and C). Physiological concentrations (10^{-10} to 10^{-9} M) of insulin prevented this decrease in telomerase activity and telomere shortening induced by high glucose (Fig. 2, B and C). However, such effects were not observed at high concentrations of insulin (10^{-8} to 10^{-6} M) (Fig. 2, B and C). The endothelial expression levels of p53, a canonical inducer of cellular senescence (Kletsas et al., 2004), and senescence marker protein-30 (SMP30), a protein that decreases with aging (Feng et al., 2004), were significantly affected by high insulin under normal and high glucose in the absence of insulin (Fig. 3, A and B). Therefore, the high-glucose-induced increase in p53 was significantly decreased and the decrease in SMP30 was significantly increased by insulin at a physiological concentration.

Phosphorylation of Akt and eNOS. No evident decrease in glucose levels in the culture medium was found, and we never detected that the glucose transporter protein GLUT4 was expressed in human endothelial cells (data not shown), which is consistent with the previous report that endothelial cells lack GLUT4 (Chisalita et al., 2006). This suggests the specificity of glucose metabolism in human endothelial cells compared with other tissues. We also investigated the effects of insulin on high-glucose-induced changes in Akt and eNOS activation in human endothelial cells. As shown in Fig. 3C, phosphorylation levels of Akt and eNOS were inhibited by high glucose, and they were prevented by insulin at both physiological and supraphysiological concentrations. These results suggest that the favorable effect of physiological insulin on endothelial senescence under high glucose cannot be attributed solely to the ability to improve the high-glucose-induced impairment of Akt/eNOS signal transduction.

A HUVECs **B HAECs**

C
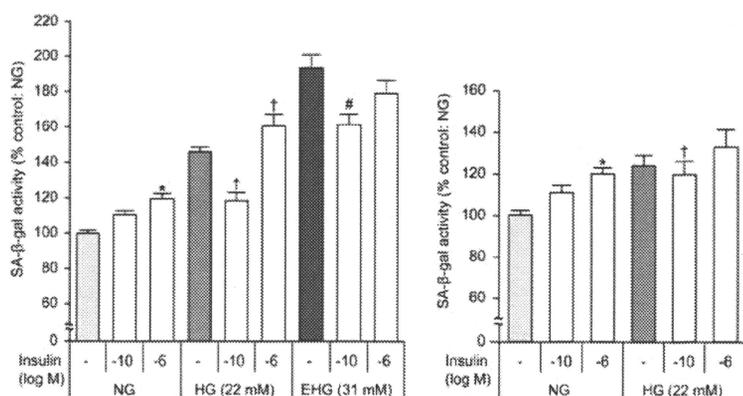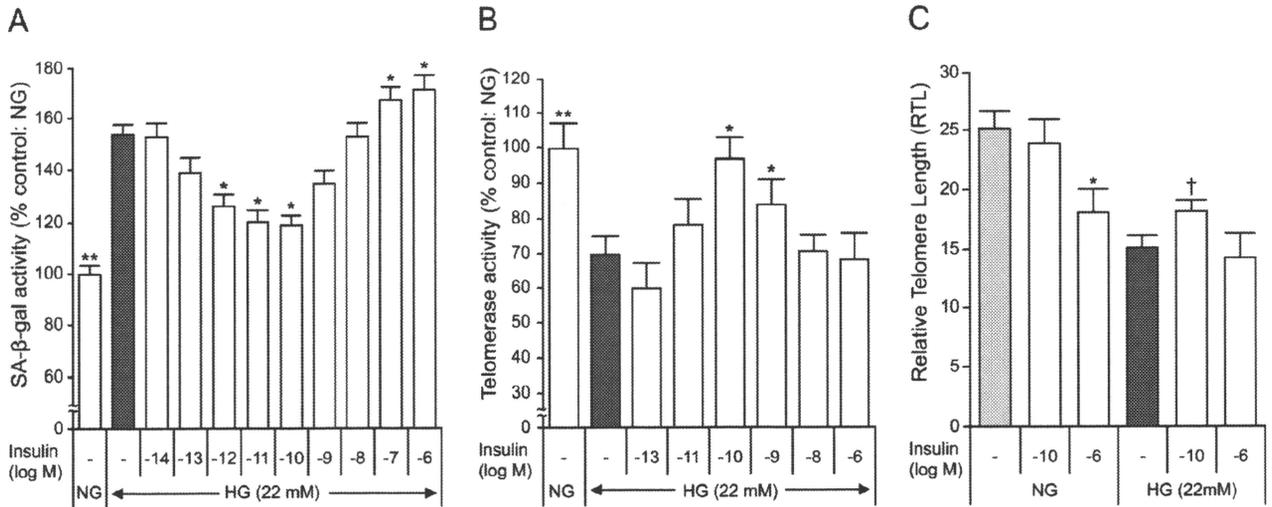
Fig. 1. Effects of glucose and insulin on senescence in HUVECs and HAECs (3 days of exposure). SA-β-gal activity was measured to evaluate cellular senescence. A, effects of low and high concentrations of insulin on SA-β-gal activity at normal (NG), high (HG), and extremely high (EHG) glucose concentrations in HUVECs (n = 6). *, P < 0.05 versus NG; †, P < 0.05 versus HG; #, P < 0.05 versus EHG. B, effects of insulin on SA-β-gal activity under NG and HG in HAECs (n = 6). *, P < 0.05 versus NG; †, P < 0.05 versus HG. C, cytochemical staining for SA-β-gal activity. NG, 5.5 mM; HG, 22 mM; EHG, 31 mM.
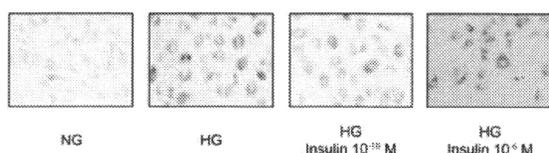
**Fig. 2.** Effects of insulin on senescence in HUVECs exposed to high glucose. A and B, concentration-dependent effects of insulin on SA-β-gal activity (3 days of exposure) (A) and telomerase activity (B) under high glucose. Telomerase activity was measured by the telomere repeat application protocol (trap) assay ($n = 6$). *, $P < 0.05$; **, $P < 0.01$ versus HG without insulin. C, effects of low and high concentrations of insulin on telomere length under normal or high glucose (28 days of exposure). Telomere length was measured to evaluate the relationship to replicative senescence ($n = 5$). *, $P < 0.05$ versus NG; †, $P < 0.05$ versus HG.
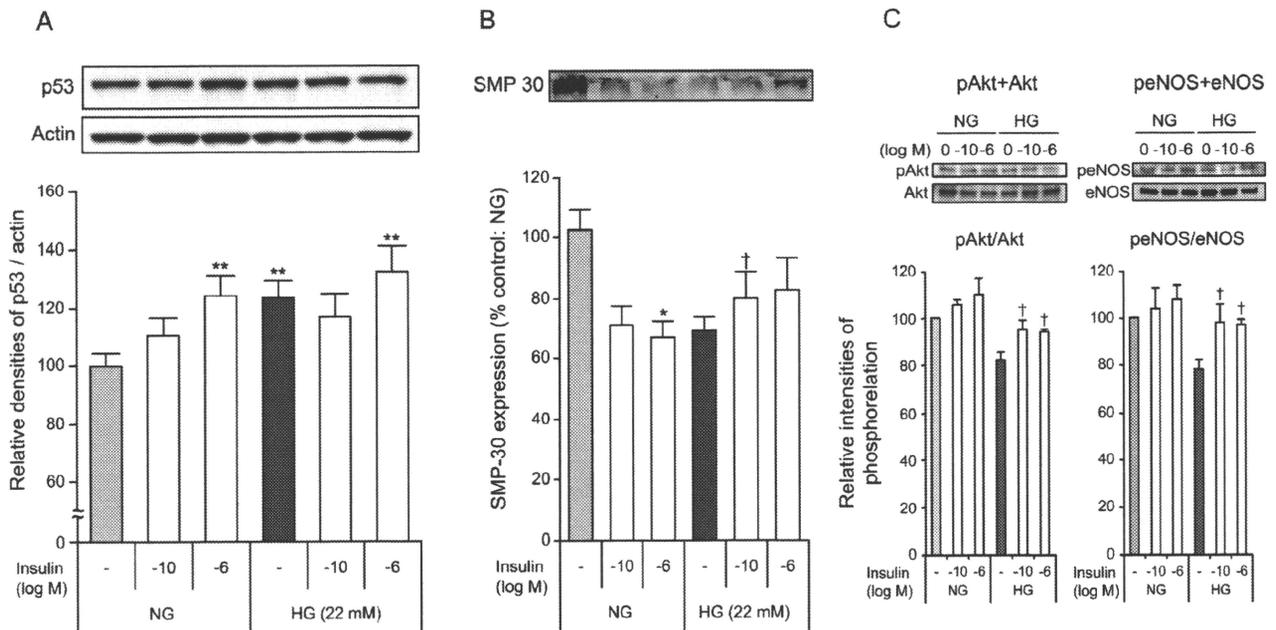
Fig. 3. Effects of insulin on p53 expression, SMP30 expression, and eNOS and Akt phosphorylation in HUVECs. A, bottom, effects of low and high concentrations of insulin on p53 expression under normal and high glucose (3 days of exposure). **, $P < 0.01$ versus NG without insulin. NG, 5.5 mM; HG, 22 mM. Top, representative Western blots of p53 and actin ($n = 6$). B, bottom, effects of low and high concentrations of insulin on SMP30 expression under normal and high glucose (3 days of exposure). *, $P < 0.05$ versus NG without insulin. †, $P < 0.05$ versus HG without insulin. NG, 5.5 mM; HG, 22 mM. Top, a representative Western blot of SMP30 ($n = 6$). C, bottom, effects of low and high concentrations of insulin on the phosphorylation of eNOS and Akt under normal and high glucose (3 days of exposure). †, $P < 0.05$ versus HG without insulin. NG, 5.5 mM; HG, 22 mM. Top, representative Western blots ($n = 6$).
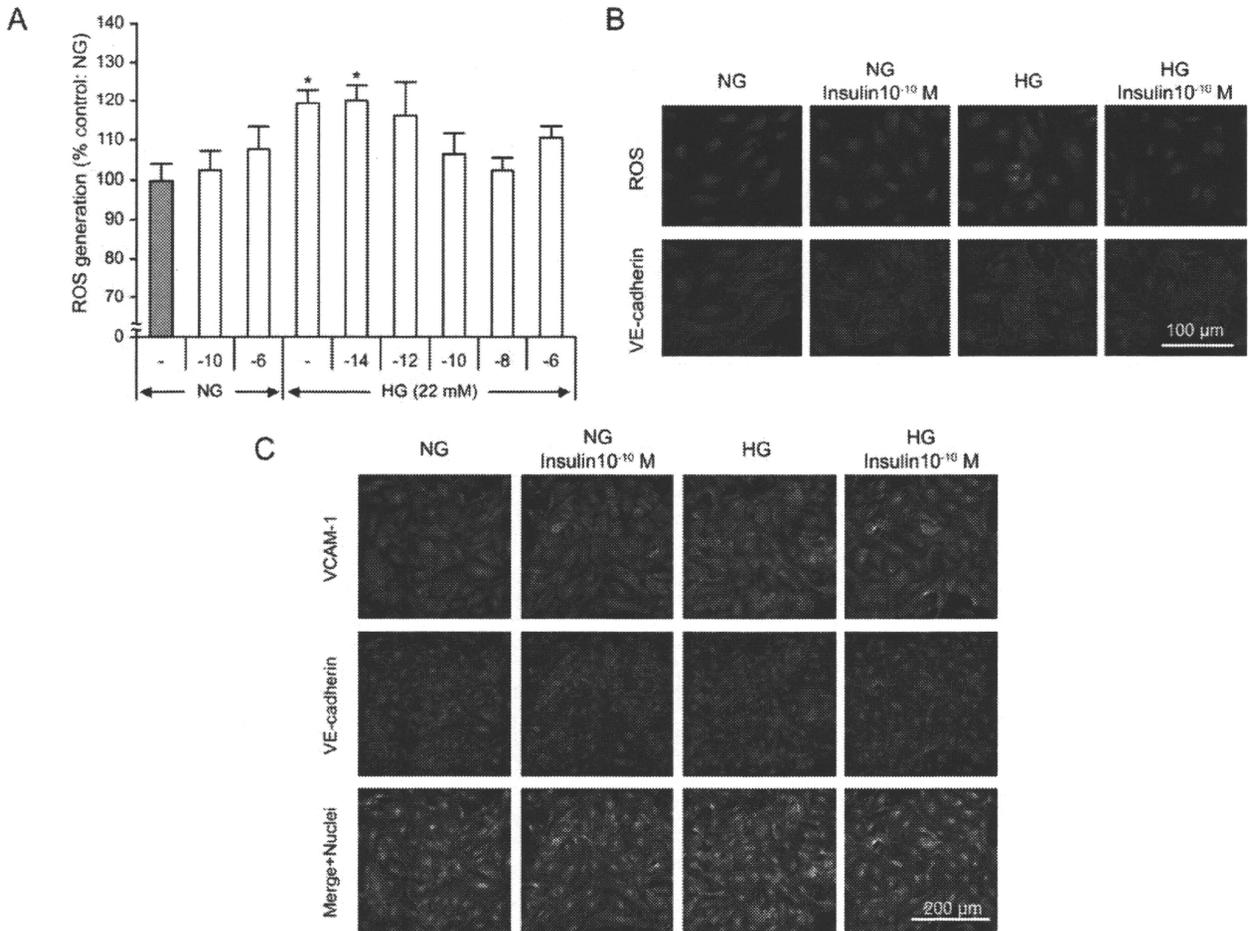
ROS Generation and VCAM-1 Expression. The exposure of HUVECs to high glucose (22 mM) for 3 days increased ROS generation (Fig. 4, A and B). Insulin did not significantly affect ROS generation under normal glucose. However, both physiological and high concentrations of insulin reduced ROS generation under high glucose (Fig. 4, A and B). The expression of VCAM-1, which is involved in the recruitment of leukocytes to inflammatory sites, under normal glucose was unchanged by

