研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Hayashi T, Kawashima S, Itoh H, Yamada N, Sone H, Watanabe H, Hattori Y, Ohrui T, Yokote K, Nomura H, Umegaki H, Iguchi A	Low HDL cholesterol is associated with the risk of stroke in elderly diabetic individuals: changes in the risk for atherosclerotic diseases at various ages.	Diabetes Care.	32	1221-1223	2009
Hisako Matsui-Hirai, Toshio Hayashi, Seiji Yamamoto, Koichiro Ina, Morihiko Maeda,Hitoshi Kotani, Akihisa Iguchi, Louis J. Ignarro, and Yuichi Hattori	Dose-Dependent Modulatory Effects of Insulin on Glucose- Induced Endothelial Senescence In Vitro and In Vivo: a Relationship between Telomeres and Nitric Oxide	THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS	337	1-9	2011
Funami J, Hayashi T, Nomura H, Ding QF, Ishitsuka-Watanabe A, Matsui-Hirai H, Ina K, Zhang J, Yu ZY, Iguchi A.	Clinical factors such as B-type natriuretic peptide link to factor VII, endothelial NO synthase and estrogen receptor alpha polymorphism in elderly women.	Life Sciences	85	316-321	2009
Atsushi Araki and Hideki Ito	Diabetes mellitus and geriatric syndromes	Geniatrics Gerontology	9	105-114	2009
Ogiwara Y, Mori S, Iwama M, Sawabe M, Takemoto M, Kanazawa N, Furuta K, Fukuda I, Kondo Y, Kimbara Y, Tamura Y, Chiba Y, Araki A, Yokote K, Maruyama N, Ito H.	Hypoglycemia due to ectopic secretion of insulin-like growth factor-I in a patient with an isolated sarcoidosis of the spleen.	Endocr J	57	325-330	2010
Kodaira C, Sugimoto M, Nishino M, Yamade M, Shirai N, Uchida S, Ikuma M, Yamada S, Watanabe H, Hishida A, Furuta T.	Effect of MDR1 C3435T polymorphism on lansoprazole in healthy Japanese subjects.	Eur J Clin Pharmacol.	65(6)	593-600	2009

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Takeda M, Yamashita T, Shinohara M, Sasaki N, Takaya T, Nakajima K, Inoue N, Masano T, Tawa H, Satomi-Kobayashi S, Toh R, Sugiyama D, Nishimura K, Yokoyama M, Hirata K, Kawashima S.	Plasma tetrahydrobiopterin/dihydrobiopterin ratio: a possible marker of endothelial dysfunction.	Circulation Journal	73(5)	955-962	2009
Yokote K, Saito Y; CHIBA.	Influence of statins on glucose tolerance in patients with type 2 diabetes mellitus: subanalysis of the collaborative study on hypercholesterolemia drug intervention and their benefits for atherosclerosis prevention (CHIBA study).	J Atheroscler Thromb.	16(3)	297-298	2009
Ohnishi S, Fujimoto M, Oide T, Nakatani Y, Tsurutani Y, Koshizaka M, Mezawa M, Ishikawa T, Takemoto M, Yokote K.	Primary lung cancer associated with Werner syndrome	Geriatr Gerontol Int	10	319-323	2010
K. Une, Y. A. Takei, N. Tomita, T. Asamura, T. Ohrui, K. Furukawa and H. Arai	Adiponectin in plasma and cerebrospinal fluid in MCI and Alzheimer's disease	European Journal of Neurology			2011
Nakamura S, Kimura M, Goto C, Noma K, Yoshizumi M, Chayama K, Kihara Y, Higashi Y.	Cigarette smoking abolishes ischemic preconditioning-induced augmentation of endothelium-dependent vasodilation.	Hypertension.	53(4)	674-681	2009
Yoshida N, Okamoto M, Nanba K, Yoshizumi M.	Transthoracic Tissue Doppler Assessment of Left Atrial Appendage Contraction and Relaxation: Their Changes with Aging	Echocardiography	27	839-846	2010

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kodama S, Saito K, Tanaka S, Maki M, Yachi Y, Asumi M, Sugawara A, Totsuka K, Shimano H, Ohashi Y, Yamada N, Sone H.	Cardiorespiratory fitness as a quantitative predictor of all-cause mortality and cardiovascular events in healthy men and women: a meta-analysis.	JAMA	301	2024-2035	2009
Yokoyama H, Sone H, Yamada D, Honjo J, Haneda M.	Contribution of glimepiride to basal-prandial insulin therapy in patients with type 2 diabetes.	Diabetes Res Clin Pract.	91(2)	148-153	2010
能登洋	糖尿病診療マニュアル	Medico	42	53-56	2011
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

Ⅲ. 研究成果の刊行物・別刷

Low HDL Cholesterol Is Associated With the Risk of Stroke in Elderly Diabetic Individuals

Changes in the risk for atherosclerotic diseases at various ages

TOSHIO HAYASHI, MD, PHD[1]
SEINOSUKE KAWASHIMA, MD, PHD[2]
HIDEKI ITOH, MD, PHD[3]
NOBUHIRO YAMADA, MD, PHD[4]
HIROHITO SONE, MD, PHD[5]
HIROSHI WATANABE, MD, PHD[6]
YOSHIYUKI HATTORI, MD, PHD[7]
TAKASHI OHRUI, MD, PHD[8]
KOUTARO YOKOTE, MD, PHD[9]
HIDEKI NOMURA, MD, PHD[10]
HIROYUKI UMEGAKI, MD, PHD[1]
AKIHISA IGUCHI, MD, PHD[1]
ON BEHALF OF THE JAPAN CDM GROUP

OBJECTIVE — To clarify the relationship between lipid levels and ischemic heart disease (IHD) and cerebrovascular disease (CVD) in diabetic individuals.

RESEARCH DESIGN AND METHODS — The Japan Cholesterol and Diabetes Mellitus Study is a prospective cohort study of 4,014 type 2 diabetic patients (1,936 women; mean ± SD age 67.4 ± 9.5 years). Lipid and glucose levels and other factors were investigated in relation to occurrence of IHD or CVD.

RESULTS — IHD and CVD occurred in 1.59 and 1.43% of participants, respectively, over a 2-year period. The relation of lower HDL or higher LDL cholesterol to occurrence of IHD in subjects <65 years old was significant. Lower HDL cholesterol was also significantly related to CVD in subjects ≥65 years old and especially in those >75 years old (n = 1,016; odds ratio 0.511 [95% CI 0.239–0.918]; $P < 0.05$). Stepwise multiple regression analysis with onset of CVD as a dependent variable showed the same result.

CONCLUSIONS — Lower HDL cholesterol is an important risk factor for not only IHD but also CVD, especially in diabetic elderly individuals.

Diabetes Care 32:1221–1223, 2009

Type 2 diabetes, dyslipidemia, and aging are independent risk factors for cardiovascular diseases. Japanese individuals have lower rates of ischemic heart disease (IHD) and higher rates of cerebrovascular disease (CVD); however, diabetic individuals have an increased risk of IHD (1,2). Risk factors for IHD or CVD in elderly diabetic individuals are not fully known (3), and the Japan Cholesterol and Diabetes Mellitus Study was formulated to evaluate them (Umin Clinical Trials Registry, clinical trial reg. no. UMIN00000516; http://www.umin.ac.jp/ctr/index.htm).

RESEARCH DESIGN AND METHODS — The Japan Cholesterol and Diabetes Mellitus Study is a single-center prospective cohort study comprised of 4,014 Japanese diabetic individuals on a consecutive outpatient basis recruited between September 2004 and March 2005 (1,936 women; mean ± SD age 67.4 ± 9.5 years [range 35–83 years]). Patients with previous IHD (myocardial infarction, unstable angina pectoris, angioplasty, or bypass grafting) or CVD (stroke) were excluded. Follow-up information was available for 98.2 and 92.3% of patients enrolled in the first and second years, respectively. Patients were divided into those aged <65 years, 65–74 years, and >75 years (n = 1,267, 1,731, and 1,016, respectively). The primary end points were onset of IHD or CVD. Plasma lipid, glucose, A1C, and other relevant levels were measured annually.

