Table 4 The relationship on gender between IHD and clinical variables

IHD: Male	**Mean(SD)**	**Hgb A1C = < 7.0 (Gp.MF)**		**7.0 < HgbA1C (Gp.MP)**	
Number of patients		1037		1022	
Number of events		20		26	
		Univariate	Multivariate	Univariate	Multivariate
Age (y.o.)	60.9 (7.9)	1.06 (0.97-1.19)		**1.10*(1.01-1.22)**	**1.11*(1.02-1.25)**
Duration of diabetes (years)	9.15 (8.22)	1.07(0.98-1.15)		1.09+(0.99-1.18)	
HemoglobinA1C(%)	7.38 (1.31)	1.25(0.44-4.60)		1.34(0.72-2.33)	
Triglyceride (mg/dl)	146.9(129.5)	1.00 (0.98-1.01)		1.00(0.98-1.01)	
LDL-Chol (mg/dl)	119.2 (34.1)	1.01 (0.99-1.04)		1.02+(1.00-1.04)	
HDL-Chol (mg/dl)	55.4 (16.2)	0.97 (0.92-1.03)		0.99(0.95-1.02)	
Systolic BP (mmHg)	133.5 (17.5)	**1.06* (1.02-1.11)**	**1.06* (1.02-1.11)**	0.99(0.94-1.04)	
Diastolic BP (mmHg)	75.5 (11.7)	0.95 (0.89-1.02)		1.03(0.95-1.12)	
Insulin user (%)	32.80%	1.74 (0.55-2.77)		**4.01**(1.15-7.38)**	**4.11**(1.19-8.12)**
IHD:Female	Mean(SD)	Hgb A1C = < 7.0 (Gp.OF)		7.0 < HgbA1C (Gp.OP)	
Number of patients		816		1140	
Number of events		19		17	
		Univariate	Multivariate	Univariate	Multivariate
Age (y.o.)	75.4 (4.3)	1.00 (0.96-1.06)		0.99 (0.95-1.05)	
Duration of diabetes (years)	10.18 (9.08)	**0.96*(0.93-0.98)**	**0.96*(0.93-0.98)**	**0.92*(0.84-0.99)**	**0.92*(0.84-0.99)**
HemoglobinA1C(%)	7.26 (1.15)	1.01 (0.78-1.31)		0.84 (0.61-1.10)	
Triglyceride (mg/dl)	129.2 (62.2)	1.01 (0.95-1.07)		0.99 (0.96-1.02)	
LDL-Chol (mg/dl)	116.1 (30.6)	1.00 (0.98-1.03)		1.00 (0.95-1.05)	
HDL-Chol (mg/dl)	54.7 (16.2)	0.97(0.92-1.02)		0.92(0.80-1.03)	
Systolic BP (mmHg)	135.9 (17.1)	1.04 (0.92-1.16)		1.05 (0.91-1.18)	
Diastolic BP (mmHg)	72.2 (10.9)	0.93 (0.85-1.03)		1.05 (0.94-1.15)	
Insulin user (%)	33.90%	1.08+(1.01-1.15)		6.27+(0.91-14.24)	
CVA: Male	Mean(SD)	Hgb A1C = < 7.0 (Gp.MF)		7.0 < HgbA1C (Gp.MP)	
Number of events		22		16	
		Univariate	Multivariate	Univariate	Multivariate
Age (y.o.)	60.9 (7.9)	0.99 (0.92-1.08)		1.05(0.92-1.25)	
Duration of diabetes (years)	9.15 (8.22)	1.03+(0.99-1.06)		1.01(0.99-1.02)	
HemoglobinA1C(%)	7.38 (1.31)	1.68(0.74-4.33)		0.92(0.72-1.24)	
Triglyceride (mg/dl)	146.9(129.5)	1.00 (0.98-1.01)		0.99(0.97-1.01)	
LDL-Chol (mg/dl)	119.2 (34.1)	1.01 (0.99-1.04)		1.02(0.98-1.05)	
HDL-Chol (mg/dl)	55.4 (16.2)	0.96+(0.91-1.01)		0.99(0.92-1.05)	
Systolic BP (mmHg)	133.5 (17.5)	1.01 (0.95-1.06)		0.99(0.92-1.08)	
Diastolic BP (mmHg)	75.5 (11.7)	0.96 (0.90-1.05)		1.05(0.93-1.22)	
Insulin user (%)	32.80%	4.44 (0.35-6.77)		1.01(0.35-1.18)	
CVA:Female	Mean(SD)	Hgb A1C = < 7.0 (Gp.OF)		7.0 < HgbA1C (Gp.OP)	
Number of events		11		21	
		Univariate	Multivariate	Univariate	Multivariate
Age (y.o.)	75.4 (4.3)	1.00 (0.93-1.12)		1.35*(1.00-1.85)	
Duration of diabetes (years)	10.18 (9.08)	**0.97*(0.95-0.99)**	**0.97*(0.95-0.99)**	**0.94*(0.88-0.98)**	**0.95*(0.89-0.99)**
HemoglobinA1C(%)	7.26 (1.15)	7.48 (0.70-22.8)		0.94 (0.65-1.30)	
Triglyceride (mg/dl)	129.2 (62.2)	1.01 (0.91-1.07)		1.01 (0.97-1.03)	
LDL-Chol (mg/dl)	116.1 (30.6)	0.96 (0.74-1.10)		1.02 (0.99-1.05)	
HDL-Chol (mg/dl)	54.7 (16.2)	0.99(0.92-1.05)		0.99(0.94-1.03)	
Systolic BP (mmHg)	135.9 (17.1)	1.02 (0.90-1.10)		0.88 (0.76-0.97)	
Diastolic BP (mmHg)	72.2 (10.9)	1.03 (0.94-1.17)		1.02 (0.91-1.16)	
Insulin user (%)	33.90%	0.93 (0.75-1.12)		**3.26*(1.12-6.24)**	**3.29*(1.13-6.42)**

+P < 0.1, *P < 0.05, **P < 0.01

Clinical characteristics of diabetic individuals and the results of the univariate and multiple regression analyses examining the association between various clinical variables and IHD and CVA risk with stratification by gender group. Odds ratios and the corresponding 95% confidence intervals, which are in parentheses following the odds ratios, are shown.

than 70. Stepwise regression analysis confirmed the association of high LDL-C and low HDL-C with a risk of IHD in the NF group; however, the significance of the LDL-C association was not confirmed by multiple regression analysis [21]. The use of anti-dyslipidemia agents, such as statins, with their pleiotropic effect may affect this difference [21]. Insulin use tended to decrease the incidence of IHD in patients in the NF group (Table 2).

Among older diabetic individuals with fair glycemic control (the OF group), lower diastolic blood pressure was associated with IHD. Insulin use was associated with IHD in the OP group. Coronary circulation depends on diastolic blood flow, and the isolated systolic hypertension (with lower diastolic blood pressure) values may reflect aortic atherosclerosis, which is common among the elderly.

Interestingly, insulin therapy was associated with IHD in the OP group. The duration of diabetes was longer among insulin users than among non-users in the NF and NP groups; however, there was no difference in diabetes duration between the OF compared to the OP group. Overall, the combination of higher plasma glucose and insulin use may progress atherosclerosis and subsequently increase the risk of IHD among elderly diabetic individuals. However, maintaining good glycemic control via insulin use could help prevent IHD. Because patients with a history of IHD and/or CVA were excluded from the study, few patients in our cohort used antiplatelet agents (< 10% of patients).

Conversely, IHD was associated with high systolic blood pressure in the MF group, age in the MP control, and short duration of diabetic history in the FF and FP groups. Insulin use was associated with IHD in the male/poor group. Females usually develop complications of atherosclerotic diseases, such as IHD or CVA, 10-15 years later than males [22], which may partially be due to the prevention of atherosclerosis progression by estrogen. However, previous reports have indicated that gender does not affect the age of onset of atherosclerotic disease in individuals with diabetes [23,24]. Our observation that a short duration of diabetic history is associated with IHD and CVA in females may reflect these phenomena. A detailed mechanism was not obvious in the present study and should be evaluated in future research. Regarding gender differences and laboratory findings in Japanese populations, it was recently suggested that hsCRP levels increase continuously across the fasting plasma glucose (FPG) spectrum, starting from the lowest FPG in both men and women, but the increase in hsCRP levels is greater in women than men. Moreover, higher CRP gamma-glutamyl transferase (GGT) levels are synergistically associated with the metabolic syndrome and insulin resistance, independently of other confounding factors in the general population [25,26].