physiological insulin treatment, but it normalized the high-glucose-induced increase in VCAM-1 expression (Fig. 4C). The expression of VE-cadherin was unaffected by any of the treatments individually or combined.

Effect of NO on Cellular Senescence. L-Arg, a NOS substrate, had no effect on the SA-β-gal activity of HUVECs incubated with high glucose (Fig. 5A). L-NAME, a NOS inhibitor, significantly increased SA-β-gal activity (Fig. 5A). In contrast, apocynin,

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Fig. 4. Effects of insulin on ROS generation and VCAM-1 expression in HUVECs exposed to high glucose for 3 days. ROS generation was detected as intracellular oxidant generation by flow cytometry. Images of intracellular ROS and VE-cadherin were obtained by immunofluorescence and confocal analysis. A, concentration-dependent effects of insulin on ROS generation under normal and high glucose ($n = 5$). *, $P < 0.05$ versus NG without insulin. NG, 5.5 mM; HG, 22 mM. B, images of intracellular ROS visualization using CM-H$_2$DCFDA. Effects of 10^{-10} M insulin on ROS generation under normal and high glucose are shown. C, immunofluorescent images for VCAM-1. Effects of 10^{-10} M insulin on VCAM-1 expression under normal and high glucose are shown. In the merged images, nuclei were counterstained with Hoechst.

an NADPH oxidase inhibitor, and AICAR, an AMP-activated protein kinase agonist, inhibited SA-β-gal activity under high glucose (Fig. 5A).

Coincident with the changes in SA-β-gal activity, L-NAME further decreased telomerase activity, but apocynin and AICAR increased this activity and prevented the effects of high glucose (Fig. 5B). Apocynin decreased ROS levels under high glucose, whereas L-Arg and L-NAME had no effect on the high-glucose-induced increase in ROS (Fig. 5C).

To further substantiate the contribution of NO in mediating the effects of glucose and insulin on cell senescence, siRNA was used to specifically knock down eNOS mRNA in HUVECs. The transfection of eNOS siRNA for 72 h successfully silenced the expression of eNOS protein and reduced NOx production compared with the negative control under normal and high-glucose conditions (Fig. 5, D and E). The increases in eNOS protein (Fig. 5D) and NOx (Fig. 6) observed under high glucose in the presence of physiological insulin were significantly reduced by eNOS siRNA (Fig. 5D).

Under normal glucose, transfection with eNOS siRNA alone marginally affected SA-β-gal activity (Fig. 5F), and physiological insulin significantly increased SA-β-gal activity with eNOS

siRNA. High concentrations of insulin significantly increased SA-β-gal activity regardless of whether eNOS siRNA was applied. However, under high glucose, treatment with eNOS siRNA further significantly enhanced SA-β-gal activity and blunted the decreased activity induced by physiological concentrations of insulin. Likewise, LY294002, a PI3-K inhibitor, eliminated the inhibitory effect of physiological insulin on SA-β-gal activity under high glucose. SA-β-gal under high glucose remained elevated even in the presence of a high concentration of insulin in the absence or presence of LY294002 (Fig. 5G).