The study was approved by institutional review boards and by the safety-monitoring board. All events were confirmed by the organizing committee annually. The guidelines of the Japan Atherosclerosis Society (2002), stating that LDL cholesterol should be <120 mg/dl and HDL cholesterol >40 mg/dl in diabetic individuals, and the American Diabetes Association criteria for diagnosis of type 2 diabetes were used (4,5).

Results are presented as means ± SD. All statistical analyses were performed using JMP software (SAS Institute, Cary, NC). Incidences were analyzed in relation to risk factors. Univariate and multiple logistic regression analysis and stepwise analysis were used. Values of $P < 0.05$ were considered significant.

RESULTS — Mean A1C, fasting plasma glucose, LDL cholesterol, triglyceride, HDL cholesterol, and systolic and diastolic blood pressure levels on registration were 7.53 ± 1.12%, 159.4 ± 52.7

From the [1]Department of Geriatrics, Nagoya University Graduate School of Medicine, Nagoya, Japan; the [2]Division of Cardiovascular and Respiratory Medicine, Department of Internal Medicine, Kobe University Graduate School of Medicine, Kobe, Japan; the [3]Tokyo Metropolitan Geriatric Hospital, Tokyo, Japan; the [4]Department of Internal Medicine, Endocrinology and Metabolism, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Japan; the [5]Department of Nutrition, Ochanomizu University, Tokyo, Japan; the [6]Department of Clinical Pharmacology and Therapeutics, Hamamatsu University School of Medicine, Hamamatsu, Japan; the [7]Department of Endocrinology and Metabolism, Dokkyo University School of Medicine, Mibu, Japan; the [8]Department of Geriatric Medicine, Tohoku University School of Medicine, Sendai, Japan; the [9]Division of Diabetes, Metabolism and Endocrinology, Department of Internal Medicine, Chiba University Hospital, Chiba, Japan; and the [10]Department of Geriatrics, Nagoya Kita Hospital, Nagoya, Japan.
Corresponding author: Toshio Hayashi, hayashi@med.nagoya-u.ac.jp.
Received 18 September 2008 and accepted 7 January 2009.
Published ahead of print at http://care.diabetesjournals.org on 8 June 2009. DOI: 10.2337/dc08-1677.

Table 1—*Adjusted multiple regression analyses of factors found to be significant by univariate regression analysis for IHD or CVD, as well as major atherogenic risk factors; total* n = 4,014

	<65 years old (*n* = 1,276)		65–74 years old (*n* = 1,731)		≤75 years old (*n* = 1,016)	
	Adjusted OR (95% CI)	*P*	Adjusted OR (95% CI)	*P*	Adjusted OR (95% CI)	*P*
IHD						
Sex	1.469 (1.02–1.94)	0.02*	1.109 (1.02–1.74)	0.04*	0.829 (0.23–3.06)	0.78
Age	1.063 (0.96–1.20)	0.28	0.991 (0.86–1.15)	0.99	0.996 (0.83–1.17)	0.87
LDL cholesterol	1.225 (1.02–2.04)	0.04*	1.001 (0.72–1.25)	0.89	0.776 (0.43–1.40)	0.40
HDL cholesterol	0.659 (0.39–0.98)	0.04*	0.939 (0.68–1.25)	0.38	0.946 (0.58–1.29)	0.23
Triglycerides	1.356 (1.00–2.02)	0.05	0.731 (0.52–1.94)	0.18	0.881 (0.46–1.70)	0.71
A1C	1.179 (0.75–1.88)	0.27	1.082 (0.76–1.55)	0.67	1.274 (0.57–2.35)	0.44
SBP	0.702 (0.49–1.09)	0.15	1.082 (0.79–1.69)	0.15	1.051 (0.58–1.89)	0.87
DBP	1.020 (0.97–1.05)	0.28	1.088 (0.73–1.27)	0.24	1.998 (0.99–4.35)	0.08
CVD						
Sex	1.158 (0.68–2.17)	0.47	1.004 (0.79–1.69)	0.82	0.847 (0.45–1.52)	0.58
Age	1.006 (0.94–1.10)	0.88	0.982 (0.82–1.14)	0.39	1.139 (0.99–1.30)	0.06
LDL cholesterol	1.099 (0.98–1.23)	0.06	1.067 (0.76–1.44)	0.51	1.128 (0.64–1.59)	0.71
HDL cholesterol	0.888 (0.64–1.48)	0.09	0.758 (0.53–0.98)	0.04*	0.511 (0.24–0.92)	0.04*
Triglycerides	1.147 (0.68–2.04)	0.62	1.070 (0.69–1.67)	0.75	1.355 (0.75–2.56)	0.32
A1C	0.996 (0.64–1.28)	0.52	1.019 (0.75–1.74)	0.54	1.015 (0.60–1.72)	0.95
SBP	1.005 (0.67–1.33)	0.86	0.991 (0.94–1.13)	0.35	1.063 (0.62–1.57)	0.75
DBP	1.109 (0.61–2.13)	0.74	1.303 (0.81–2.09)	0.27	1.045 (0.68–1.5)	0.59

	IHD				CVD			
	<65 years old	65–74 years old	≤75 years old	Total	<65 years old	65–74 years old	≤75 years old	Total
HDL cholesterol (mg/dl)								
<44	2.31	2.49	1.68	2.14	1.13	1.99	2.62*	2.01
44–53	1.45	1.45	1.64	1.50	1.05	1.84	2.15*	1.64
54–63	1.25	1.41	0.98	1.23	1.44	0.80	0.88*	1.04
≥64	0.42	1.69	0.99	1.19	1.0	0.80	0.45*	0.72

Data were adjusted for sex. The ratio of male to female subjects is 1:1. *Statistically significant (P <0.05). DBP, diastolic blood pressure; SBP, systolic blood pressure.

mg/dl, 120.3 ± 32 mg/dl, 140.6 ± 108.3 mg/dl, 55.8 ± 18.0 mg/dl, 136.5 ± 17.1 mmHg, and 75.1 ± 11.1 mmHg, respectively. Insulin and oral agents for diabetes were prescribed for 19.9 and 70.5% of individuals, respectively. Dyslipidemia was seen in 79.1%, and antihyperlipidemic drugs were prescribed in 59.0%. Mean lipid and glucose metabolism levels did not change significantly over the 2-year study period.

In the first and second years, 83 and 69 vascular events occurred, respectively. IHD and CVD occurred in 0.80 and 0.71% of total patients per year. The relationship between IHD or CVD and background factors such as LDL cholesterol levels in each age-group was analyzed by univariate logistic regression.

Sex, age, LDL cholesterol, HDL cholesterol, and triglyceride were significantly related to IDH in patients aged <65 years. Age, sex, history of hypertension, and antihypertensive drugs were related in patients aged between 65 and 74 years, and sex and systolic and diastolic blood pressure were related in patients aged > 75 years. CVD and LDL cholesterol were related in patients aged <65 years, and HDL cholesterol and systolic blood pressure were related in patients aged >75 years.

We performed multiple regression analysis with factors found to be significant by univariate regression analysis for IHD or CVD and other atherogenic risk–related factors (A1C, etc.) in three age-groups (Table 1). LDL and HDL cholesterol were associated with IHD in patients aged < 65 years but not in other age-groups. Sex was associated with IHD in individuals aged <74 years. HDL cholesterol was also associated with CVD in individuals between aged between 65 and 74 years and >75 years.