CVA risk factors

The incidence of CVA is higher in East Asian individuals, such as the Japanese, than in Caucasians. Thus, the incidence of CVA was higher in the present study than those reported in previous Western studies, but it was comparable to that observed in prior Japanese studies, such as JDCS [27]. The differences in eating habits, diabetic complications, and older average age observed in the present study may have led to these differences.

Among patients in the NP group, low HDL-C was associated with CVA. This result is consistent with previous reports; however, the relationship between the HDL-C level and CVA has been described recently [9,28].

Among patients in the OF group, insulin use was associated with CVA (OR = 2.09). Although the underlying mechanism is still unknown and the higher frequency of CVA in Japan may affect the data, our preliminary findings show that hypoglycemia occurred more frequently among individuals in the OF group than those in the OP group. Hypoglycemia increases stroke risk [5,29]. In the strict glucose control group of the ACCORD study, morbidity rates increased in the form of severe hypoglycemia and weight gain.

In the OP group, glycemic control was worse among insulin users than among non-users. The duration of diabetes did not differ between the OF and OP groups. There has been a drastic increase in the elderly population in the last several decades. Therefore, understanding the characteristics of lifestyle-related diseases in the elderly is important to maintain their good health. Postprandial hyperglycemia is common in the elderly, and hyperosmolar nonketotic hyperglycemia often complicates the course of diabetes in the elderly. Insulin therapy reduces glucose toxicity and is necessary in some elderly diabetic patients. Strict blood glucose control including insulin therapy is necessary to prevent the progression of diabetic microangiopathies. However, insulin induces smooth muscle cell proliferation and may lead to the progression of atherosclerosis [30]. Muis et al. focused on type 1 diabetes patients (instead of type 2) to minimize the effects of insulin resistance [31]. They found that the cumulative dose of regular insulin was significantly related to carotid intima-media thickness. They observed a similar relationship between the use of intermediate-acting insulin with carotid intima-media thickness and concluded that the cumulative dose of insulin was a risk factor for atherosclerosis. Insulin contributes to cellular senescence and causes aging in organisms, such as mice [32,33]. The detrimental effects of insulin therapy, such as hypoglycemia leading to stroke, was more evident in the elderly in the present study. Unfortunately, a detailed analysis was not possible because of the fact that patients' insulin regimens (dose and type of insulin) change

frequently. Future studies should examine the effects of insulin. CVA was associated with short duration of diabetes in the FF and FP groups, and insulin use was associated in the FP group. The results that short duration of diabetic history is associated with CVA in the female may be explained similarly as to IHD, as drastic effect of menopause [34].

Limitations

Treatment for diabetes was based on data recorded at the time of enrollment. Patients were followed for 2 years, and we could not analyze the detailed mechanisms underlying insulin therapy on the risk of IHD and CVA.

Conclusions

The present study suggests that the risk factors for IHD and CVA in diabetic individuals change with age and gender and perhaps with a patient's degree of glycemic control. Insulin use has a potential role in preventing IHD but may also be a risk factor for CVA among the diabetic elderly. Therefore, although the treatment of diabetes is obviously important, insulin therapy for glycemic control should be carefully considered in those elderly patients. Treatment modalities that reduce the adverse effects of insulin without sacrificing its glycemic controlling effects would be of particular interest in the treatment of elderly diabetic individuals.

Abbreviations list

IHD: ischemic heart disease; CVA: cerebrovascular accident; T2DM: type2 diabetes mellitus; UKPDS: the United Kingdom Prospective Diabetes Study; ACCORD: Action to Control Cardiovascular Risk in Diabetes; ADVANCE: Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation; JCDM: Japan Cholesterol and Diabetes Mellitus Investigation; NF: under 70 years of age with fair glycemic control; NP: under 70 years of age with poor glycemic control; OF: over 70 years of age with fair control; OP: over 70 years of age with poor control; MF: males with fair control; MP: males with poor control; FF: females with fair control; FP: females with poor control.

Acknowledgements

We thank Hisako Matsui-Hirai for her assistance with the statistical analysis and also tank Dr. Ginger T., Ms. Anna T., Ms. Laura D., and American Journal Experts for their assistance with the correct usage of the English language. This study was supported by the Ministry of Health, Labor and Welfare in Japan.

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Authors' contributions

TH, SK, HI, HW, TO, HS, YK, YH, MY and KI participated in the design of the study and carried out the cohort study in their hospitals and related hospitals. HN and KK participated in the design of the study and performed the statistical analysis. TH also conceived of the study, and participated in its coordination. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 30 August 2011 Accepted: 6 October 2011
Published: 6 October 2011

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doi:10.1186/1475-2840-10-86
Cite this article as: Hayashi *et al.*: Age, gender, insulin and blood glucose control status alter the risk of ischemic heart disease and stroke among elderly diabetic patients. *Cardiovascular Diabetology* 2011 10:86.

0022-3565/11/3373-591–599$25.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
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JPET 337:591–599, 2011

Vol. 337, No. 3
177584/3686193
Printed in U.S.A.

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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Received November 24, 2010; accepted February 23, 2011

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.

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

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan [Grant 195910403].
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.110.177584.

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-4*H*-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_2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester; VE, vascular endothelial; NG, normal glucose; HG, high glucose; EHG, extremely high glucose.

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 the Action to Control Cardiovascular Risk in Diabetes trial, 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., 2006), 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 (Bodnar 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-aminofluorscein 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 μM) 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)

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 95 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.

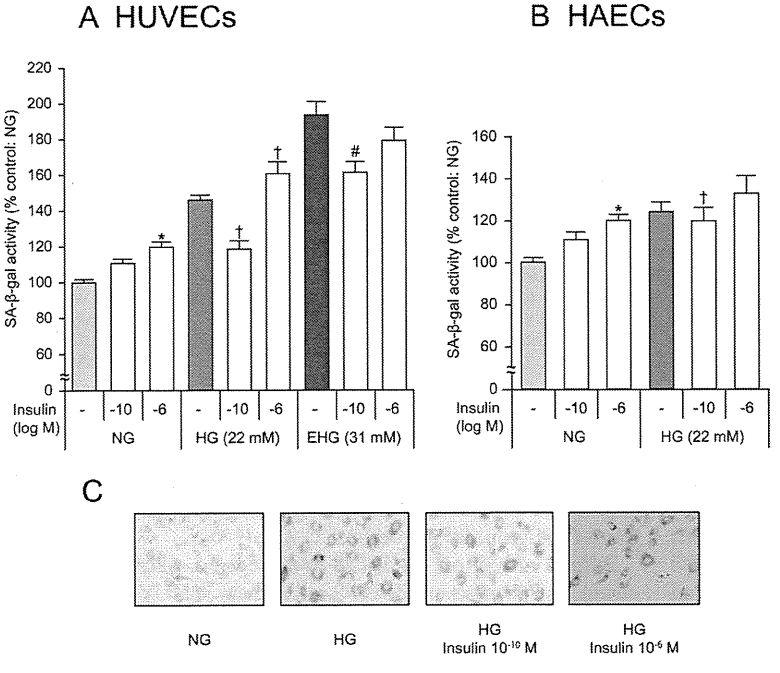

Fig. 1. Effects of glucose and insulin on senescence in HUVECs and HAECs (3 days of exposure). SA-β-gal activity was measured to evaluate cellular senescence. A, effects of low and high concentrations of insulin on SA-β-gal activity at normal (NG), high (HG), and extremely high (EHG) glucose concentrations in HUVECs (n = 6). *, $P < 0.05$ versus NG without insulin; †, $P < 0.05$ versus HG without insulin; #, $P < 0.05$ versus EHG without insulin. 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 of glucose in culture medium.