Aged Diabetic Rats and Vascular Senescence. We generated young (8 weeks old) and old adult (82 weeks old) diabetic rats using STZ. The plasma glucose levels in aged rats were 102 ± 12 mg/dl in the control group, 429 ± 117 mg/dl in the diabetic group, and 153 ± 39 mg/dl in the insulin-treated diabetic group. Insulin levels were 0.75 ± 0.46, 0.18 ± 0.10, and 3.53 ± 1.13 ng/ml, respectively. The plasma glucose and insulin levels in control, diabetic, and insulin-treated diabetic groups of young rats were not significantly different from the respective groups of aged rats (data not shown). SA-β-gal-stained cells in the aortic endothelium are shown in Fig. 7. In young rats, no significant SA-β-gal-

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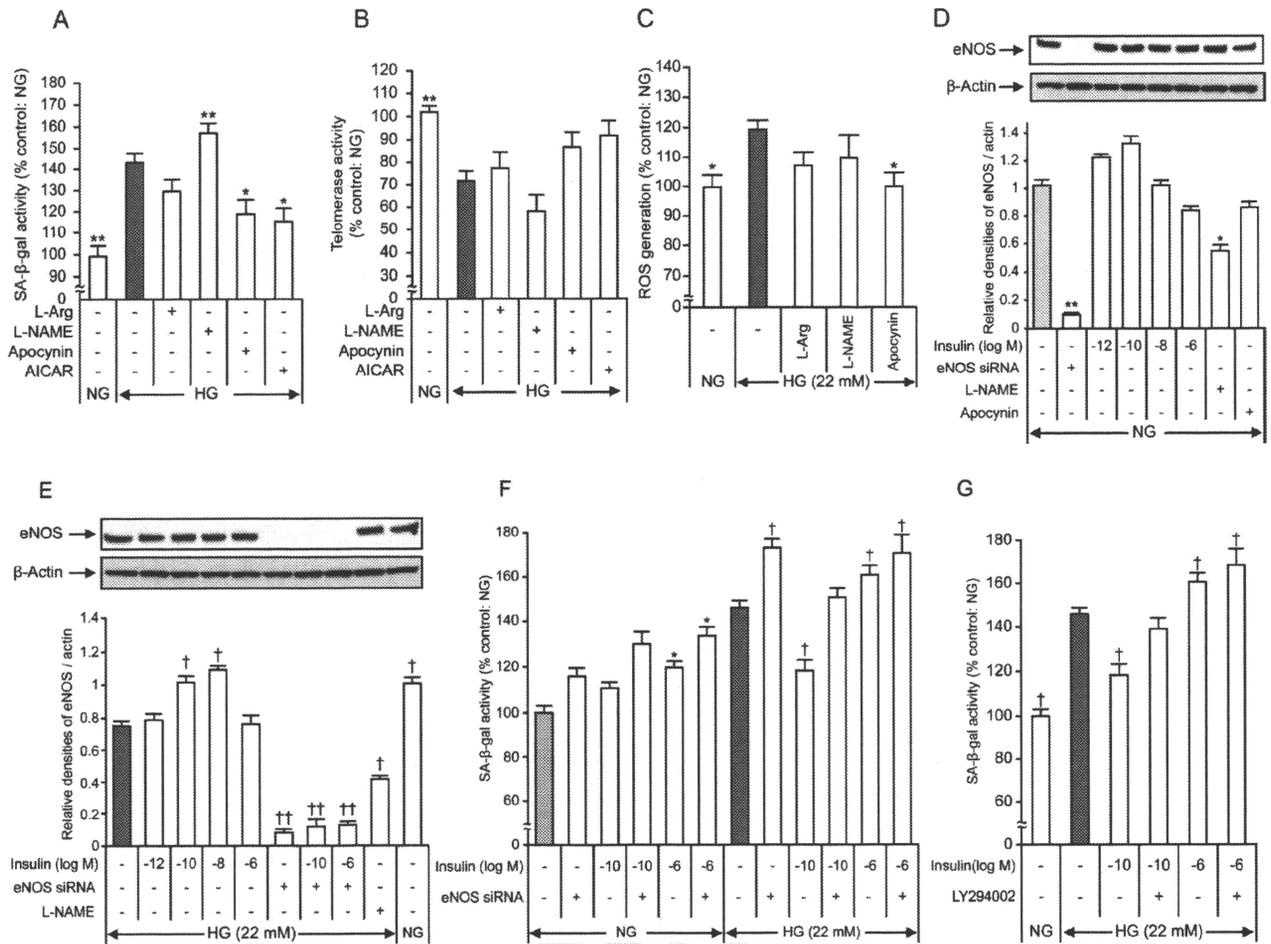
Fig. 5. Analysis of the possible mechanisms underlying the effects of high glucose and insulin in endothelial senescence in HUVECs. A, B, and C, effects of L-Arg, L-NAME, apocynin, and AICAR on the changes in SA-β-gal activity (A), telomerase activity (B), and ROS generation (C) were examined in HUVECs exposed to high glucose for 72 h ($n = 6$). *, $P < 0.05$; **, $P < 0.01$ versus HG. NG, 5.5 mM; HG, 22 mM. D and E, bottom, effects of insulin on eNOS protein expression and cellular senescence in HUVECs for 3 days. Concentration-dependent effects of insulin on eNOS protein expression under normal and high glucose are presented with eNOS siRNA transfection. For comparison, the effects of L-NAME and apocynin are shown. Nonsilencing control siRNA was used as a negative control, and scrambled siRNA was used as a control. *, $P < 0.05$; **, $P < 0.01$ versus NG. †, $P < 0.05$; ††, $P < 0.01$ versus HG. Top, representative Western blots of eNOS and β-actin ($n = 5$). F and G, modulation by eNOS siRNA and LY294002 of effects of low and high concentrations of insulin on SA-β-gal activity under normal and high glucose ($n = 5$). *, $P < 0.05$ versus NG; †, $P < 0.05$ versus HG. NG, 5.5 mM; HG, 22 mM.
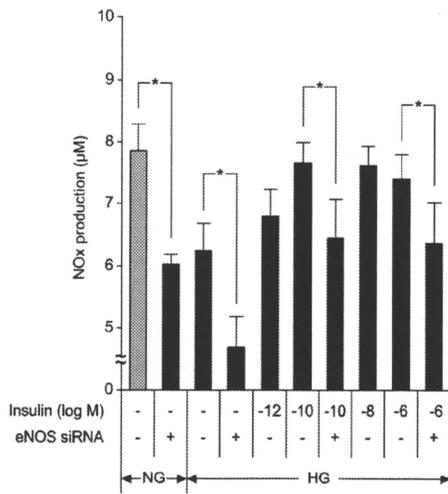
stained cells were observed in the endothelial cells of aortas in each group (Fig. 7, A and B). However, aged diabetic rats exhibited an increased ratio of SA-β-gal-stained cells, and insulin decreased the ratio to nearly the same level observed in age-matched control rats (Fig. 7, C and D).

Discussion

This study demonstrated the interactive effects of insulin and glucose on cellular senescence and both an NO-dependent and -independent regulatory pathway. High-glucose-induced replicative senescence in endothelial cells was reversed by physiological concentrations of insulin through NO-dependent and telomere-related mechanisms. We also confirmed the effect of insulin on high-glucose-induced endothelial senescence in vivo using aged STZ-induced diabetic rats with or without insulin treatment.

We were especially interested in the role of endothelial cell senescence in the development of diabetic vascular disease.

Senescent endothelial cells were accompanied by impaired endothelial function, such as NO release, which would cause the migration and adhesion of vascular monocytes as the first step of atherosclerosis. The migration and proliferation of smooth muscle cells in media is the second step and shows the features of proliferative diseases, such as atherosclerosis and diabetic microvascular disease. Telomere extension by the overexpression of telomerase does not affect stress-induced senescence (Gorbunova et al., 2002) but prevents replicative senescence (Bodnar et al., 1998). Therefore, the change in telomerase activity, subsequent to the change in telomere length induced by high glucose, reflected replicative senescence. The increase in p53 and decrease in SMP30 were similar to the change in telomerase activity. Ordinary stimuli, such as hydrogen peroxide in cellular senescence experiments, causes stress-induced senescence within 30 min and conformational changes occur in telomeres instead of telomere shortening (Breitschopf et al., 2001; Ota et al., 2008). However, a high-glucose stimulus is gentler and closer to

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Fig. 6. Effects of insulin on basal NOx production in HUVECs under normal and high glucose conditions. NOx contents in the medium were measured with an automated NO detector high-performance liquid chromatography system. Cells were incubated for 3 days under normal or high glucose. ∗, $P < 0.05$. NG, 5.5 mM; HG, 22 mM.

pathophysiological conditions, such as diabetes mellitus. High-glucose-induced endothelial senescence has the characteristics of both replicative and stress-induced senescence.

In our previous study, HUVEC proliferation rate showed a tendency to decline in senescent cells, and L-NAME inhibited the proliferation of HUVECs (Hayashi et al., 2006). High glucose also affected HUVEC proliferation, which revealed a moderate inhibition (data not shown).

In this study, high glucose reduced NO and increased oxidative stress. Its cellular senescent effects were partially reversed by the NADPH oxidase inhibitor apocynin or the AMP-activated protein kinase agonist AICAR. Apocynin is also a superoxide scavenger, but the discrimination of the role of apocynin

on the specificity of NADPH oxidase inhibition was difficult in the present study (Williams and Griendling, 2007). Oxidized low-density lipoproteins inhibit endothelial telomerase activity (Breitschopf et al., 2001). Likewise, long-term exposure of HUVECs to mild oxidative stress caused by perturbation of the glutathione redox cycle results in accelerated telomere erosion (Parrinello et al., 2003; Polytarchou and Papadimitriou, 2005). Oxidative stress may also stimulate replicative- and stress-induced senescence. It is noteworthy that individuals with shorter white blood cell telomeres showed a 2.8-fold higher coronary risk than the highest quartile for telomere length after adjusting for age (Brouilette et al., 2003). Lifestyle and atherosclerotic risk affects telomere length in blood cells. We showed the interactions of glucose and insulin on telomere length, which may lead to changes in coronary risk burden. VCAM-1 is activated during inflammatory processes and plays an important role in atherosclerosis, reflects endothelial senescence induced by high glucose and insulin, and identifies the close relationship between atherosclerosis and endothelial senescence.