Stepwise multiple regression analysis was performed using factors that were found to be significant by univariate regression analysis for IHD or CVD and other atherogenic risk–related factors. HDL and LDL cholesterol were associated with IHD in individuals aged <65 years (HDL cholesterol odds ratio 0.79 [95% CI 0.58–0.96; P = 0.04] and LDL cholesterol 0.60 [0.33–0.99; P = 0.04]). HDL cholesterol was associated with CVD in individuals aged between 65 and 74 years and ≥75 years (65–74 years 0.73 [0.56–0.94; P = 0.04] and ≥75 years 0.60 [0.35–0.91; P = 0.01]).

The relation of age or HDL cholesterol to IHD and CVD was evaluated in quartile categories. HDL cholesterol levels were inversely correlated with IHD in individuals aged <65 years (hazards ratio 0.633 [95% CI 0.428–0.975]) but not in other groups. The relationship between CVD and HDL cholesterol was prominent in those aged >75years but not in other age-groups (Table 1). There were no sex-related differences in the relationship of HDL cholesterol with CVD. There was no relationship between LDL, triglyceride, fasting blood glucose, or A1C and the frequency of CVD.

CONCLUSIONS — This study represents one of the largest-scale attempts to examine IHD and CVD in middle-aged and elderly diabetic individuals. In the U.S., evidence suggests that middle-aged diabetic individuals have an IHD risk similar to that for individuals with myocardial infarction (6). However, this risk may not exist in elderly diabetic individuals. Many guidelines to prevent atherothrombotic diseases recommend strict control of LDL cholesterol in diabetic patients but the same guideline for HDL cholesterol control (40 mg/dl) as that used for nondiabetic subjects (4–7).

A novel finding was that type 2 diabetic elderly individuals had frequent CVD, and incidence rates were associated with HDL cholesterol. Few data were available for the relationship among elderly, type 2 diabetes, and CVD (8,9).

There have been three large-scale clinical studies of statins that included participants aged up to 75 years (10–12). Although they reported that statins exerted effects on IHD (including in diabetic individuals), effects were not pronounced. (Prosper reported that statins induced a 16% decrease in IHD without any effects on CVD.) The data suggest that because LDL cholesterol decreased, simple LDL cholesterol control may not prevent IHD or CVD in elderly individuals. Our study shows the importance of HDL cholesterol in CVD in elderly diabetic individuals and in IHD in middle-aged diabetic individuals. If HDL cholesterol is well controlled in elderly diabetic patients, then CVD and IHD might be decreased to the levels found in middle-aged cohorts. Patients prescribed statins whose HDL cholesterol was <40 mg/dl showed the same risk (data not shown). Although medicated patients may be more conscious of diseases, HDL cholesterol is a strong risk factor and masks the effects of statins.

In conclusion, HDL and LDL cholesterol were risk factors for IHD in diabetic patients aged <65 years. In addition, HDL cholesterol was a risk factor for CVD in elderly diabetic subjects, especially those aged >75 years. HDL cholesterol may help prevent CVD in elderly diabetic subjects. Risk factors for IHD and CVD appear to change with advancing age.

Acknowledgments— This study was supported by the Japanese Ministry of Health, Welfare and Labor.

No potential conflicts of interest relevant to this article were reported.

References

1. Kubo M, Kiyohara Y, Kato I, Tanizaki Y, Arima H, Tanaka K, Nakamura H, Okubo K, Iida M. Trends in the incidence, mortality, and survival rate of cardiovascular disease in a Japanese community: the Hisayama study. Stroke 2003;34:2349–2354
2. Sone H, Mizuno S, Ohashi Y, Yamada N. Type 2 diabetes prevalence in Asian subjects. Diabetes Care 2004;27:1251–1252
3. Valensi P, Pariès J, Brulport-Cerisier V, Torremocha F, Sachs RN, Vanzetto G, Cosson E, Lormeau B, Attali JR, Maréchaud R, Estour B, Halimi S. Predictive value of silent myocardial ischemia for cardiac events in diabetic patients: influence of age in a French multicenter study. Diabetes Care 2005;28:2722–2727
4. Hata Y, Mabuchi H, Saito Y, Itakura H, Egusa G, Ito H, Teramoto T, Tsushima M, Tada N, Oikawa S, Yamada N, Yamashita S, Sakuma N, Sasaki J, the Working Committee on JAS Guideline for Diagnosis and Treatment of Hyperlipidemias. Report of the Japan Atherosclerosis Society (JAS) Guideline for Diagnosis and Treatment of Hyperlipidemia in Japanese adults. J Atheroscler Thromb 2002;9:1–27
5. American Diabetes Association Consensus Panel. Guidelines for computer modeling of diabetes and its complications. Diabetes Care 2004;27:2262–2265
6. IDF Clinical Guidelines Task Force. Global guideline for type 2 diabetes: recommendations for standard, comprehensive, and minimal care. Diabet Med 2006; 23:579–593
7. Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Executive summary of the third report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). JAMA 2001;285: 2486–2497
8. Marshall SM, Flyvbjerg A. Prevention and early detection of vascular complications of diabetes. BMJ 2006;333:475–480
9. Ratner R, Goldberg R, Haffner S, Marcovina S, Orchard T, Fowler S, Temprosa M, the Diabetes Prevention Program Research Group. Impact of intensive lifestyle and metformin therapy on cardiovascular disease risk factors in the diabetes prevention program. Diabetes Care 2005;28: 888–94
10. Shepherd J, Blauw GJ, Murphy MB, Bollen EL, Buckley BM, Cobbe SM, Ford I, Gaw A, Hyland M, Jukema JW, Kamper AM, Macfarlane PW, Meinders AE, Norrie J, Packard CJ, Perry IJ, Stott DJ, Sweeney BJ, Twomey C, Westendorp RG, the PROSPER Study Group. PROspective Study of Pravastatin in the Elderly at Risk (PROSPER): a randomized controlled trial. Lancet 2002;360:1623–1630
11. Heart Protection Study Collaborative Group. MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomized placebo-controlled trial. Lancet 2002;360:7–22
12. Sever PS, Poulter NR, Dahlöf B, Wedel H, Collins R, Beevers G, Caulfield M, Kjeldsen SE, Kristinsson A, McInnes GT, Mehlsen J, Nieminen M, O'Brien E, Ostergren J. Reduction in cardiovascular events with atorvastatin in 2,532 patients with type 2 diabetes: Anglo-Scandinavian Cardiac Outcomes Trial–lipid-lowering arm (ASCOT-LLA). Diabetes Care 2005;28: 1151–1157

tapraid3/zpt-pt/zpt-pt/zpt00611/zpt9384d11z | xppws | S=1 | 3/16/11 | 12:09 | 4/Color Figure(s): 1, 4, 7 | Art: 177584 | Input-KAF

0022-3565/11/3373-1–9$25.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Copyright © 2011 by The American Society for Pharmacology and Experimental Therapeutics
JPET 337:1–9, 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

Hisako Matsui-Hirai, Toshio Hayashi, Seiji Yamamoto, Koichiro Ina, Morihiko Maeda, Hitoshi Kotani, Akihisa Iguchi, Louis J. Ignarro, and Yuichi Hattori

Department of Geriatrics, Nagoya University Graduate School of Medicine, Nagoya, Japan (H.M.-H., T.H., K.I., M.M., H.K., A.I.); Department of Molecular and Medical Pharmacology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan (S.Y., Y.H.); and Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California, Los Angeles, California (L.J.I.)

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.

AQ: A

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.

AQ: L

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, AQ: B 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 AQ: C 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 AQ: D (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 me- AQ: E dium-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, AQ: F 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 AQ: G (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)

tapraid3/zpt-pt/zpt-pt/zpt00611/zpt9384d11z | xppws | S=1 | 3/16/11 | 12:09 | 4/Color Figure(s): 1, 4, 7 | Art: 177584 | Input-KAF

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.

AQ: H

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).

F1, AQ: I

F2

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.

F3

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.