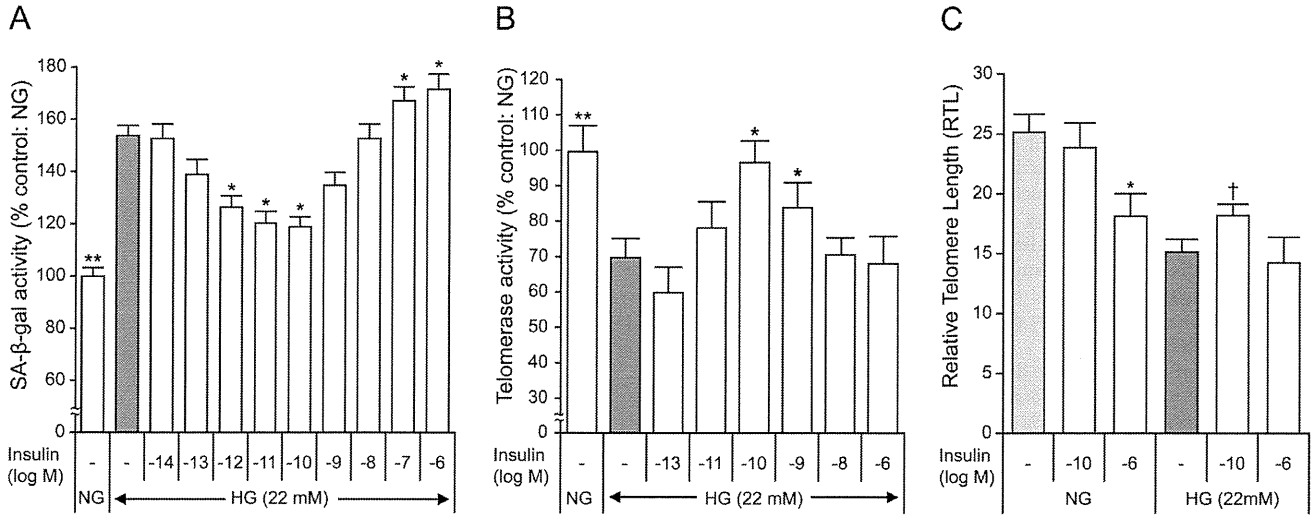

Fig. 2. Effects of insulin on senescence in HUVECs exposed to high glucose. A and B, concentration-dependent effects of insulin on SA-β-gal activity (3 days of exposure) (A) and telomerase activity (B) under high glucose. Telomerase activity was measured by the telomere repeat application protocol (trap) 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.

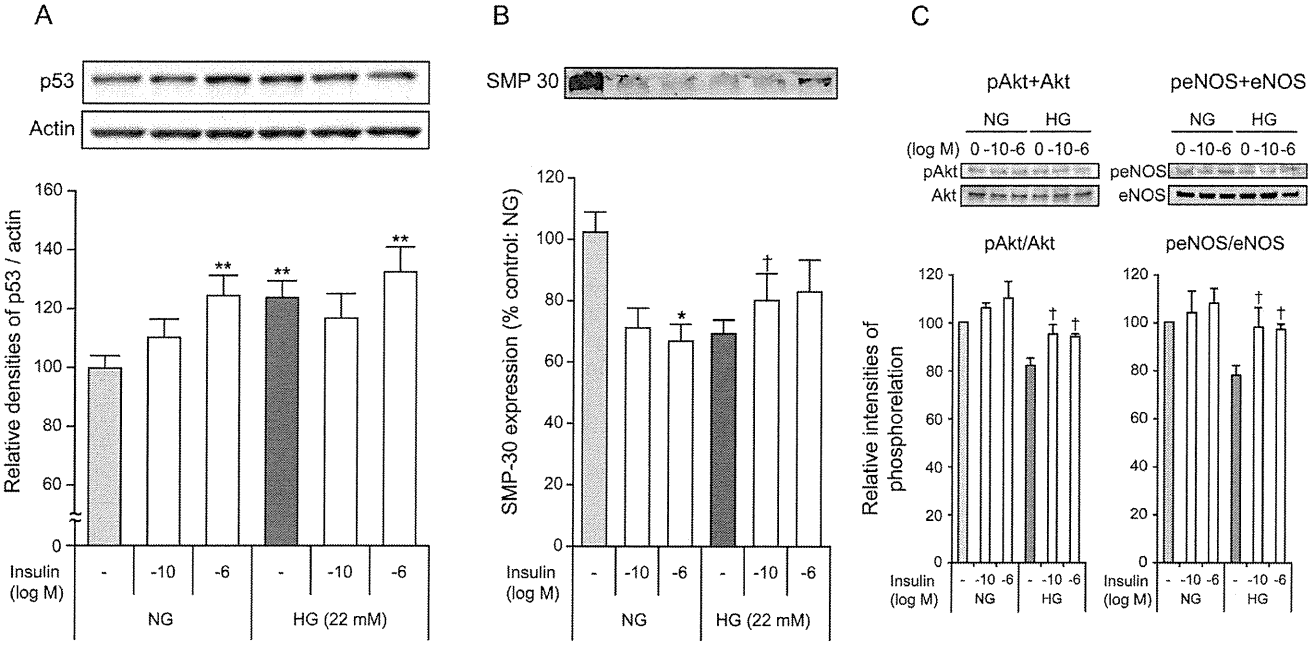

Fig. 3. Effects of insulin on p53 expression, SMP30 expression, and eNOS and Akt phosphorylation in HUVECs. A, bottom, effects of low and high concentrations of insulin on p53 expression under normal and high glucose (3 days of exposure). **, $P < 0.01$ versus NG without insulin. NG, 5.5 mM; HG, 22 mM. 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,

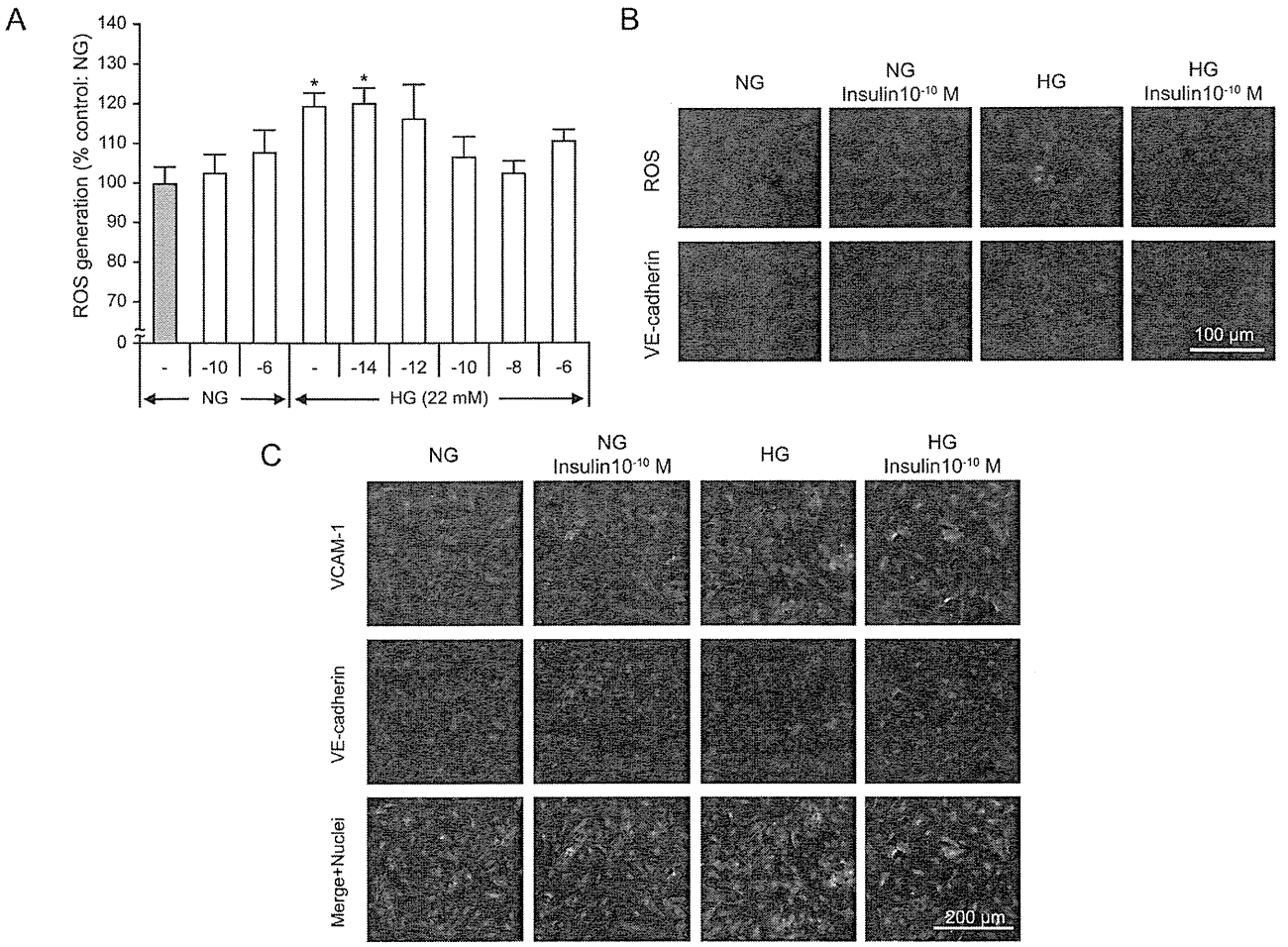

Fig. 4. Effects of insulin on ROS generation and VCAM-1 expression in HUVECs exposed to high glucose for 3 days. ROS generation was detected as intracellular oxidant generation by flow cytometry. Images of intracellular ROS and VE-cadherin were obtained by immunofluorescence and confocal analysis. A, 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_2DCFDA. 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 (Figs. 5, D and E, and 6). The increases in eNOS protein (Fig. 5E) and NOx (Fig. 6) observed under high glucose in the presence of physiological insulin were significantly reduced by eNOS siRNA (Figs. 5E and 6).