In this study, physiological concentrations of insulin accelerated cellular senescence under normal glucose, but they retarded it under high glucose. Under normal glucose, telomerase activity can be post-transcriptionally regulated by various molecules, including protein kinase C, extracellular signal-regulated kinase 1/2, and Akt/protein kinase B, in endothelial cells (Miyauchi et al., 2004). The phosphorylation of Akt leads to the phosphorylation and inactivation of forkhead transcription factor FOXO3a, which consequently decreases MnSOD and increases ROS (Miyauchi et al., 2009). This mechanism is speculated for insulin under normal glucose and, it is noteworthy that the NO-mediated reaction is not large under normal glucose. However, physiological insulin retarded the senescence in an NO-dependent manner under high glucose because eNOS siRNA and inhibitors of

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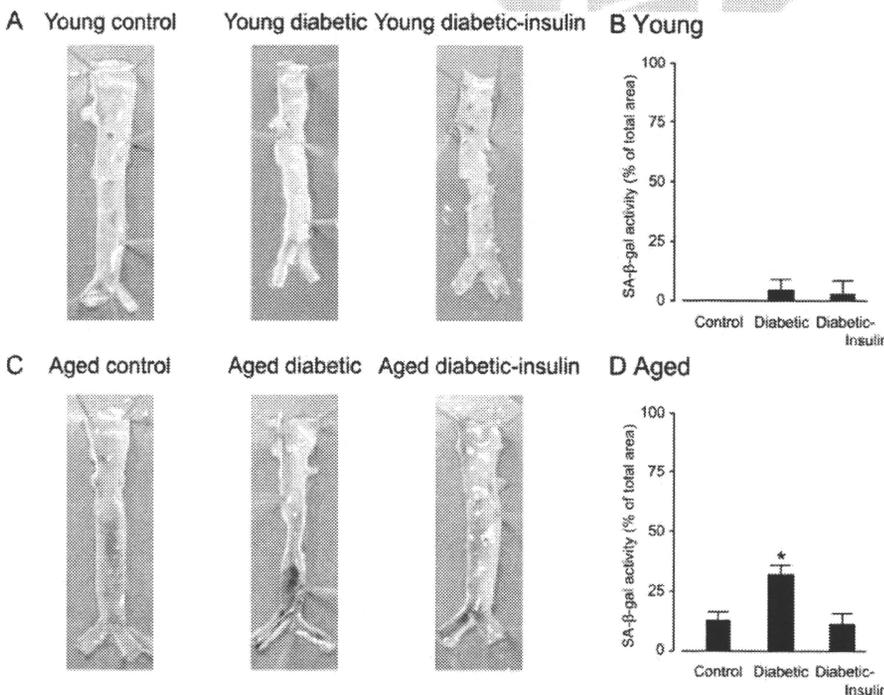
Fig. 7. SA-β-gal activity in diabetic rat vessels. Diabetes was induced in young (8 weeks old) and aged rats (82 weeks old) by an STZ injection. SA-β-gal-positive staining was observed in the intimal side of aortas of aged diabetic rats. Insulin treatment for 7 days reduced its staining. A, representative photographs of SA-β-gal-positive staining in the intimal side of aortas of young rats. B, relative ratio of SA-β-gal positively stained cells in the intimal side of aortas of young rats. C, representative photographs of SA-β-gal-positive staining in the intimal side of aortas of aged rats. D, relative ratio of SA-β-gal positively stained cells in the intimal side of aortas of aged rats. ∗, $P < 0.05$ versus control.

the PI3-K pathway eliminated the antisenescence effects of physiological insulin. Although an effect of insulin on eNOS has been reported, little is known regarding its effect on cellular senescence under high glucose. Plasma insulin levels are variable ($\sim10^{-8}$ M) because of eating and other stimuli, including chemical injections. High concentrations of insulin ($\sim2\times10^{-7}$ M) are observed temporarily after an injection of large insulin doses in some diabetic patients (Epel et al., 2004). These plasma concentrations may be similar to the concentrations in the endothelial cells environment in our study.

Another finding of this study is that high concentrations of insulin promoted senescence independently of glucose concentrations. The mechanisms of this effect may differ from the underlying action of physiological insulin. The effect of a high concentration of insulin on the high-glucose-induced impairment of eNOS phosphorylation was the same as that of a physiological concentration of insulin. The concentrations of insulin at $>10^{-8}$ M activate not only insulin receptors but also IGF receptors (Abu-Lebdeh et al., 2006). IGF signaling promotes senescence and shortened life spans in *C. elegans* and mice. Insulin promotes endothelial senescence, as determined by indirect assays (e.g., p53/p21 transcriptional activity) (Miyauchi et al., 2004), at normal glucose levels, a concept that is supported by the results of this study. The effect of supraphysiological insulin on the IGF receptor pathway may mask its insulin receptor-mediated, eNOS-dependent beneficial action on endothelial cell senescence. The results with supraphysiological concentrations of insulin would provide some insight into the pathophysiology of insulin resistance.

These dual effects of insulin on cellular senescence have implications for how the concentration of insulin needed for control of glucose in diabetics may contribute to endothelial damage and promote vascular disease. Insulin may contribute to the antiatherogenic effect and the pathogenesis of atherosclerosis as a result of insulin resistance and the consequent high concentrations of insulin.

Diabetic macroangiopathy may occur under the same conditions as cellular senescence with increased superoxide from NADPH oxidase and an impairment of NO production (Thomas et al., 1995). We found a significant effect of the NADPH oxidase inhibitor apocynin on cellular senescence under high glucose. However, apocynin may have the potential to be an antioxidant by itself (Heumüller et al., 2008). From this standpoint, the results with apocynin may be associated with an increase in NO bioavailability rather than a specific inhibition of NADPH oxidase.

ROS, such as O_2^-, decrease the telomerase activity that precedes replicative senescence, and this may be caused by the actions of NADPH oxidase and the uncoupling of eNOS (Thomas et al., 1995). However, Akt, which is phosphorylated by NO, maintains human telomerase in an active state in the nucleus, thereby preventing telomere shortening (Guzik et al., 2002). In this study, physiological insulin activated telomerase by an NO/Akt-dependent mechanism under high glucose.

Finally, we found that aged diabetic rats showed greatly increased SA-β-gal-positive staining in aortas and that insulin treatment decreased the staining to nearly the same level observed in age-matched control rats. We have previously demonstrated significant SA-β-gal-positive staining in ath-

erosclerotic legions of the intimal side of human thoracic aorta (Hayashi et al., 2006). The question remains as to why staining was seen in abdominal and not in thoracic aortas of aged diabetic rats in the present study. At present, we do not have a clear understanding of this observation. The significance of this observation awaits further study.

The present study highlighted the effect of glucose and the concentration-dependent effects of insulin on endothelial senescence. High-glucose-induced endothelial senescence had the characteristics not only of stress-induced senescence but also of replicative senescence. These results give credence to the notion that physiological concentrations of insulin delay cellular senescence through an NO-dependent and telomere-related mechanism and may retard atherosclerosis formation under high glucose. This NO-dependent action of insulin may result from an interference with the redox balance of endothelial cells (Kang et al., 1999). In contrast, all concentrations of insulin under normal glucose or high concentrations of insulin under high glucose promoted cellular senescence in an eNOS-independent manner. These unique dual effects of insulin offer an important clue for the pathophysiological basis of endothelial cell senescence in diabetes and aging.

Authorship Contributions   AQ: K

Participated in research design: Hayashi, Iguchi, Ignarro, and Hattori.

Conducted experiments: Matsui-Hirai, Hayashi, Yamamoto, Ina, Maeda, and Kotani.

Performed data analysis: Matsui-Hirai, Hayashi, Yamamoto, and Hattori.

Wrote or contributed to the writing of the manuscript: Matsui-Hirai, Hayashi, and Hattori.

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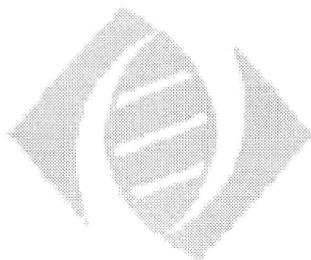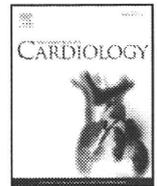
A hydroxymethylglutaryl coenzyme a reductase inhibitor improves endothelial function within 7 days in patients with chronic hemodialysis

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ABSTRACT

Background: Atherosclerosis-related diseases are leading causes of morbidity among patients undergoing hemodialysis. The effects of hydroxymethylglutaryl coenzyme A reductase inhibitors (statins) on the endothelial function of hemodialyzed patients are not known.