COLOR

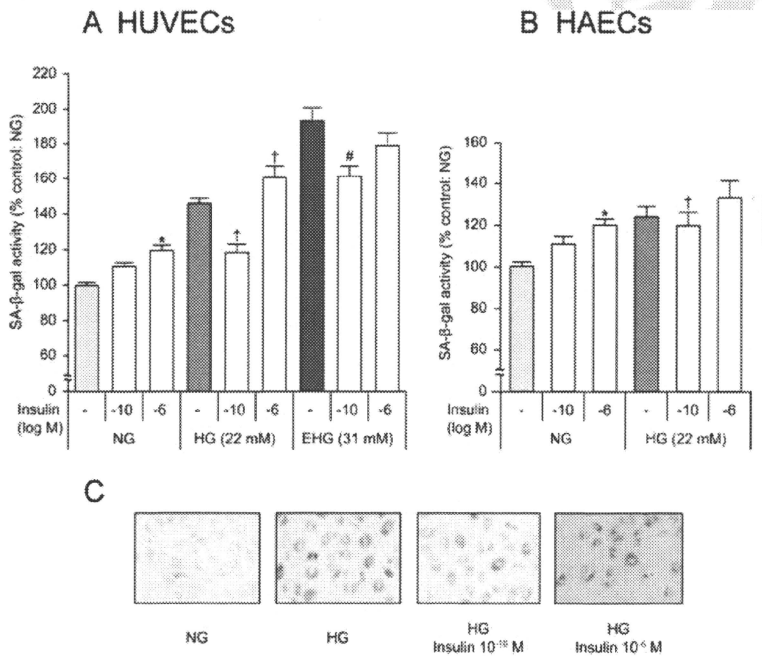

Fig. 1. Effects of glucose and insulin on senescence in HUVECs and HAECs (3 days of exposure). SA-β-gal activity was measured to evaluate cellular senescence. A, effects of low and high concentrations of insulin on SA-β-gal activity at normal (NG), high (HG), and extremely high (EHG) glucose concentrations 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.

tapraid3/zpt-pt/zpt-pt/zpt00611/zpt9384d11z | xppws | S=1 | 3/16/11 | 12:09 | 4/Color Figure(s): 1, 4, 7 | Art: 177584 | Input-KAF

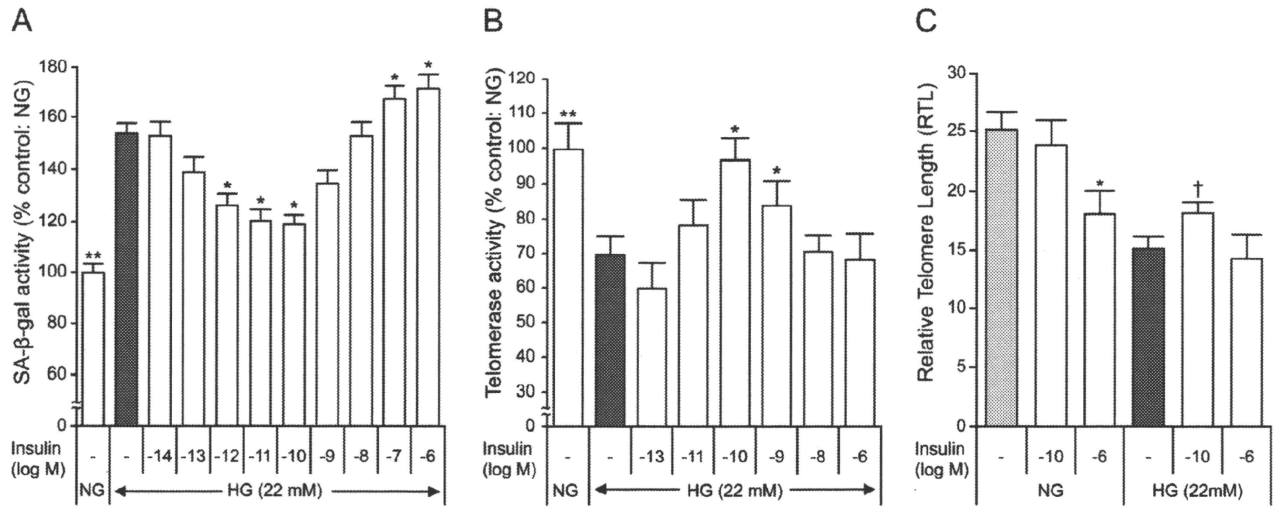

Fig. 2. Effects of insulin on senescence in HUVECs exposed to high glucose. A and B, concentration-dependent effects of insulin on SA-β-gal activity (3 days of exposure) (A) and telomerase activity (B) under high glucose. Telomerase activity was measured by the telomere repeat application protocol (trap)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

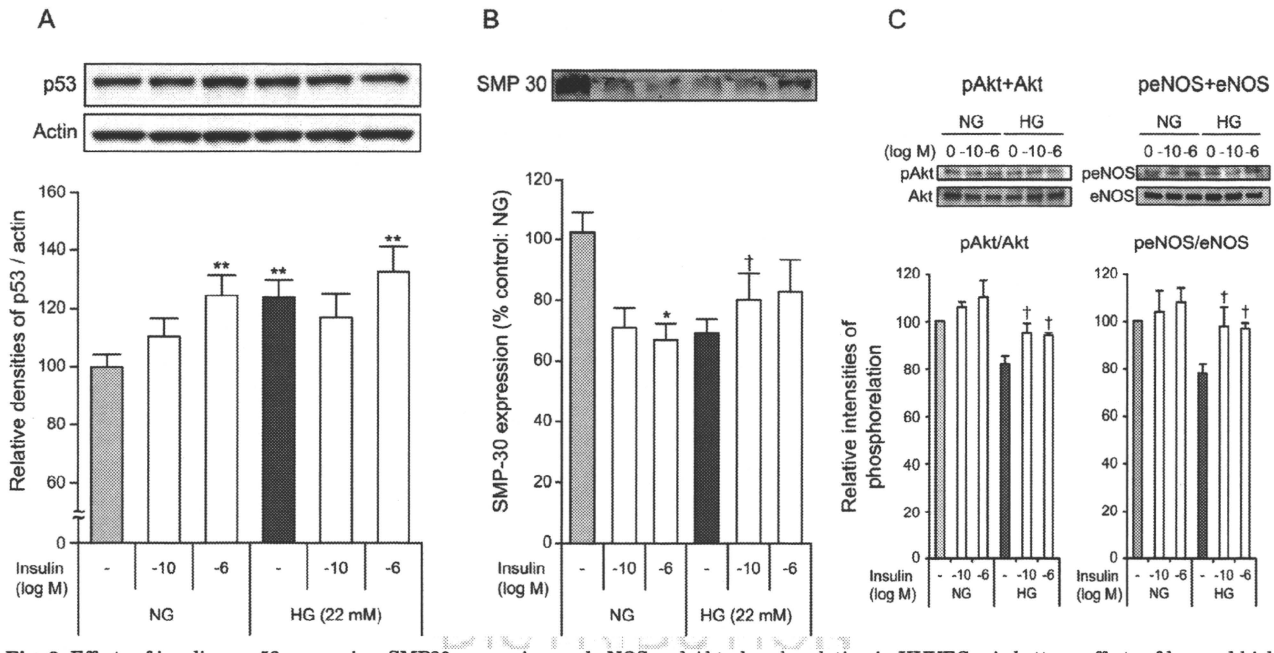

Fig. 3. Effects of insulin on p53 expression, SMP30 expression, and eNOS and Akt phosphorylation in HUVECs. A, bottom, effects of low and high concentrations of insulin on p53 expression under normal and high glucose (3 days of exposure). **, $P < 0.01$ versus NG without insulin. NG, 5.5 mM; HG, 22 mM.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