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

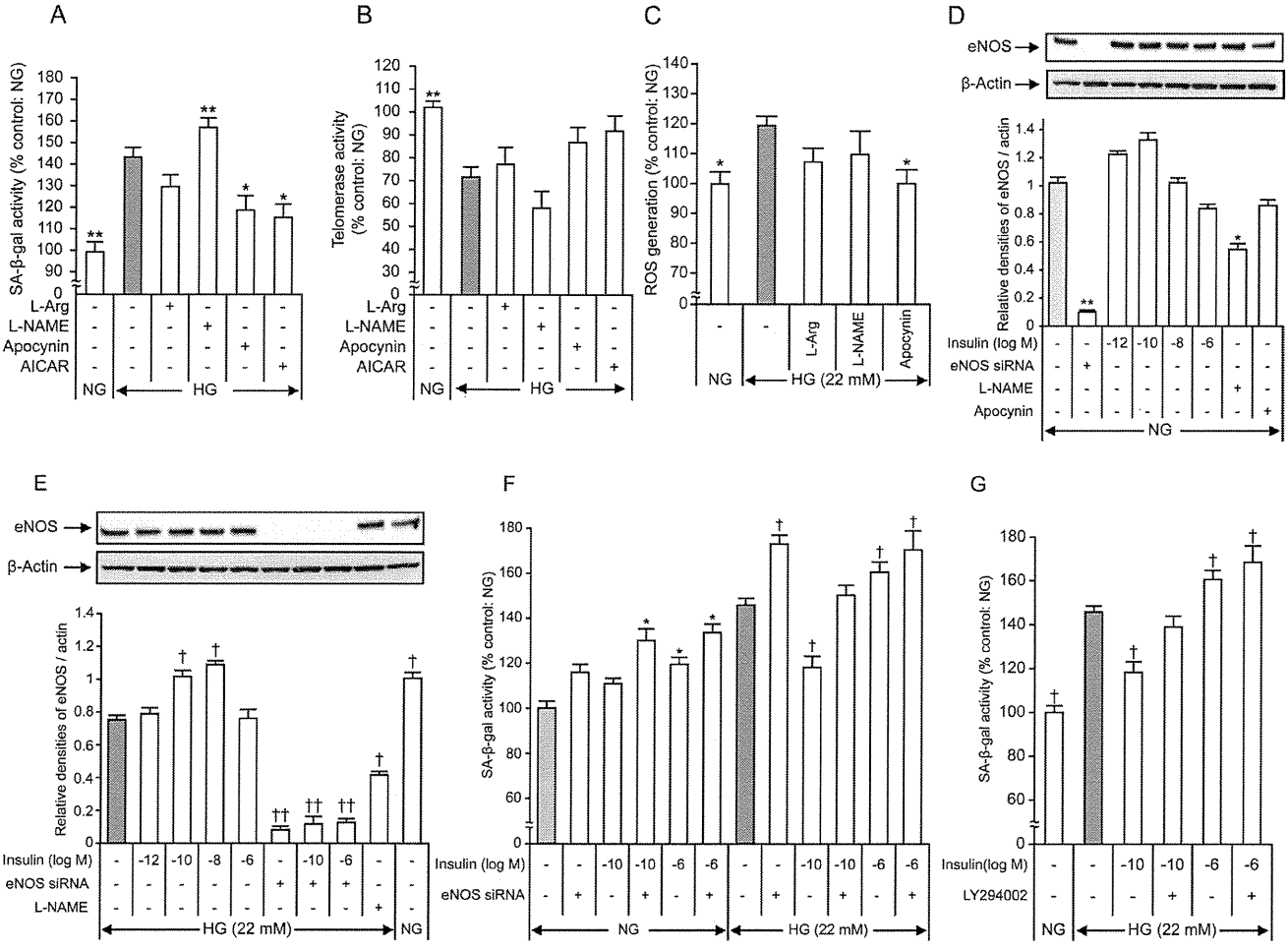

Fig. 5. Analysis of the possible mechanisms underlying the effects of high glucose and insulin in endothelial senescence in HUVECs. A, B, and C, effects of L-Arg, L-NAME, apocynin, and AICAR on the changes in SA-β-gal activity (A), telomerase activity (B), and ROS generation (C) were examined in HUVECs 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 without insulin. †, $P < 0.05$; ††, $P < 0.01$ versus HG without insulin. 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 without insulin; †, $P < 0.05$ versus HG without insulin. NG, 5.5 mM; HG, 22 mM.

are shown in Fig. 7. In young rats, no significant SA-β-gal-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).

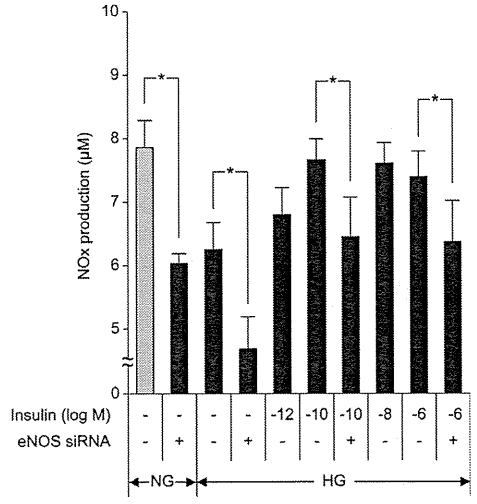

Fig. 6. Effects of insulin on basal NOx production in HUVECs under normal and high glucose conditions. NOx contents in the medium were measured with an automated NO detector high-performance liquid chromatography system. Cells were incubated for 3 days under normal or high glucose. *, $P < 0.05$. NG without insulin, 5.5 mM; HG without insulin, 22 mM.

However, a high-glucose stimulus is gentler and closer to 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., 2004). 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 in-

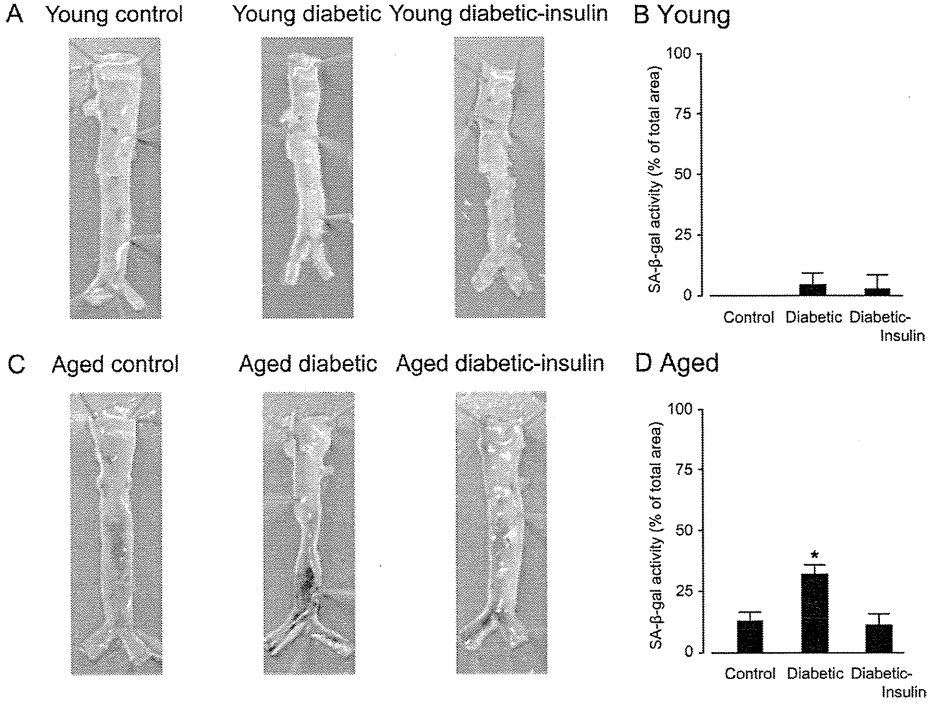

Fig. 7. SA-β-gal activity in diabetic rat vessels. Diabetes was induced in young (8 weeks old) and aged rats (82 weeks old) by an STZ injection. SA-β-gal-positive staining was observed in the intimal side of aortas of aged diabetic rats. Insulin treatment for 7 days reduced its staining. A, representative 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.

sulin retarded the senescence in an NO-dependent manner under high glucose because eNOS siRNA and inhibitors of 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 ($\sim 10^{-8}$ M) because of eating and other stimuli, including chemical injections. High concentrations of insulin ($\sim 2 \times 10^{-7}$ 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 atherosclerotic 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.