Methods and results: For 16 weeks, we prescribed simvastatin (low dose: 5 mg or moderate dose: 10 mg) to 28 patients (low dose: $n = 14$, 61.2 ± 8.6 years, moderate dose: $n = 14$, 60.8 ± 10.2 years) and chose 9 patients (61.5 ± 5.2 years) without prescriptions as controls. We compared the effects of statin on lipids, flow-mediated endothelium-dependent and nitroglycerin-induced endothelium-independent dilatation (%FMD, %NTD), and markers of oxidant stress and atherosclerosis. Serum HDL-cholesterol and triglycerides did not change significantly in any of the three groups; however, LDL-cholesterol was decreased at 16 weeks in both simvastatin groups. The %FMD and plasma NOx increased at 1 and 16 weeks in both statin groups, but not in the control group ($P<0.01$). The %NTD did not change. Oxidized LDL, VCAM-1, and 8-isoprostane decreased significantly after 16 weeks in both statin groups; however, TNF-α and interleukin 6 did not change. In the control group, no significant changes in these parameters were observed. Multiple regression analyses showed that the (short) period of hemodialysis and (young) age are significant factors associated with %FMD improvement.

Conclusions: A statin improved impaired endothelial function in the arteries of chronic dialysis patients, in part by enhancing NO bioavailability within one week. Improved endothelial function is in line with the anti-atherosclerotic effects observed in patients undergoing chronic hemodialysis.

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1. Introduction

Atherosclerosis-related diseases such as myocardial infarction and ischemic heart disease-related heart failure are the leading causes of morbidity among patients undergoing hemodialysis in developed countries, such as the United States or Japan [1]. It is well known that lipid-lowering therapy, especially the use of hydroxymethylglutaryl coenzyme A reductase inhibitors (statins), decreases the risk of coronary events in both primary and secondary prevention [2,3]. The anti-atherosclerotic effects of statins are thought to be attributable to changes in plasma lipid levels (i.e., decreased LDL cholesterol and increased HDL cholesterol) [2,3]. We have reported that treatment of diabetic patients with a statin resulted in improved endothelial function before the appearance of its effects on lipids, in other words, in three days [4]. Statins are known to up-regulate endothelial nitric oxide synthase (eNOS) in cultured endothelium and in the endothelium of the aorta of rabbits fed a high-cholesterol diet [5,6]. The direct action of

statins on the atherosclerotic arteries of rabbits, without lowering plasma lipids, has also been studied [6]. However, to our knowledge, there are no existing studies on this direct action in atherosclerotic arteries of patients undergoing hemodialysis. The present study focuses on the effect of statins on endothelial function, especially flow-mediated dilatation and nitric oxide (NO)-related endothelial function in hemodialytic atherosclerotic arteries of humans. We selected simvastatin, which is thought to have a long and strong tissue affinity [7]. Because we anticipated difficulty in improving endothelial function in patients undergoing hemodialysis, we examined two treatment groups, one of which received a low dose (5 mg/day, the usual dose in Japan) and the other a moderate dose of simvastatin (10 mg/day).

2. Materials and methods

2.1. Patients

Endothelial function was assessed in 37 hemodialysis patients (aged 60.6 ± 9.2 years, 17 males, and 20 females) with or without mild hyperlipidemia (LDL cholesterol, 95.9 ± 37.1 mg/dl, 72.9 to 172.8 mg/dl). The participants were ambulatory and were patients at our medical clinics (Souen Chuo Hospital, Sapporo, Japan; Nakashibetsu Public Hospital, Nakashibetsu, Japan; and Kyouritsu Hospital, Nagoya, Japan). They had not been prescribed

Table 1
Biochemical profile, *P<0.05 between low and moderate dose of statin.

	Statin (low dose)	Statin (moderate dose)	Control
Male/female (number)	6/8	6/8	4/5
Age (y.o.)	61.2 (8.6)	60.8 (10.2)	61.5 (5.2)
BMI	24.9 (2.7)	24.7 (2.8)	25.0 (2.0)
Period of H.D. (months)	**32.4 (14.1)**	**64* (44.2)**	**51 (28.5)**
Origin of H.D. (%)			
Diabetes mellitus	57.1	43.0	55.5
Glomerulonephritis	21.5	21.5	22.2
Hypertension	**0**	**7.1***	**0**
Others	14.3	28.6	22.2
Complication (%)			
Ischemic heart disease	28.7	28.7	22.2
Hypertension	64.3	64.3	55.5
Diabetes mellitus	64.3	43.0	44.4
Smoking	21.5	14.3	22.2
Medication affecting endothelial functions (%)			
ACEis/ARBs	28.7	28.7	33.3
Other anti-hypertensive drugs	50.0	57.1	55.5
diuretics	21.5	21.5	22.2
nitrates	0	0	0
Anti-platelet/anti-coagulant	0	0	0
Plasma lipids			
Total chol. (mg/dl)	135.5 (36.0)	176.1 (46.1)	149.4 (44.4)
Triglyceride (mg/dl)	99.8 (57.4)	133.8 (62.0)	106.3 (40.1)
LDL chol. (mg/dl)	**83.2 (34.3)**	**118.0* (33.8)**	**91.1 (30.2)**
HDL chol. (mg/dl)	32.4 (11.7)	32.1 (6.2)	33.4 (7.8)
Cytokines and others			
sVCAM-1 (ng/ml mg prot,)	880.2 (168.2)	1000.4 (151.9)	919.2 (139.0)
TNFα (pg/ml)	**5.0 (1.9)**	**9.8* (5.4)**	**6.3 (3.2)**
IL-6 (pg/ml)	7.9 (3.3)	3.6 (1.4)	6.3 (2.6)
8epi ISP (ng mg protein/ml)	21.4 (6.4)	40.1 (6.8)	29.1 (5.7)
Oxidized LDL (mg/ml)	24.3 (33.5)	29.4 (34.6)	27.0 (42.4)
Vascular and NO related profile			
Baseline diameter (mm)	3.01 (0.18)	2.94 (0.19)	2.95 (0.22)
Peak diameter (mm)	3.17 (0.25)	3.09 (0.25)	3.10 (0.24)
%FMD	5.6 (1.0)	4.8 (0.9)	5.2 (0.9)
GTN-induced peak diameter (mm)	3.31 (0.19)	3.24 (0.21)	3.26 (0.20)
%NTG-D	10.9 (1.1)	10.0 (0.9)	10.2 (0.9)
NOx (μM)	129.2 (16.8)	111.6 (18.1)	120.4 (15.1)

Bold emphasis and * show the significant differences between moderate dose of statin group and other two (low dose and control) groups.

The numbers are the mean ± SD, or the percent of each groups. *P<0.05 vs. data in low dose of statin treatment.

Abbreviations: Statin (low dose): simvastatin 5 mg/day group, statin (moderate dose): simvastatin 10 mg/day group. control: no prescription group.

BMI:Body Mass Index, H.D.:hemodialysis, ACE: angiotensin-converting enzyme, ARB: angiotensin receptor blocker, LDL: low-density lipoprotein, HDL: high-density lipoprotein, chol.:cholesterol, 8epi ISP: 8epiisoprostan, FMD: flow-mediated dilation, NTG-D: nitroglycerin-mediated dilation.

lipid-lowering drugs for at least 6 weeks prior to the study. None had suffered acute coronary events for at least three months prior to the study. Based on the plasma LDL levels of the patients, they were randomly assigned to treatment in the low-dose simvastatin group or the control group (baseline LDL<100 mg/dl: 5 mg/d of simvastatin; n = 14, 6 men, LDL 82.3 ± 34.3 mg/dl and no prescription; n = 9, 4 men, LDL 91.1 ± 30.2 mg/dl) or to the moderate-dose simvastatin group (10 mg/day; n = 14, 6 men, baseline LDL<100 mg/dl, LDL 118.0 ± 33.8 mg/dl). Prescription treatment lasted 16 weeks. All patients provided informed consent, agreed to the protocols, and were willing to participate in the study. Ineligible patients included those who had not taken any estrogen for >12 weeks. The study was approved by the ethics committee of Nagoya University Graduate School of Medicine. The participants had received hemodialysis for 4.1 ± 1.2 years, their average systolic and diastolic blood pressure was 127.4 ± 11.7/78.2 ± 9.8 mm Hg, and their complicated diseases included ischemic heart disease, hypertension, and diabetes mellitus (Table 1). Seven patients were smokers (Table 1). Their use of medications that can affect endothelial function, such as angiotensin converting enzyme inhibitor (ACEI), angiotensin receptor blockers (ARBs), other antihypertensive agents, and diuretics, is indicated in Table 1. Diabetic nephropathy was most frequent underlying renal disease. The patient profiles, including other backgrounds, are shown in Table 1. Patients in the moderate-dose group had received hemodialysis for a longer term, on average, and most of them suffered from hypertension.

Between the low and moderate-dose groups and the controls, there were no significant differences among other parameters, including prescribed agents.