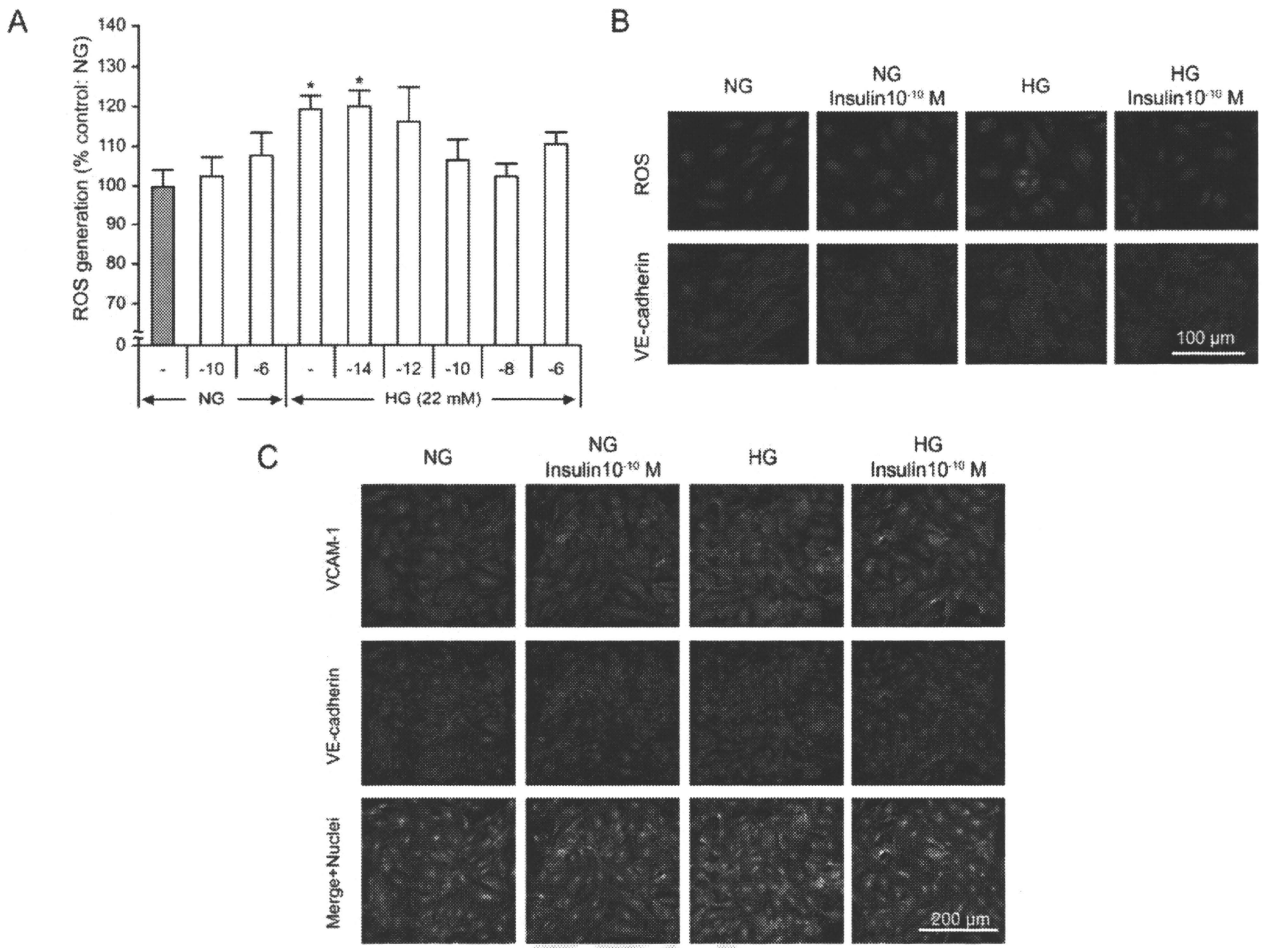

Fig. 4. Effects of insulin on ROS generation and VCAM-1 expression in HUVECs exposed to high glucose for 3 days. ROS generation was detected as intracellular oxidant generation by flow cytometry. Images of intracellular ROS and VE-cadherin were obtained by immunofluorescence and confocal analysis. A, 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.

COLOR

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). F6

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- F7

tapraid3/zpt-pt/zpt-pt/zpt00611/zpt9384d11z | xppws | S=1 | 3/16/11 | 12:09 | 4/Color Figure(s): 1, 4, 7 | Art: 177584 | Input-KAF

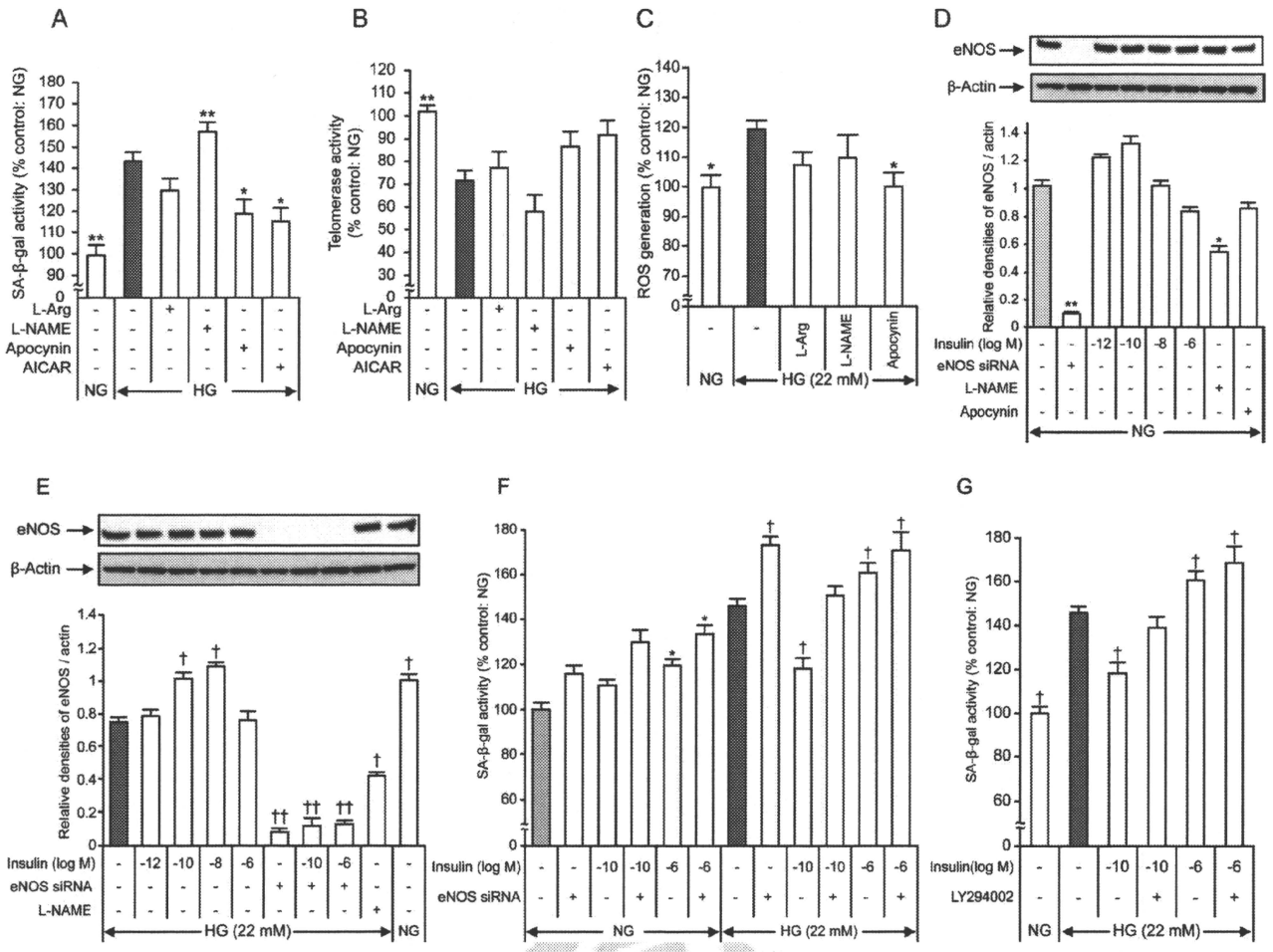

Fig. 5. Analysis of the possible mechanisms underlying the effects of high glucose and insulin in endothelial senescence in HUVECs. A, B, and C, effects of L-Arg, L-NAME, apocynin, and AICAR on the changes in SA-β-gal activity (A), telomerase activity (B), and ROS generation (C) were examined in HUVECs 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