Acknowledgments

We thank Kengo Tomita for excellent help in creating the figures for this article.

Authorship Contributions

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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Life Sciences 88 (2011) 425–431

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

The role of insulin growth factor on atherosclerosis and endothelial function: The effect on hyperlipidemia and aging

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ARTICLE INFO

Article history:
Received 9 August 2010
Accepted 11 December 2010
Available online 8 January 2011

Keywords:
Insulin-like growth factor-1
Atherosclerosis
IGF binding protein 3
Endothelial senescence
Nitric oxide

ABSTRACT

Aims: Insulin/insulin-like growth factor (IGF-1) signaling is important for a variety of age-related processes. However, whether or not it affects atherosclerosis is unknown.
Main methods: Six groups of 6 male New Zealand white rabbits were treated for 12 weeks under the following conditions: Groups YC and YIGF: Young rabbits (10 weeks old) were fed regular chow w/wo IGF-1 (Somazon0.1 mg/kg/day, s.c.). Groups HC and HIGF: young rabbits were fed HCD (0.5% cholesterol plus regular chow) w/wo IGF-1. Groups OC and OIGF: old rabbits (120 weeks old) were fed regular chow w/wo IGF-1.
Key findings: Plasma lipid levels, endothelial responses and morphological findings did not differ between groups YIGF and YC. Animals in group HC had increased plasma lipid levels and atheromas. In group HIGF, IGF led to atheromas with increased plasma insulin growth factor binding protein 3 (IBP3), inducible nitric oxide synthase (iNOS) expression and nitrotyrosine staining, macrophage staining, SM1 staining and SM embryo staining compared to HC. Basal nitric oxide (NO) release evaluated by plasma NO metabolites (NOx) and cGMP levels were lowest in the HIGF group.
Significance: Overall, IGF-1 promoted atherosclerosis by affecting endothelial function and aging. These findings indicate that Insulin/IGF1 may contribute to atherogenesis in the elderly.

Introduction

Insulin treatment is administered to prevent hyperglycemia in both type I and type II diabetic individuals. One adverse effect of insulin treatment is that it enhances the progression of atherosclerosis by promoting increased circulation of monocytes, endothelial cell adhesion and subsequent migration of monocytes into the sub-endothelial layers (Bayes-Genis et al., 2000). Insulin/insulin-like growth factor (IGF-1) signal transduction is important not only for glucose metabolism, but also for the aging process and atherogenesis. Insulin/IGF-1 promotes aging in *C. elegans* and mice via activation of PI3 kinase and FOXO/DAF16 pathways (Miyauchi et al., 2004). Recent clinical trials, including ACCORD and ADVANCE, indicated that diabetic patients have an increased risk of cardiovascular disease, and that insulin-enhanced atherogenesis may be causal. Furthermore, the cardiovascular risks associated with insulin therapy are more evident in the elderly, indicating that insulin signaling is important during both atherosclerosis and aging (ACCORD study group, 2008; ADVANCE Collaborative Group et al., 2008). However, how insulin affects aging and atherogenesis is unknown.

We sought to determine how IGF-1 affects atherosclerosis and aging. To this end, we evaluated the effects of IGF-1 on young and old rabbits that were or were not fed a cholesterol-enriched diet (0.5% cholesterol, HCD), and we then examined the expression of nitric oxide synthase (NOS), insulin binding protein 3 and modulation of IGF-1 expression.

Materials and methods

Chemicals and solutions

Acetylcholine chloride (ACh), prostaglandin F2α (PGF2α) and indomethacin were purchased from Sigma/Aldrich Chemical Co. (St. Louis, MO). Nitroglycerin (NTG) (10% w/w triturate in lactose) was from Nihon Kayaku Co. (Tokyo, Japan). Krebs' Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11 mM glucose, and 0.002 mM EDTA (disodium ethylendiamine-tetraacetic acid), pH 7.4) was saturated with 95% O_2/5% CO_2. All concentrations are those in the final bath.

Animals

Male New Zealand white (NZW) rabbits weighing 3–3.5 kg were used. All animals were fed regular chow for 2 weeks. Then, rabbits were divided into 6 groups (6 rabbits/group) and fed the following diets for 12 weeks: Groups YC and YIGF, young rabbits (10 weeks old)

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doi:10.1016/j.lfs.2010.12.021

were fed regular chow with(YIGF) or without (YC) IGF-1 (Somazon 0.1 mg/kg/day, s.c.); Groups HC and HIGF, young rabbits were fed regular chow plus 0.5% cholesterol (HCD) with (HIGF) or without (HC) IGF-1; Groups OC and OIGF, old rabbits (120 weeks old) were fed regular chow with (OIGF) or without (OC) IGF-1. The animals were housed in individual cages and saline or IGF-1 were delivered via subcutaneous injection. The animal protocol was formally approved by the Animal Research Committee of the Nagoya University Graduate School of Medicine.

Determination of plasma lipid and insulin/IGF concentrations

An aliquot of blood was collected from each animal and added to tubes containing EDTA just before animals were anesthetized and euthanized. Total cholesterol and triglyceride levels were measured using enzymatic assays as described previously (Lipid Research Clinics, 1982). HDL-cholesterol levels were determined after precipitation with phosphotungstate-MgCl2. Plasma concentrations of IGF-1 and IGFBP3 were examined as described previously (Lee et al., 1991). Plasma concentrations of nitric oxide metabolite (NOx: $NO_2^- + NO_3^-$) and insulin binding protein 3 were examined as described previously (Hayashi et al., 2000).

Vascular responses

Rabbits were anesthetized with pentobarbital (50 mg/kg, i.v.) and euthanized via exsanguination. The thoracic aorta was then carefully removed from the orifice of the left first costal artery down to the portion enclosed by the diaphragm. The aorta was then cut into 2-mm-wide transverse rings. Isometric tension was measured as described previously (Hayashi et al., 1992). The rings were stretched to optimal force, which was predetermined as the contractile response to 122 mM KCl, mounted in organ chambers, and bathed in Krebs–Henseleit solution at 37 °C. Prostaglandin F2α induced sub-maximal force (2.6×10^{-6} M). Endothelium-dependent relaxation induced by acetylcholine (ACh) was determined by the responsiveness of the endothelium-intact aortic rings. Endothelium-independent relaxation by Nitroglycerin (NTG) was determined by the responsiveness of the endothelium-denuded rings. In some experiments, indomethacin (5×10^{-6} M) was added for 60 min prior to the experiment to rule out prostanoid contributions.

Histological evaluation of aortic atherosclerosis

After 12 weeks of the aforementioned feeding conditions, the animals were euthanized with pentobarbital (100 mg/kg i.v.). The descending thoracic aorta was quickly dissected and either frozen in liquid nitrogen or preserved in formaldehyde. Cross sections of the descending thoracic aorta were stained with hematoxylin-eosin to examine the endothelial lining or van Gieson's elastic stain to determine the thickness of the intima. Morphometric analysis was performed as described by Weiner et al. (1986). Briefly, the complete section of each block was projected onto a vertical surface with a projecting microscope. Six samples from each rabbit aorta were analyzed using an objective lens. The contours of the lumen and the internal elastic lamina were traced, and the tracings were digitized using a graphics tablet. The surface involvement by an atherosclerotic lesion was calculated by dividing the lesion circumference by the circumference of the internal elastic lamina. The circumferences of the lesion area and normal area were defined as circumferences of each parts of the internal elastic lamina. The area occupied by atherosclerotic lesions was defined as the %area bound by the lumen and the internal elastic lamina. The control luminal area was calculated from the perimeter of the internal elastic lamina as described previously (Hayashi et al., 2006).

Immunohistochemical analysis

Cross sections of the descending thoracic aorta were deparaffinized with xylene and dehydrated with graded alcohol. Specimens were pre-incubated in methanol containing 0.3% hydrogen peroxide and washed with phosphate-buffered saline (PBS). Specimens were permeabilized with 0.1% triton ×100 in PBS and washed with PBS. Specimens were then blocked with normal horse serum and incubated with a primary monoclonal antibody (for smooth muscle cell α-actin, monocyte/macrophage, inducible NOS (iNOS), nitrotyrosine, SM1, SM2, and SM) diluted in PBS for 60 min. Samples were then washed with PBS. Negative controls were incubated in irrelevant antibodies. Nuclei were counterstained with methyl green. Each field was scored for the number of positively stained cells (for each antibody) in plaques, and all cells in the plaques were calculated and analyzed statistically as described previously (Hayashi et al., 2003). Five digital images were obtained from each section with a 3CCD color camera (JVC, Victor Company of Japan, Japan) and a Leitz microscope (Wetzlar, Germany). The intensity and distribution patterns of the staining reactions were evaluated by two blinded, independent observers (HH and TH) using a semi-quantitative staining score (graded as 0 = none, 1 = weak, 2 = moderate and 3 = strong). The cells with a mean staining score greater than 2 were considered to be positively stained cells.