2.2. Vascular function

Flow-mediated dilatation (FMD) and dilatation by nitroglycerin were determined according to a method described previously [8]. Briefly, the diameter of the right brachial artery was measured by a high-resolution ultrasound cardiograph (SONOS 2000, Hewlett Packard). Blood pressure was monitored every 2 min. To produce reactive hyperemia, blood flow to the forearm was prevented by inflation of the cuff on the arm to 250 mm Hg for 5 min. The diameter was measured from the anterior to the posterior interface between the media and adventitia and was calculated from 3 cardiac cycles synchronized with the R-wave peaks on the ECG. The measurement obtained at 60 s after cuff release showed maximal dilatation. The diameter change was expressed as the percent change relative to the diameter during the initial resting scan (%FMD). Fifteen minutes later, a resting scan was recorded and a sublingual nitroglycerin spray (300 μg, Toa Eiyou Co.) was administered. Three minutes later, the last scan was performed. The diameter change was expressed as the percent dilatation by nitroglycerin (%NTD). In our study, the interobserver variability for repeated measurements of resting arterial diameter was 0.05 ± 0.02 mm. The intraobserver variability for repeated measurements of resting arterial diameter was 0.02 ± 0.02 mm. In other words, the reproducibility (<0.1% difference) of the %FMD was greater than 96.3%. Vascular function was studied before commencing treatment, and then after 1 week and 16 weeks of treatment; it was studied in the morning of the day of hemodialysis, and it was performed just before hemodialysis during overnight fast status.

2.3. Blood sampling

Blood sampling was performed on the morning of the ultrasound examination (day of hemodialysis) under overnight fast status. Serum total cholesterol, triglyceride, and HDL cholesterol concentrations were measured [9]. Plasma nitrite and nitrate levels (NO_2^- and NO_3^-) were measured with an automated NO detector/high-performance liquid chromatography system (ENO10, Eicom Co., Kyoto, Japan), as previously reported [10]. In brief, nitrite and nitrate levels in the patient's plasma were separated by a reverse-phase separation column, and nitrate was reduced to nitrite in a reduction column. Nitrite was mixed with a Griess reagent, and the absorbance at 540 nm was measured by a flow-through spectrophotometer. The concentration of interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), soluble vascular cell adhesion molecule 1 (sVCAM-1), and 8-isoprostane (8-epi-prostaglandin F2) were assessed by ELISA kits (Cytoscreen Immunoassay Kit, Bioxytech 8-isoprostane assay kit, Oxis International, Inc). Plasma Ox-LDL was assayed using a Kyowa Medex MX kit (Kyowa Medex, Inc., Tokyo), which is a sandwich-type enzyme immunoassay using anti-oxidized phosphatidylcholine monoclonal antibody (DLH3) and anti-human apolipoprotein-B antibody [11,12].

2.4. Safety measures

All adverse events were recorded at each examination. Physical examinations, hematology, and serum chemistry assays (liver and renal function and creatine phosphokinase) were conducted throughout the study.

2.5. Statistical analyses

Data are presented as the mean ± SD of each group of patients. P<0.05 was considered to indicate statistical significance in all analyses. All statistical analyses were performed using JMP software (version 6, SAS Institute Inc., Cary, NC). Differences between categorical baseline characteristics were tested by the chi-square test. In addition, the parameters of interest were tested for statistical difference by ANOVA between the three different groups (low dose, moderate dose, and control groups).

Multivariate logistic regression analyses were done with adjustment for baseline characteristics such as sex.

3. Results

Table 1 shows the baseline profiles for each group. The period of hemodialysis, LDL cholesterol, and TNF-α were different between subjects receiving low (5 mg/day) doses of simvastatin or subjects in the control group and subjects receiving moderate (10 mg/day) doses of simvastatin (Table 1). No other differences were observed in the values for each condition shown in Table 1. Serum lipid concentrations (total cholesterol, triglycerides, and HDL cholesterol) remained unchanged in all patients in response to 1 week of treatment with simvastatin, and LDL cholesterol was decreased at 16 weeks in both statin groups (Table 2). The sVCAM-1 level decreased significantly at 1 week in both statin treatment groups (especially in the low-dose group), but not in the control group (Table 2). However, TNF-α and IL-6 did not change during the course of the experiment (Table 2). No abnormal data were noted in the other biochemical measures, including creatine phosphokinase levels, throughout the treatment term in either group (data not shown).

The endothelium-dependent flow-mediated dilatation (%FMD) in those receiving simvastatin significantly increased at 1 week in both statin groups (low dose: 5.7% to 8.7% in 1 week, P<0.01, and 10.1% in 16 weeks, P<0.001, moderate dose: 4.5% to 5.7% in 1 week, P<0.01, and 7.9% in 16 weeks, P<0.001 Fig. 1A). No difference in the response to nitroglycerin (%NTG) was demonstrated after 16 weeks of treatment (Fig. 1B). The %FMD

Table 2
Change of lipids and cyotokine concentrations by statin treatment.

Statin treatment	Low dose (5 mg/day)			Moderate dose (10 mg/day)			Control		
	Before	1 wk	16 wks	Before	1 wk	16 wks	Before	1 wk	16 wks
Total chol. (mg/dl)	135.5 ± 36.0	122.1 ± 46.3	120.3 ± 41.4	171.1 ± 46.1	151.0 ± 45.4	**128.2* ± 23.1**	149.4 ± 42.4	153.1 ± 49.1	152.5 ± 48.5
Triglycerides (mg/dl)	99.8 ± 57.4	95.2 ± 52.5	101.4 ± 48.5	133.8 ± 62.0	116.7 ± 55.7	103.0 ± 44.1	108.3 ± 40.6	113.2 ± 52.1	115.0 ± 54.3
HDL chol. (mg/dl)	32.4 ± 11.7	34.2 ± 12.8	35.3 ± 13.6	32.1 ± 6.2	31.8 ± 7.1	35.8 ± 9.4	33.1 ± 7.9	32.9 ± 7.2	33.6 ± 9.0
LDL chol. (mg/dl)	83.2 ± 34.3	71.1 ± 33.0	**64.7* ± 28.3**	109.6 ± 33.8	89.0 ± 33.7	**74.5* ± 30.6**	93.5 ± 31.2	94.1 ± 35.3	95.0 ± 32.8
sVCAM-1 (ng/ml mg protein)	880.2 ± 168.2	**686.2* ± 132.1**	**619.2* ± 210.4**	1027.4 ± 151.9	798.8 ± 115.4	**765.6* ± 115.1**	922.6 ± 137.2	938.2 ± 166.0	940.1 ± 169.1
TNFα (pg/ml)	5.0 ± 1.9	5.3 ± 2.3	4.5 ± 1.1	9.1 ± 5.4	9.0 ± 5.4	8.9 ± 3.1	6.6 ± 3.4	6.4 ± 3.7	6.4 ± 3.1
IL-6 (pg/ml)	7.9 ± 3.3	7.7 ± 2.5	7.9 ± 2.3	4.6 ± 1.4	4.9 ± 2.1	5.6 ± 2.0	6.5 ± 2.6	6.6 ± 2.5	6.4 ± 2.8

Bold emphasis and * show the significant differences vs. value in before treatment.
Low dose(5 mg/day): simvastatin 5 mg/day group, moderate dose(10 mg/day): simvastatin 10 mg/day group. Control: no prescription group. The numbers are the mean ± SD. *P<0.05 vs. the value in before treatment. Abbreviations: low dose (5 mg/day): simvastatin 5 mg/day group, moderate dose/10 mg/day): simvastatin 10 mg/day group. Control: no prescription group.
LDL: low-density lipoprotein, HDL: high-density lipoprotein, chol.:cholesterol.
Before: before treatment, 1 wk; treatment with simvastatin for 1 week, 16 wks; treatment with simvastatin for 16 weeks.

after 16 weeks of treatment in both simvastatin groups tended to be higher than in patients in both groups at week 1 (Fig. 1A). In the control group, no changes were observed in %FMD or %NTG. There was no significant relationship between the degree of LDL lowering and improved endothelial function; this may suggest a direct effect of statin other than its lipid-lowering effect. However, the basal conditions, such as plasma LDL cholesterol and the period of hemodialysis, were different between the low and moderate statin groups (Table 1), and direct comparison between these two groups was difficult. The plasma nitrite/nitrate (NOx) levels also tended to become higher in patients receiving simvastatin (Fig. 2A), and low-dose simvastatin administration also caused an increase in NOx (mM) (120.4 ± 15.6 in 0 week, 77. 2 ± 5.2 in 1 week, 159.4 ± 7.61 in 16 weeks) (P<0.05). The 8-Epi-isoprostan (ng mg protein/ml)was decreased at 1 and 16 weeks in both statin treatment groups (low dose: 21.4 ± 6.4 in 0 week, 13. 2 ± 2.6 in 1 week, 10.1 ± 2.4 in 16 weeks), but it did not change in the control group (Fig. 2B). Oxidized LDL (mg/ml) was also decreased at 16 weeks in both statin groups (low dose: 245 ± 33.4 in 0 week, 220. 2 ± 19.2 in 1 week, 163.4 ± 17.6 in 16 weeks) (P<0.05 in data of 0 weeks or that of 8 weeks vs. that of 16 weeks) (Fig. 2C). However, oxidized LDL did not remain significantly lower after statin treatment when corrected for LDL cholesterol levels, and it is not evident whether a decrease in oxidized LDL or in LDL affects %FMD more. Considering dose differences with regard to the effect of statin treatment, no additional effect of a high dose of statin was observed in the levels of % FMD, NOx, sVCAM-1, 8-isoprostane, TNF-α, or IL-6. The effect of statin therapy on %FMD, NOx, and 8-epi-isoprostane tended to be greater in the low-dose group compared to the moderate-dose group without reaching the significance threshold. Since the effect of statin therapy on plasma cholesterol in this group was less pronounced, these data might favor a direct mechanism of statin therapy.