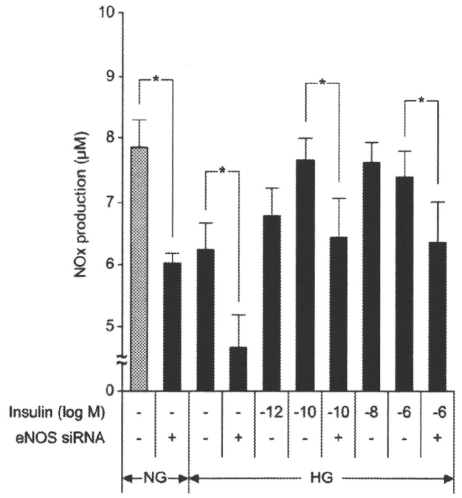

Fig. 6. Effects of insulin on basal NOx production in HUVECs under normal and high glucose conditions. NOx contents in the medium were measured with an automated NO detector high-performance liquid chromatography system. Cells were incubated for 3 days under normal or high glucose. *, $P < 0.05$. NG,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

AQ: J

COLOR

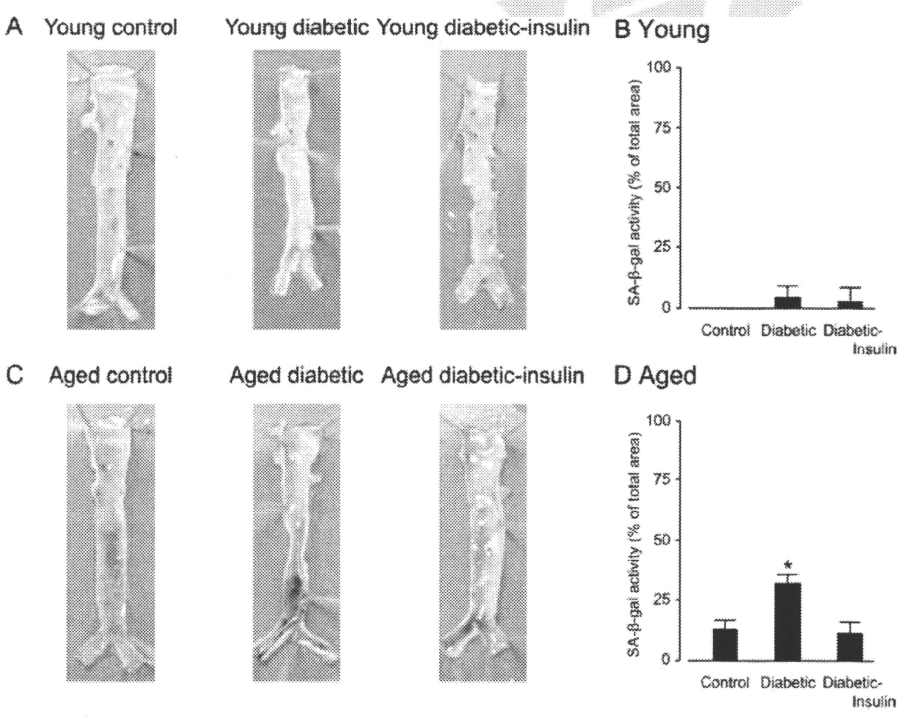

Fig. 7. SA-β-gal activity in diabetic rat vessels. Diabetes was induced in young (8 weeks old) and aged rats (82 weeks old) by an STZ injection. SA-β-gal-positive staining was observed in the intimal side of aortas of aged diabetic rats. Insulin treatment for 7 days reduced its staining. A, representative 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 ($\sim 10^{-8}$ 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 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.

References

Abu-Lebdeh HS, Barazzoni R, Meek SE, Bigelow ML, Persson XM, and Nair KS (2006) Effects of insulin deprivation and treatment on homocysteine metabolism in people with type 1 diabetes. *J Clin Endocrinol Metab* **91:**3344–3348.

Action to Control Cardiovascular Risk in Diabetes Study Group, Gerstein HC, Miller ME, Byington RP, Goff DC Jr, Bigger JT, Buse JB, Cushman WC, Genuth S, Ismail-Beigi F, et al. (2008) Effects of intensive glucose lowering in type 2 diabetes. *New Engl J Med* **358:**2545–2559.

Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, and Wright WE (1998) Extension of life-span by introduction of telomerase into normal human cells. *Science* **279:**349–352.

Breitschopf K, Zeiher AM, and Dimmeler S (2001) Proatherogenic factors induce telomerase inactivation in endothelial cells through an Akt-dependent mechanism. *FEBS Lett* **493:**21–25.

Brouilette S, Singh RK, Thompson JR, Goodall AH, and Samani NJ (2003) White cell telomere length and risk of premature myocardial infarction. *Arterioscler Thromb Vasc Biol* **23:**842–846.

Canela A, Vera E, Klatt P, and Blasco MA (2007) High-throughput telomere length quantification by FSH and its application to human population studies. *Proc Natl Acad Sci USA* **104:**5300–5305.

Chandra J, Hackbarth J, Le S, Loegering D, Bone N, Bruzek LM, Narayanan VL, Adjei AA, Kay NE, Tefferi A, et al. (2003) Involvement of reactive oxygen species in adaphostin-induced cytotoxicity in human leukemia cells. *Blood* **102:**4512–4519.

Chisalita SI, Nitert MD, and Arnqvist HJ (2006) Characterization of receptors for IGF-I and insulin; evidence for hybrid insulin/IGF-I receptor in human coronary artery endothelial cells. *Growth Horm IGF Res* **16:**258–266.

Epel ES, Blackburn EH, Lin J, Dhabhar FS, Adler NE, Morrow JD, and Cawthon RM (2004) Accelerated telomere shortening in response to life stress. *Proc Natl Acad Sci USA* **101:**17312–17315.

Feng D, Kondo Y, Ishigami A, Kuramoto M, Machida T, and Maruyama N (2004) Senescence marker protein-30 as a novel antiaging molecule. *Ann NY Acad Sci* **1019:**360–364.

Fox CS, Coady S, Sorlie PD, Levy D, Meigs JB, D'Agostino RB Sr, Wilson PW, and Savage PJ (2004) Trends in cardiovascular complications of diabetes. *JAMA* **292:**2495–2499.

Fukatsu A, Hayashi T, Miyazaki-Akita A, Matsui-Hirai H, Furutate Y, Ishitsuka A,

Hattori Y, and Iguchi A (2007) Possible usefulness of apocynin, an NADPH oxidase inhibitor, for nitrate tolerance: prevention of NO donor-induced endothelial cell abnormalities. *Am J Physiol Heart Circ Physiol* **293:**H790–H797.

Gorbunova V, Seluanov A, and Pereira-Smith OM (2002) Expression of human telomerase (hTERT) does not prevent stress-induced senescence in normal human fibroblasts but protects the cells from stress-induced apoptosis and necrosis. *J Biol Chem* **277:**38540–38549.

Guzik TJ, Mussa S, Gastaldi D, Sadowski J, Ratnatunga C, Pillai R, and Channon KM (2002) Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase. *Circulation* **105:**1656–1662.

Hayashi T, Ishikawa T, Naito M, Kuzuya M, Funaki C, Asai K, Hidaka H, and Kuzuya F (1991) Low level hyperlipidemia impairs endothelium-dependent relaxation of porcine coronary arteries by two mechanisms. Functional change in endothelium and impairment of endothelium-dependent relaxation by two mediators. *Atherosclerosis* **87:**23–38.