Data analysis

The results were expressed as mean ± SEM. The SPSS/PC software package, version 13.0 (SPSS, Munich, Germany), was used for data collection, processing and statistical analysis for all experiments. Statistical analysis was performed using the nonparametrical Wilcoxon's signed rank test for comparison of the means. The Spearman rho coefficient was used to determine significant correlations between the analyzed substances within the distinct groups. P-values that were <0.05 were considered statistically significant.

Results

Blood chemistry

All animals appeared to be healthy throughout the duration of the study. No significant differences in serum HDL-cholesterol, total serum protein or body weight were determined among the six groups over the course of the study (Table 1). In animals fed normal chow (Groups YC, YIGF, OC and OIGF), there was no difference in total cholesterol levels compared to basal values. The addition of 0.5% cholesterol to the normal diet (Groups HC and HIGF) led to significantly increased total cholesterol levels compared to baseline values (Table 1). Treatment with IGF-1 did not significantly affect plasma lipids levels in this study (Groups YIGF, HIGF and OIGF); however, it did lead to an increase in plasma IGF-1 levels. YIGF and HIGF animals displayed increased IGF-1 concentrations, accompanied by significant increases in plasma IGFBP3 (Table 1). Conversely, YC and HC animals displayed low levels of IGF-1 and IGFBP3 (Table 1). IGF-1 and IGFBP3 levels were slightly increased in plasma from HC and OC rabbits compared to those in YC rabbits, and they were significantly increased in OIGF plasma (Table 1).

NO related marker and vascular responses

Plasma NOx and cGMP concentrations were lower in HC compared to YC animals (Table 1). The same values were much lower in HIGF compared to HC animals. To address aging, plasma NOx concentration and plasma cGMP concentrations were also assessed. These values were lower in OC rabbits compared to YC rabbits. Furthermore, the values were much lower in OIGF animals. Acetylcholine-induced NO

Table 1
Biochemical profile of each group of rabbits.

	TC m/dl	TG mg/dl	HDL mg/dl	TP g/dl	BW Kg	NOx nM	cGMP pg/ml	IGF-1 ng/ml	IGF-BP3 mg/ml
YC	102 (11)	42(4.1)	37(2.3)	8.0(0.7)	3.05(0.31)	34.0 (3.2)	30.5(2.6)	198.5(32.1)	1.08(0.33)
YIGF	97 (10)	40(5.0)	38(2.6)	8.1(0.9)	3.12(0.26)	30.5 (4.1)	32.4(3.4)	788* (49)	5.94**(0.84)
HC	1423*(105)	48(6.1)	34(2.5)	6.9(0.7)	2.78(0.24)	23.4*(3.2)	17.2*(3.1)	219(21)	2.03*(0.58)
HIGF	1409*(115)	49(5.5)	36(2.6)	7.2(0.8)	2.96(0.29)	20.5*(3.1)	14.6**(2.8)	912*(76)	8.12**(0.76)
OC	88 (9)	38(4.2)	31(2.1)	6.9(0.7)	3.41(0.19)	28.2*(3.1)	23.4*(2.8)	182(12.5)	2.01*(0.35)
OIGF	85 (10)	39(4.1)	29(2.0)	7.1(0.6)	3.51(0.23)	25.5*(4.2)	21.8*(2.3)	704*(35)	6.02**(0.93)

Rabbits were treated with the indicated conditions for 15 weeks. The mean ± SEM was determined for 6 rabbits/condition. The unpaired student test was used to determine statistical significance.
* $p<0.05$ vs. YC.
** $p<0.01$ vs. YC.

release levels displayed similar results (Fig. 1); however, nitroglycerine-induced endothelium-independent responses were similar in all groups of animals including HC and HIGF animals (Fig. 2).

Histological examination of atherosclerosis

No remarkable atheromatous lesions were observed in animals that were fed regular chow (YC, YIGF, OC and OIGF). However, histological examination of the thoracic aortae revealed increased numbers of atheromatous lesions (as determined by the mean percentage of luminal encroachment and the mean lesion area) in animals that received a cholesterol-enriched diet and IGF-1 (HIGF) compared to those that received a cholesterol-enriched diet without IGF-1 (HC)(Fig. 3). The atherosclerotic area was increased by 30% in the IGF treated group (HIGF) compared to the HCD group (HC) (Fig. 3).

Immunocytochemical analysis — smooth muscle cell α-actin, monocyte/macrophage, inducible nitric oxide synthase, and nitrotyrosine ($ONOO^-$)

A significant increase in the atherosclerotic area, as well as the relative number of macrophages, was observed in animals in the IGF-1 treated hyperlipidemic group (HIGF) compared to the saline-treated hyperlipidemic group (HC) (Fig. 4 upper). The number of smooth muscle cell α-actin positive cells was not different between the HC and HIGF groups (Fig. 4. middle). The atheroma in the aorta expressed high levels of SM1 and SM embryo but SM2 in HC and HIGF animals (Fig. 4 lower). It is well known that SM1 staining area is almost all overlapping of smooth muscle cell α-actin positive cells, SM2 staining area is contractile smooth muscle cells and that SM embryo staining area is intimal thickening composed of synthetic smooth muscle cells. Expression of SM1 and SM embryo, but not SM2, was further increased in HIGF aortae, especially in the sub-intima (Fig. 5 upper). These data indicate that the atherosclerosis in HIGF is the status of proceeding of progression of atherosclerosis. Nitrotyrosine and iNOS (data not shown) staining area is almost coincided and the area was increased in the IGF treated group (HIGF) compared to the control group (HC) (Fig. 5 lower), indicating that the most advanced atherosclerotic regions of HIGF aortas are necrotic. Nitrotyrosine staining area is speculated to equal to the area of peroxynitrite expression. Our observations that iNOS expression is increased in T-cells and macrophages of advanced atherosclerotic plaques are consistent with previously reported data (Hayashi et al., 1999; Esaki et al., 1997).

Discussion

Endothelial dysfunction is characterized by impaired endothelium derived nitric oxide (EDNO) production and impaired endothelium-dependent vasodilatation; and it plays an integral part in the initiation

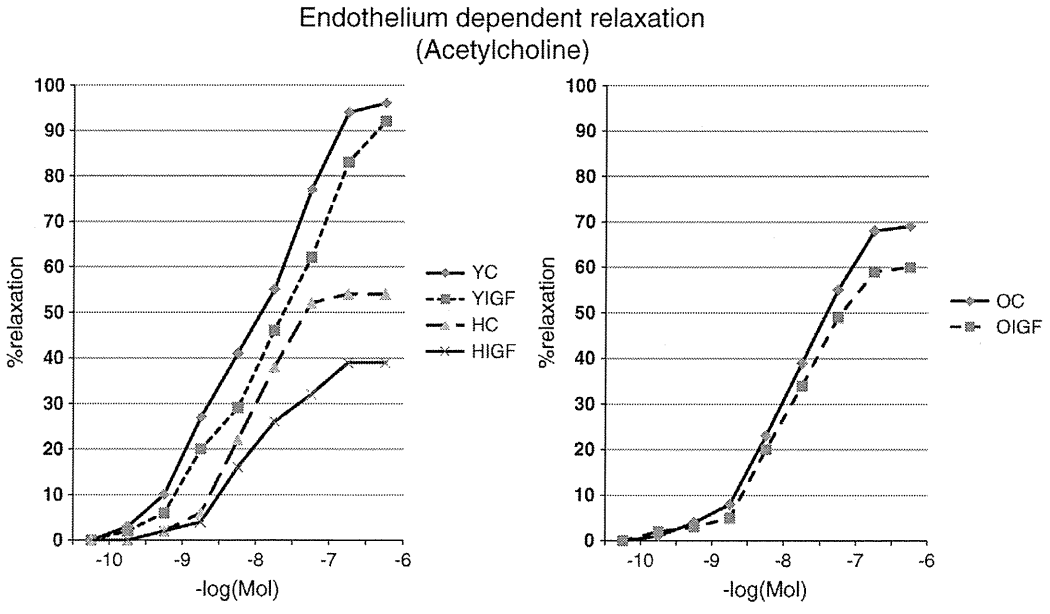

Fig. 1. Endothelium-dependent relaxation. Left: cumulative concentration–response curves to acetylcholine during contraction evoked by PGF2α in the thoracic aortae of rabbits of Groups YC, YIGF, HC and HIGF: young rabbits (10 weeks old) fed regular chow with (YIGF) or without (YC) IGF-1 and young rabbits fed HCD (0.5% cholesterol plus regular chow) with (HIGF) or without (HC) IGF-1. Right: cumulative concentration–response curves to acetylcholine during contraction evoked by PGF2α in the thoracic aortae of rabbits of Groups OC and O-IGF: old rabbits (120 weeks old) fed regular chow with (OIGF) or without (OC) IGF-1. *$p<0.05$.