As the improvement of % FMD did not depend on (change of) LDL levels, we did multiple regression analyses inserting data in 0 weeks found to be significantly different between low dose and moderate dose groups. We found the (short) period of hemodialysis and (young) age are significant factors associated with %FMD improvement.

4. Discussion

The guidelines of the Japan Atherosclerosis Society (2007) and NCEP (2006) state that LDL should be below 120–130 mg/dl and HDL should be greater than 40 mg/dl [13,14] in individuals with end-stage renal disease. We tried to investigate the additional effect of statin on a further decrease in LDL and other effects in patients undergoing hemodialysis. Simvastatin improved the impaired endothelial function of dialysis patients by decreasing oxidized LDL, improving the lipid profile and, at least in part, enhancing NO bioavailability.

Various mechanisms other than lipid lowering have been proposed to account for the anti-atherosclerotic effects of statins, including antioxidant activity and enhanced NO activity, as direct effects of statins on cells comprising the vascular wall [4–6,10]. Statins increase eNOS activity both in vitro and in vivo [5,6,15]. Because NO has many
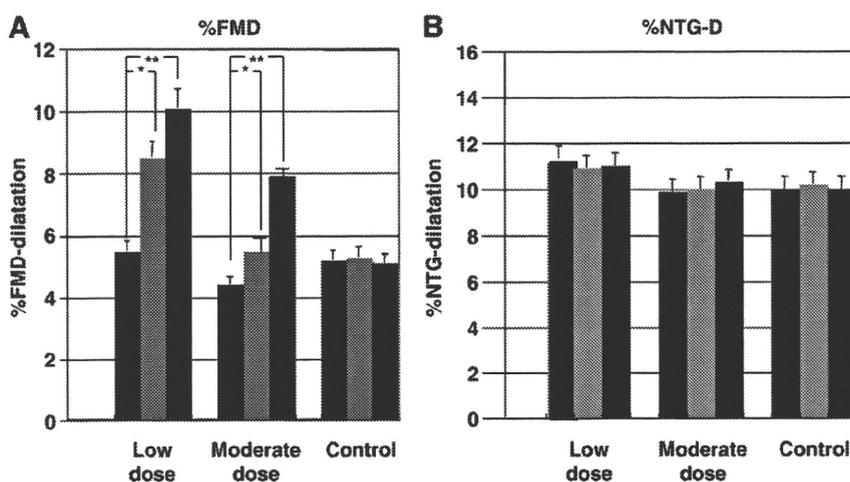
Fig. 1. A. Endothelial function assessed by measuring dilatation of the brachial artery using high-resolution vascular ultrasound in response to reactive hyperemia (FMD: endothelial-dependent flow mediated dilatation). The percent increase in vessel diameter induced by FMD (%FMD) is shown. *P<0.05. B, Endothelial-independent function assessed by measuring dilatation in response to sublingual nitroglycerin (NTG) infusion. The percent increase in diameter induced by nitroglycerin is shown (%NTG-D). No significant differences were observed following simvastatin treatment compared to before treatment. Data are expressed as the mean ± SD. Low dose: simvastatin 5 mg/day group, moderate dose: simvastatin 10 mg/day group. Control: no prescription group. The explanation of each bar graph in the group. Left; before treatment, middle; treatment with simvastatin for 1 week, and right; treatment with simvastatin for 16 weeks.
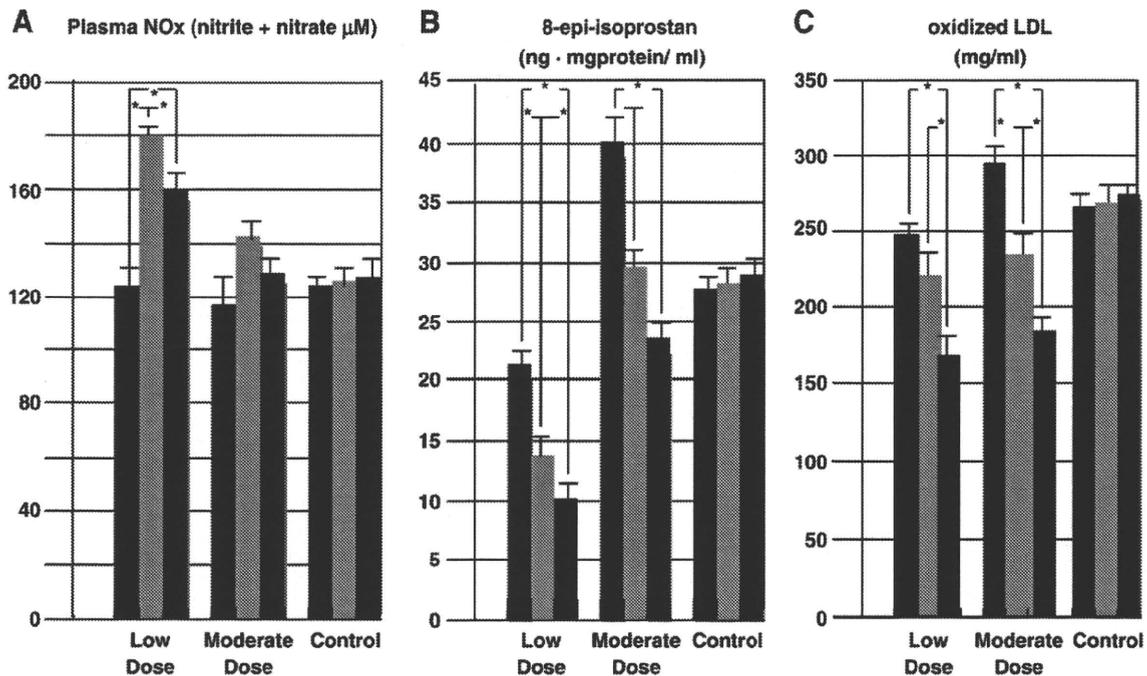
Fig. 2. Plasma concentration of nitrite/nitrate (NOx; A), isoprostane (B) or oxidized LDL (C) before and after simvastatin treatment or in the control group. Data are expressed as the mean ± SD. *P<0.05. Low dose: simvastatin 5 mg/day group, moderate dose: simvastatin 10 mg/day group. Control: no prescription group. The explanation of each bar graph in the group. Left; before treatment, middle; treatment with simvastatin for 1 week, and right; treatment with simvastatin for 16 weeks.

anti-atherosclerotic effects, such as inhibition of monocyte migration, the increased activity of eNOS in response to statins may partially explain its anti-atherosclerotic effects.

The %FMD has been studied extensively in recent years, and it is believed to reflect NO function in vessels [16]. Impairment of %FMD has been reported to precede coronary artery disease, and the %FMD is known to be low in atherosclerotic arteries [17]. Atherosclerosis is very severe in hemodialytic patients, regardless of their original diseases [18]. We noted that the %FMD in the patients evaluated in our study was low. The improvement in %FMD in hemodialysis patients through short-term statin treatment may be due to improved microvascular circumstances or blood fluidity rather than improvement in the atherosclerotic conduit vessel itself [19,20]. The fact that statistical analyses show that the period after introducing hemodialysis is a significant determinant may support this concept.

There are few reports on the effects of statin in patients undergoing hemodialysis [21–25]. Although the number of cardiac events may be reduced, total cardiovascular events, mortality, and total mortality were reported to be the same with or without statin [21–23]. In the present study, statin treatment does not seem to increase HDL cholesterol or reduce plasma triglycerides. Although this may be due to the specific composition of the study population under hemodialysis, it may remain a noteworthy observation and may be related to the effect of statin as mentioned above. Six weeks of atorvastatin treatment (40 mg/day) was reported to improve small artery compliance, but not FMD, in patients in stages 3–5 stages of CKD and hemodialysis [24], although the number of hemodialysis patients was small and a more detailed analysis may be necessary. Furthermore, five months of treatment with pravastatin in patients with chronic dialysis did not have a significant effect on surrogate markers of endothelial function, such as IL-6, sVCAM-1, sICAM-1, etc. Although the CKD and hemodialysis study [25] did not measure NO-related products and we cannot compare the data directly, the kind of statin and the condition of patients with dialysis, such as the period of dialysis, might account for the discrepancy. The difference in the present study's data with regard to the grade of FMD improvement between low and moderate-dose groups may support this explanation.