Hayashi T, Matsui-Hirai H, Miyazaki-Akita A, Fukatsu A, Funami J, Ding QF, Kamalanathan S, Hattori Y, Ignarro LJ, and Iguchi A (2006) Endothelial cellular senescence is inhibited by nitric oxide: implications in atherosclerosis associated with menopause and diabetes. *Proc Natl Acad Sci USA* **103:**17018–17023.

Hayashi T, Yano K, Matsui-Hirai H, Yokoo H, Hattori Y, and Iguchi A (2008) Nitric oxide and endothelial cellular senescence. *Pharmacol Ther* **120:**333–339.

Heumüller S, Wind S, Barbosa-Sicard E, Schmidt HH, Busse R, Schröder K, and Brandes RP (2008) Apocynin is not an inhibitor of vascular NADPH oxidases but an antioxidant. *Hypertension* **51:**211–217.

Ignarro LJ and Napoli C (2004) Novel features of nitric oxide, endothelial nitric oxide synthase, and atherosclerosis. *Curr Atheroscler Rep* **6:**281–287.

Kang SS, Kwon T, Kwon DY, and Do SI (1999) Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reserve transcriptase subunit. *J Biol Chem* **274:**13085–13090.

Kletsas D, Pratsinis H, Mariatos G, Zacharatos P, and Gorgoulis VG (2004) The proinflammatory phenotype of senescent cells: the p53-mediated ICAM-1 expression. *Ann NY Acad Sci* **1019:**330–332.

Kurz DJ, Decary S, Hong Y, and Erusalimsky JD (2000) Senescence-associated β-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. *J Cell Sci* **113:**3613–3622.

MacLaren A, Black EJ, Clark W, and Gillespie DA (2004) c-Jun-deficient cells undergo premature senescence as a result of spontaneous DNA damage accumulation. *Mol Cell Biol* **24:**9006–9018.

Minamino T and Komuro I (2007) Vascular cell senescence: contribution to atherosclerosis. *Circ Res* **100:**15–16.

Miyauchi H, Minamino T, Tateno K, Kunieda T, Toko H, and Komuro I (2004) Akt negatively regulates the in vitro lifespan of human endothelial cells via a p53/p21-dependent pathway. *EMBO J* **23:**212–220.

Miyazaki-Akita A, Hayashi T, Ding QF, Shiraishi H, Nomura T, Hattori Y, and Iguchi A (2007) 17β-Estradiol antagonizes the down-regulation of endothelial nitric-oxide synthase and GTP cyclohydrolase I by high glucose: relevance to postmenopausal diabetic cardiovascular disease. *J Pharmacol Exp Ther* **320:**591–598.

Muniyappa R, Montagnani M, Koh KK, and Quon MJ (2007) Cardiovascular actions of insulin. *Endocr Rev* **28:**463–491.

Murcia AM, Hennekens CH, Lamas GA, Jiménez-Navarro M, Rouleau JL, Flaker GC, Goldman S, Skali H, Braunwald E, and Pfeffer MA (2004) Impact of diabetes on mortality in patients with myocardial infarction and left ventricular dysfunction. *Arch Intern Med* **164:**2273–2279.

Ota H, Eto M, Kano MR, Ogawa S, Iijima K, Akishita M, and Ouchi Y (2008) Cilostazol inhibits oxidative stress-induced premature senescence via upregulation of Sirt1 in human endothelial cells. *Arterioscler Thromb Vasc Biol* **28:**1634–1639.

Parrinello S, Samper E, Krtolica A, Goldstein J, Melov S, and Campisi J (2003) Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. *Nat Cell Biol* **5:**741–747.

Polytarchou C and Papadimitriou E (2005) Antioxidants inhibit human endothelial cell functions through down-regulation of endothelial nitric oxide synthase activity. *Eur J Pharmacol* **510:**31–38.

Rodriguez BL, Curb JD, Burchfiel CM, Huang B, Sharp DS, Lu GY, Fujimoto W, and Yano K (1996) Impaired glucose tolerance, diabetes, and cardiovascular disease risk factor profiles in the elderly. The Honolulu Heart Program. *Diabetes Care* **19:**587–590.

Stout RW (1990) Insulin and atheroma. 20-yr perspective. *Diabetes Care* **13:**631–654.

Thomas J, Linssen M, van der Vusse GJ, Hirsch B, Rösen P, Kammermeier H, and Fischer Y (1995) Acute stimulation of glucose transport by histamine in cardiac microvascular endothelial cells. *Biochim Biophys Acta* **1268:**88–96.

Williams HC and Griendling KK (2007) NADPH oxidase inhibitors: new antihypertensive agents? *J Cardiovasc Pharmacol* **50:**9–16.

Yokoi T, Fukuo K, Yasuda O, Hotta M, Miyazaki J, Takemura Y, Kawamoto H, Ichijo H, and Ogihara T (2006) Apoptosis signal-regulating kinase 1 mediates cellular senescence induced by high glucose in endothelial cells. *Diabetes* **55:**1660–1665.

Address correspondence to: Dr. Toshio Hayashi, Department of Geriatrics, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan. E-mail: hayashi@med.nagoya-u.ac.jp

Life Sciences 85 (2009) 316–321

Contents lists available at ScienceDirect

Life Sciences

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

Clinical factors such as B-type natriuretic peptide link to factor VII, endothelial NO synthase and estrogen receptor α polymorphism in elderly women

Jun Funami [1], Toshio Hayashi *, Hideki Nomura, Qun-Fang Ding [2], Asako Ishitsuka-Watanabe, Hisako Matsui-Hirai, Koichiro Ina, Jie Zhang [3], Ze-Yun Yu [4], Akihisa Iguchi [5]

Department of Geriatrics, Nagoya University Graduate School of Medicine, Tsuruma-cho 65, Showa-ku, Nagoya, Aichi 466-8550, Japan

ARTICLE INFO

Article history:
Received 9 March 2009
Accepted 18 June 2009

Keywords:
Natriuretic peptide
Estrogen receptor
Factor V Leiden
Endothelial nitric oxide synthase
Single nucleotide polymorphism
Thrombosis

ABSTRACT

Aims: This study evaluated the presence of genetic mutations in relation to thrombosis or atherosclerosis in elderly women.

Main methods: This is an observational study of 93 Japanese women with a mean age of 80.9 years recruited from outpatient clinics of Nagoya University and its related hospitals. Ten single nucleotide polymorphisms (SNPs) were studied. Each gene studied acts in or is related to either blood coagulation (factor V Leiden, prothrombin G20210A, factor XIII Val34Leu, factor VII Arg353Gln, MTHFR C677T, β-fibrinogen G-455A, PAI-1 4G/5G), metabolic syndrome-related pathways (PPARα Leu162Val), or endothelium/estrogen system (eNOS Glu298Asp, ERα IVS1-401). SNPs were analyzed for their relation to clinical values including lipids, B-type natriuretic peptide (BNP), fasting plasma glucose, tumor necrosis factor-α, interleukin-6, cyclic GMP, and nitric oxide metabolites.

Key findings: Comparisons between the distributions of different genotypes and clinical values showed three relationships. First, factor VII Arg353Gln and HDL-cholesterol (HDL-C) were linked to Arg/Arg carriers at higher levels ($P=.049$). The HDL-C to LDL-cholesterol ratio supported this link ($P=.027$). Second, eNOS Glu298Asp and triglycerides were linked to Glu/Glu carriers at higher levels ($P=.031$). Third, ERα IVS1-401 and BNP were related to CC genotype at lower levels ($P=.031$). Additionally, the last two relations showed that genotype does not influence the demarcation line of biomarkers, but the plasma/serum levels of biomarkers instead.

Significance: Correlations of factor VII Arg353Gln with HDL-C and eNOS Glu298Asp with triglycerides are new findings. Polymorphisms in the endothelium/estrogen system and the heart failure marker BNP are also correlated, with ERα IVS1-401 being the first identified marker. SNPs may be helpful for understanding the pathophysiology of atherosclerotic diseases in elderly women.