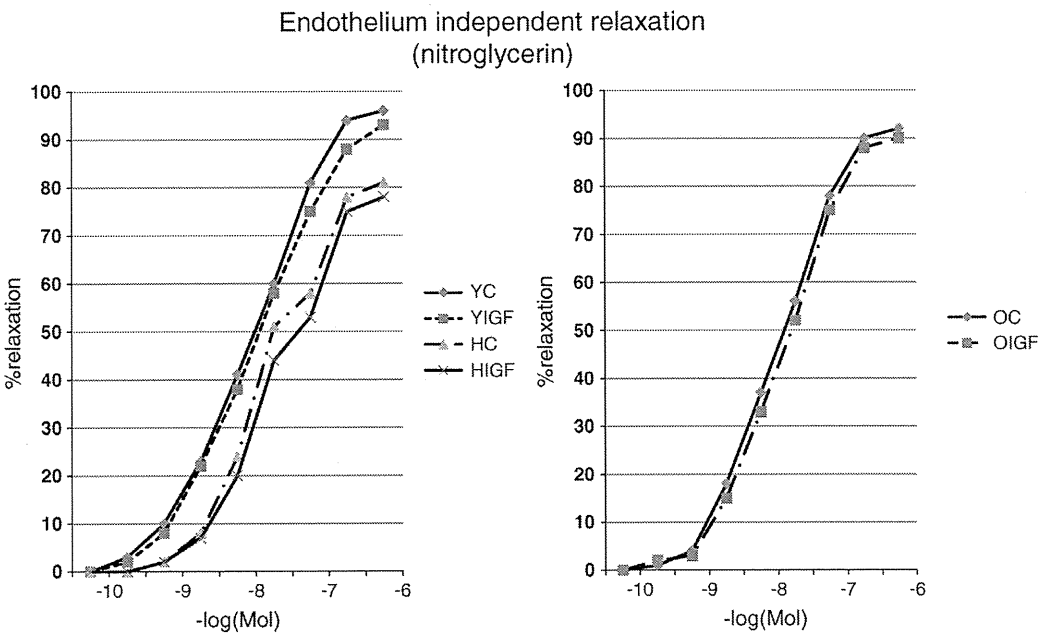

Fig. 2. Endothelium-independent relaxation. Left: cumulative concentration–response curves to nitrolycerin during contraction evoked by PGF2α in the thoracic aortae of rabbits of Groups YC, YIGF, HC and HIGF: young rabbits (10 weeks old) fed regular chow with (YIGF) or without (YC) IGF-1 and young rabbits fed HCD (0.5% cholesterol plus regular chow) with (HIGF) or without (HC) IGF-1. Right: cumulative concentration–response curves to nitroglycerin during contraction evoked by PGF2α in the thoracic aortae of rabbits of Groups OC and O-IGF: old rabbits (120 weeks old) fed regular chow with (OIGF) or without (OC) IGF-1. $^{*}p<0.05$.

of atherosclerosis. These abnormalities subsequently lead to increase monocyte-endothelial cell adhesion and atherosclerosis (Ross, 1999). Monocyte-endothelial cell adhesion leads to increased macrophage recruitment, and macrophages express inducible nitric oxide synthase (iNOS) (Hayashi et al., 2004). Increased iNOS expression leads to increase NO expression and superoxide (O_2^-) production, which leads to peroxynitrite accumulation and endothelial cell death (Beckmann et al., 1994).

Advanced age is a major risk factor for atherosclerosis, but how aging induces vascular pathologies is unclear. Activation of Insulin/IGF-1 signaling promotes aging in *C. elegans* and mice (Miyauchi et al., 2004; Kurosu et al., 2005). Interestingly, inhibition of Insulin/IGF-1 signaling lengthens the life spans of *C. elegans* and mice; however, in human beings, it may reflect insulin resistance. At high concentrations, IGF-1 acts in the same pathway as insulin (Chisalita et al., 2006). Because the effect of IGF-1 in atherosclerosis is unknown, we attempted to elucidate the role of IGF-1 in atherosclerosis.

In the present study, only animals that received cholesterol-enriched chow with or without IGF-1 supplementation developed atherosclerosis. We also observed a concomitant increase in iNOS expression in these animals, which is known to decrease NO synthesis in endothelial cells and inhibit atherosclerosis.

IGF-I is a single chain aminopeptide composed of 70 amino acids. It mediates growth hormone activity in bone and somatic cells and has a long half-life. IGF-I is produced by various organs, and it combines with IGF-BP3 primarily in plasma. The action and

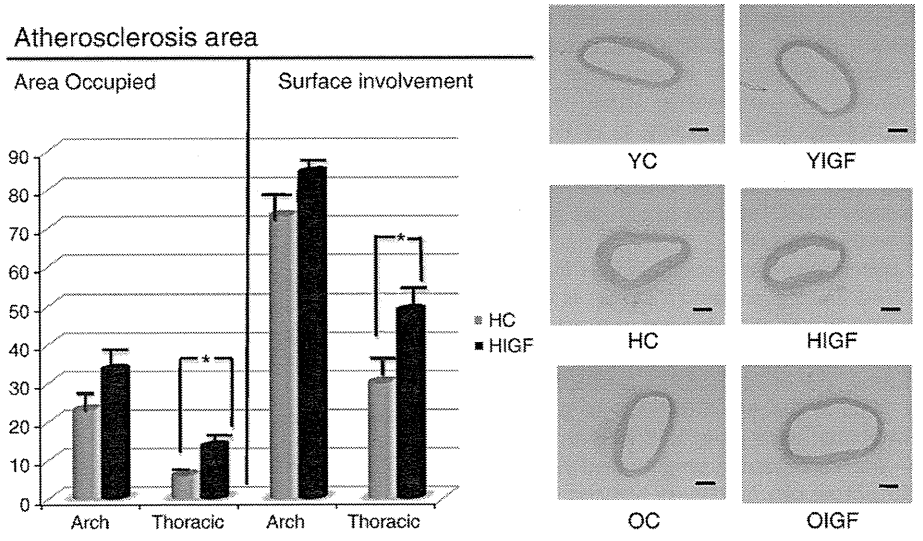

Fig. 3. Histological evaluation of the atherosclerotic area of the thoracic aortae as indicated by the surface involvement and mean lesion area (% occupied lesion) (left panels) and representative photographs (right panels). Arch: Aortic arch, Thoracic:Thoracic arteryGroups YC and YIGF: young rabbits (10 weeks old) fed regular chow with (YIGF) or without (YC) IGF-1. HC and HIGF: young rabbits fed HCD (0.5% cholesterol plus regular chow) with (HIGF) or without (HC) IGF-1. OC and O-IGF: old rabbits (120 weeks old) fed regular chow with (OIGF) or without (OC) IGF-1. $^{*}p<0.05$, $^{**}p<0.01$.Original magnification, ×40. Bar is 200 nm.