However, there is no information on the effect of the period of hemodialysis and endothelial function on the previous study's results [26]. Renal failure with or without hypercholesterolemia might further worsen endothelial function because of the presence of asymmetric dimethylarginine and/or uremic toxin [26]. Inhibition of arginine synthesis by urea is a mechanism of arginine deficiency in renal failure that leads to increased hydroxyl radical generation. However, the effect of uremic toxin should be decreased by hemodialysis. Endothelial function, as measured by %FMD, improved in the hemodialytic patients receiving simvastatin after only seven days, and the same trend was observed for plasma nitrite/nitrate, supporting the hypothesis that simvastatin improves endothelial NO function itself. This is the first report that an improvement in %FMD was observed in patients receiving hemodialysis.

In a thrombogenic event such as myocardial infarction, the thrombosis occurs due to impaired endothelial function and atherosclerosis caused by activation of cytokines or adhesion molecules such as VCAM-1 [26,27]. In the present study, VCAM-1, oxidized LDL, and isoprostane were decreased by statin. Improved endothelial function, such as increased %FMD, decreased adhesion molecules, and decreased free radicals may prevent vascular thrombogenic events [27–29]. When statins improve %FMD levels via a direct effect, an improvement in atherogenic molecules and free radicals as well as lipid lowering should result in further improvement of endothelial function. Although we reported improved %FMD in as short a time as three days [4], a short-term effect of statin independent of plasma lipid levels, the possibility of a pleiotropic effect without any relation to plasma lipid is interesting. Taken together, the detailed mechanism of the improvement in %FMD might be different between what has been previously reported for diabetics and what we determined in the present study for hemodialytic patients. In the present study, %FMD levels were greater after 16 weeks than after 7 days of treatment. The continuous improvement in %FMD levels after statin treatment for 16 weeks may mean that both mechanisms (direct and indirect effects) contribute to this action [5,6,30–32]. The data for 8-isoprostan, a marker of reactive oxygen species, support this hypothesis. Consequently, the bioavailability of

simvastatin in vessel walls is increased and the direct effect may more easily occur.

The period of hemodialysis and the TNF-α level might constitute the difference in background between study participants receiving low or moderate doses of statin. Since LDL levels in each group became the same after treatment and TNF-α levels did not change after statin treatment in the present study, it is possible that the term of hemodialysis may reflect the severity of atherosclerosis. These differences may mask the different effects of different doses of statin, although the mechanism requires further clarification.

In the present study, 8-isoprostane, an oxidant marker, was also decreased after 16 weeks. In atherosclerotic arteries, the increase in oxygen radicals and their decrease in response to statin treatment are well known. Statins could decrease O_2^- release from rabbit aortas when rabbits were fed regular chow [33,34]. It is possible that an increase in NO down-regulates an O_2^- releasing enzyme, such as NADPH oxidase [35–37]. A decrease in the levels of O_2^- releasing enzyme may contribute to a prolonged NO lifespan and indirectly improve endothelial function [37,38].

Our data may partially support simvastatin's anti-atherosclerotic effects, especially with regard to restoration of endothelial function, which may relate to the stabilization of atheroma. There is a common ground between the changes in levels of adhesion molecules and endothelial function resulting from statin treatment in this study and the anti-atherosclerotic effect of the statin.

5. Limitations of the study

There were a limited number of patients in the present study. The slight tendency toward lipid profile changes was observed even seven days after treatment, but this may have been inevitable because the hemodialysis was performed three times per week and the conditions used to measure %FMD and %NTG may need to be adjusted. These effects are not guaranteed to continue for years. Recently, atorvastatin was reported to have no beneficial effect during the end stage of renal failure, and the merit of statin may be limited only to the arteries of patients during the early term of chronic hemodialysis without severe atherosclerosis. However, there were many differences in conditions in the pertinent study, such as the amount prescribed or the frequency of adverse effects. Further examination of whether these effects persist is needed.

In conclusion, in elderly patients undergoing hemodialysis, seven days of treatment with simvastatin may improve endothelial function of atherosclerotic arteries. Changes such as those observed in %FMD and adhesion molecules induced by simvastatin could prevent A–V shunt trouble or cardiovascular diseases due to thrombotic occlusion in patients with chronic hemodialysis.

Acknowledgments

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Visceral Fat Accumulation and Metabolic Risk Factor Clustering in Older Adults

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

DESIGN: Cross-sectional study

SETTING: A community clinic in Tokyo, Japan.

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

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

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

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

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

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

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

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

METHODS

Subjects

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

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

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

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

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

VFA Measurement

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

Definition of Metabolic Risk Factors

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

Homeostasis Model Assessment of Insulin Resistance and Serum Adiponectin Level

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

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

Statistical Analysis

The subjects were divided into four groups according to individual calculated VFA values in men and women. High BP, high triglycerides, low HDL-C, and high blood glucose were used as metabolic risk factors. The number of metabolic risk factors was calculated as their sum (0–4). Data

were expressed as means ± standard deviations or standard errors. The statistical significance of differences was assessed using unpaired t-tests for two groups and analysis of variance for three or more groups, followed by the Fisher protected least significant difference test to compare each group. Multiple regression analysis was performed to determine independent factors for the number of metabolic risk factors. The correlation of VFA with HOMA-IR or serum adiponectin level was analyzed using the Pearson correlation coefficient.

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

RESULTS

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

Table 1. Clinical Characteristics of Study Population

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

* $P < .001$ vs men.
† Components of the metabolic syndrome were diagnosed according to the definition of the International Diabetes Federation: high blood pressure = systolic blood pressure ≥130 mmHg, diastolic blood pressure ≥85 mmHg, or treatment with antihypertensive drug; high serum triglycerides = fasting serum triglyceride level ≥150 mg/dL or treatment with fibrates; low high-density lipoprotein cholesterol (HDL-C) = serum HDL-C level <40 mg/dL in men and <50 mg/dL in women; high blood glucose = fasting plasma glucose ≥100 mg/dL or treatment with antidiabetic drugs.
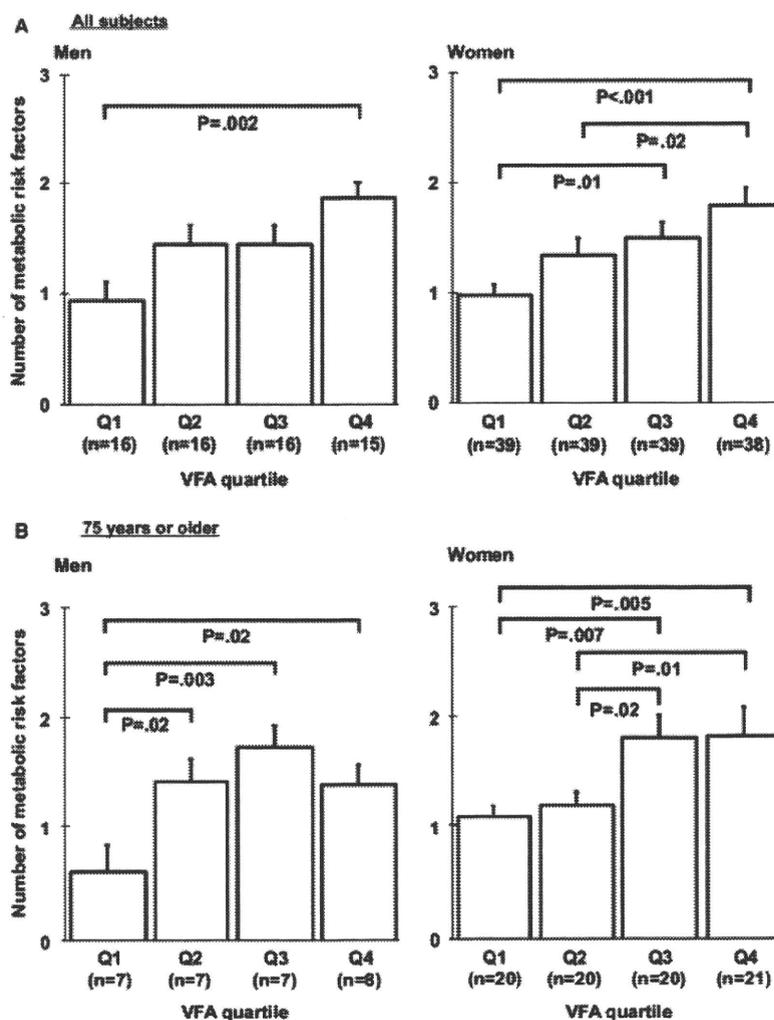SD = standard deviation.

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

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

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

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

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

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

*Correlation coefficient $(R) = 0.515$, coefficient of determination $(R^2) = 0.265$, $P < .001$.
†$R = 0.393$, $R^2 = 0.154$, $P < .001$.
VFA = visceral fat area; BMI = body mass index.
Metabolic risk factors indicate components of the metabolic syndrome except abdominal obesity according to the definition of the International Diabetes Federation.