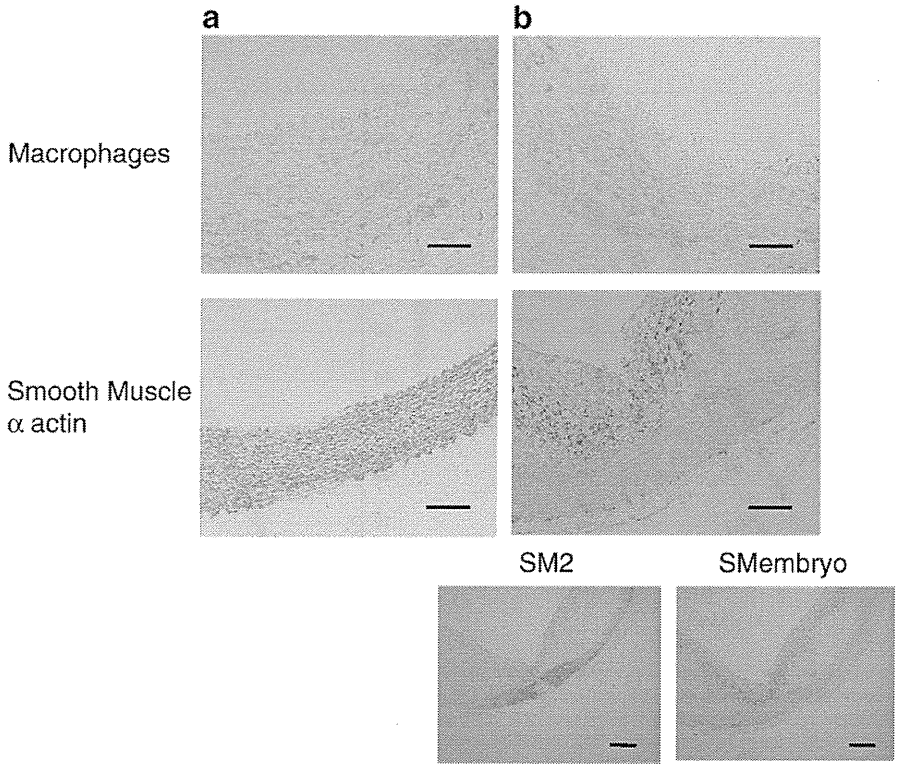

Fig. 4. Immunohistochemical analysis was performed to assess macrophage and smooth muscle cell numbers in thoracic aortae of each groups.Upper panels: Representative photographs of thoracic aorta from treatment groups (a) HC and (b) HIGF. Macrophages were detected in both the core and fibrous cap regions of sections stained with a monoclonal antibody that recognizes rabbit macrophages in groups (a) HC and (b) HIGF.Middle panels: Smooth muscle cell α-actin was detected in media and sub-intimal atherosclerotic plaque areas of thoracic aortae in (a) HC and (b) H-IGF rabbits. No significant difference in the staining area was observed between the two groups.Lower panels: Expression of SM2 (contractile type of smooth muscle in media) and SMembryo (synthetic type of smooth muscle in sub-intimal atherosclerotic plaque area) in thoracic aorta of HIGF rabbit. Original magnification, ×100. Bar is 50 nm.

stability of IGF-I is not affected by exercise, stress, sleep or diet, and basal concentrations do not typically fluctuate (Kawachi et al., 2005; Butt and Williams, 2001). However, the relationship between IGF-1 and atherosclerosis with regard to aging is unknown. IGF-I shares significant structural and functional similarities with insulin, and it is implicated in insulin resistance pathologies and cardiovascular disease (Abbas et al., 2008).

The IGF-1 receptor (IGF-1R) is known to promote aortic vascular smooth muscle cell (VSMC) growth, migration and extracellular matrix deposition. Age-related differences in activation of Akt/FOXO3a and ERK1/2 pathways are apparent in VSMC, and they appear to be a result of increased IGF-1 signaling. Li et al. (2008) showed that IGF-1R is constitutively active in VSMC from old rats, while low IGF-1R activity was observed in young VSMC. Thus, activation of IGF-1R influences VSMC function in old rats and may contribute to increased atherosclerosis risk.

Whether or not this association relates to alterations in plaque growth and stability or IGF-I binding protein (IGFBP-3) is unclear. Martin et al. (2008) assessed the relationship between circulating IGF-I levels and subclinical atherosclerosis and plaque stability 63–82 yr old patients. In total, 269 of 310 (86.8%) participants had at least one carotid or femoral plaque. IGFBP-3 was positively associated with plaque instability (odds ratio: 1.38; 0.99–1.93). The group showed that IGFBP-3 levels regulated programmed cell death and survival of VSMC in the fibrous cap. Autophagy was also involved in programmed VSMC death. TNF-α signals through c-jun N-terminal kinase and protein kinase B to induce microtubule-associated protein 1 light chain 3 (MAPLC-3) and Beclin-1 expression, and this pathway correlated with autophagic cell death of plaque VSMC. Conversely, IGF-1 signaled through Akt to inhibit MAPLC-3 expression. The group concluded that the expression of autophagy genes can be influenced by IGF-1 and is involved in regulation of plaque stability (Jia et al., 2006).

Data from Martin et al. (2008) indicates that IGF-1 signaling negatively regulates IGFBP-3 signaling. In the present study, IGFBP-3 concentration correlated with IGF-1 concentration. It is possible that our results differed from Marin, et al. because our treatment period was shorter and we treated animals with a high IGF-1 concentration compared to physiological concentrations.

Conclusion

Our results suggest that IGF promotes atherogenesis and decreases endothelial function independent of dyslipidemia and the cumulative effects of aging. Our results indicate that insulin/IGF supplementation may promote cell proliferation during atherosclerosis. IGF-mediated inhibition of arginase expression during aging may represent an additional mechanism whereby IGF enhances atherogenesis. These studies are important for evaluating the mechanism of atherosclerotic disease development in elderly patients.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

This study was supported in part by the Grant-in-Aid No. 16406001 from the Ministry of Education, Science and Culture of Japan (to TH).

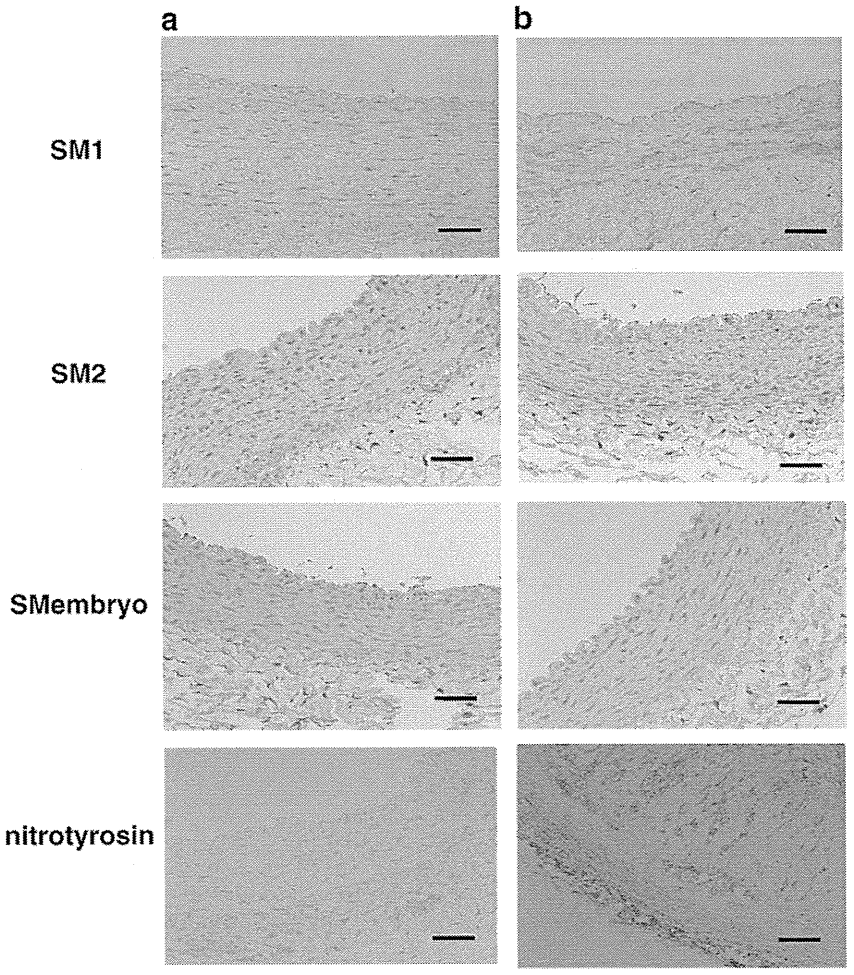

Fig. 5. Immunohistochemical analysis was performed to assess phenotype of smooth muscle cells and nitrotyrosine (nearly equal to peroxynitrite positive) staining area in thoracic aortae of each groups. Representative photographs of thoracic aorta from groups (a) HC and (b) HIGF.Upper panels: SM1 was detected in smooth mucscle cells in media and subintimal area in both groups. SM1 staining area in subintima is wider in aorta from (b) HIGF than those from (a) HC reflecting the severity of atherosclerosis.Middle upper panels: SM2 was detected in smooth muscle of media and no significant difference was observed between (a) HC and (b)HIGF.Middle lower panels: SM embryo was detected in smooth muscle of sub-intimal atherosclerotic plaque areas of thoracic aortae in (a) HC and (b) H-IGF rabbits. SMembryo staining area in subintima is wider in aorta from (b) HIGF than those from (a) HC reflecting the severity of atherosclerosis.Lower panels: Expression of nitrotyrosine in sub-intimal atherosclerotic plaque areas of thoracic aortae. Nitrotyrosine staining area in subintima is wider in aorta from (b) HIGF than those from (a) HC reflecting the severity of atherosclerosis. Original magnification, ×100.Bar is 50 nm.

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