Introduction

A thrombogenic state is an important risk factor for atherosclerotic diseases such as myocardial infarction and stroke. Thrombosis has been suggested to be an adverse effect associated with postmenopausal hormone-replacement therapy (HRT) (Fisher et al. 1998; Ettinger et al. 1999; Rosendaal et al. 2002).

Genetic mutation factors have been associated with an elevated risk associated with thrombosis. For example, factor V Leiden (also known as Arg506Gln, R506Q, or G1691A) has been associated with a 6.69-fold enhancement of the risk posed by thrombosis (Cushman et al. 2004). This single nucleotide polymorphism (SNP) is a guanine (G) to adenine (A) transition at the second base of the codon for amino acid position 506 in exon 10 of the factor V (symbol; F5) gene. In total, 5% of Caucasians are carriers of this SNP, which is known to make the protein resistant to inactivation by activated protein C (Bertina et al. 1994). This SNP is found in 20% of venous thrombosis patients (Rosendaal et al. 1995). Regional differences exist; East Asians do not carry factor V Leiden (Rees et al. 1995), which could be one of the reasons for the lower incidence of thrombosis in East Asians, although this remains unclear. Another noted SNP seen in Caucasians is prothrombin G20210A, which is a G to A transition at position 20210 of the prothrombin (symbol; F2) gene. It is observed among 6% of

* Corresponding author. Tel.: +81 52 744 2364; fax: +81 52 744 2371.
E-mail address: hayashi@med.nagoya-u.ac.jp (T. Hayashi).
[1] Present address: Department of Pharmacology, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan.
[2] Present address: Department of Geriatrics, West China Hospital of Sichuan University, Chengdu, Sichuan 610041, China.
[3] Present address: Yunnan Provincial Institute of Medical Information, 205 Renmin West Road, Kunming City, China.
[4] Present address: Yunnan Provincial Chinese Medicine Hospital, Kunming City, China.
[5] Present address: Faculty of Medical Welfare, Aichi Shukutoku University, Nagoya, Aichi 464-8671, Japan.

0024-3205/$ – see front matter © 2009 Published by Elsevier Inc.
doi:10.1016/j.lfs.2009.06.009

venous thrombosis patients (Poort et al. 1996). For these two blood clotting factors, a meta-analysis has found a moderate association with coronary disease (Ye et al. 2006), implying that, for these and other clotting factors, a genetic study should be performed.

Several SNPs related to thrombosis and atherosclerotic risk have been proposed. Factor XIII Val34Leu shows a protective effect against venous thromboembolism (Mikkola et al. 1994; Wells et al. 2006). Factor VII Arg353Gln (R353Q or G10976A) leads to a reduction of its protein levels with a lower risk of cardiovascular disease (Green et al. 1991; Lane et al. 1992; Hunault et al. 1997; Girelli et al. 2000). MTHFR C677T leads to higher homocysteine levels and is thus a risk factor for coronary artery diseases (Frosst et al. 1995; Ma et al. 1996). β-fibrinogen G-455A (HaeIII) is associated with higher plasma fibrinogen levels (Thomas et al. 1991; Iso et al. 1995; van' t Hooft et al. 1999). PAI-1 4G/5G, which is the 4G allele related to elevation of circulating PAI-1, is associated with cardiovascular disease (Dawson et al. 1991; Eriksson et al. 1995; Hoekstra et al. 2004). PPARα Leu162Val (L162V) is associated with increased transcriptional activation of itself and the elevation of serum lipid levels (Vohl et al. 2000; Sapone et al. 2000). eNOS Glu298Asp (E298D) is associated with the possibility of impaired endothelial function (Philip et al. 1999; Guzik et al. 2001). Lastly, ERα IVS1-401 (IVS1-397 or PvuII), which is a transition in the intervening sequence 1 at position-401, is associated with cardiovascular disease (Herrington et al. 2002b; Nordström et al. 2003).

In atherosclerotic lesions, lipid accumulation in the plaque intima (Kramsch et al. 1971), increased cytokine levels (Rus et al. 1991, 1996), and an increased BNP level are all detected (Casco et al. 2002). It is believed that nitrite and nitrate, which are metabolites of nitric oxide (NOx) in vascular disease, are important factors that should be assessed (Palmer et al. 1987). BNP and NO elevates intracellular cGMP level (Palmer et al. 1987; Chinkers et al. 1989), indicating the importance of this second messenger. From a clinical point of view, it is important to identify biomarkers that can be used to predict both disease and longevity (Nomura et al. 2002; Hayashi et al. 2007). However, the use of SNPs as biomarkers is not well understood to date. Our aim is to evaluate genetic factors in elderly women in order to identify biomarkers that correlate with SNPs. The experimental design of this report was restricted to an East Asian population that does not possess factor V Leiden, because it is an easier model for screening candidate factors associated with thrombosis or atherosclerotic risk. In addition, analyses focusing on postmenopausal elderly women are rarely performed, and thus may result in an improved fundamental understanding of older people.

Material and methods

Subjects

We enrolled 104 Japanese female subjects over 60 years of age who were admitted to the Department of Geriatrics in an outpatient clinic of Nagoya University Hospital, Nagoya City (Japan), between May 2004 and January 2005. All subjects gave written informed consent. Proper authorization for the study was obtained from the Ethics Committee of the Nagoya University Graduate School of Medicine. As the data of serum or plasma collection or amount of assay for DNA analyses were insufficient in 11 patients, we finally analyzed the data of 93 patients.

Serum or plasma collection and measurement

Serum or plasma was collected from fasting blood samples after centrifugation at 3000 rpm at 4 °C, and stocked at −30 °C until measurement. BNP and cGMP concentrations were derived from the blood sample analysis (SRL Laboratories, Japan) as measured by a specific immunoassay. Tumor necrosis factor-α and interleukin-6 were measured using the Quantikine HS Kit (R & D Systems, USA). NOx was determined by high-performance liquid chromatography (Hayashi et al. 2007). Other clinical biochemical factors, such as LDL-C, HDL-C, and triglyceride levels, were also assessed.

DNA isolation and genotyping

DNA was isolated from whole blood using the QIAamp DNA Blood Mini Kit (QIAGEN, Düsseldorf, Germany) and genotyped with the Mutector Dual Well Test Kit (TrimGen, Maryland, USA) according to the manufacturer's instructions. The Mutector kit was designed for mutation detection among known nucleotide substitutions using a 96-well strip plate, and can confirm all three genotypes (wild, mutant homozygous, and heterozygous types). Both positive and negative controls were provided by the manufacturer. In brief, a complimentary detection primer is designed and immobilized on the wells whose 3' end terminates just before the target base (i.e. order made by offering the specified sequence information). The polymerase chain reaction product from the preceding step is added to wells together with labeled nucleotides and extension primer for either mutant or wild strand. Primer for mutant strand makes extension when the target base is mutant type but not wild, and vice versa. As a result, labeled nucleotides are incorporated and a colorimetric reaction is observed and measured by 405 nm on a microplate reader.

Selection of SNPs

All ten analyzed SNPs have referential SNP cluster identification numbers (RefSNP ID) provided by the NCBI (National Center for Biotechnology Information, Maryland). They are: factor V Leiden (rs6025), prothrombin G20210A (rs1799963), factor XIII Val34Leu (rs3024472), factor VII Arg353Gln (rs6046), MTHFR C677T (rs1801133), β-fibrinogen G-455A (rs1800790), PAI-1 (-675) 4G/5G (rs1799889), PPARα Leu162-Val (rs1800206), eNOS Asp298Glu (rs1799983), and ERα IVS1-401 (rs2234693).

Statistical analyses

The association of genotype distribution with clinical factors, represented as mean ± SEM, was analyzed using Microsoft Excel enhanced software with either an unpaired Student's *t*-test or a Mann–Whitney *U*-test, depending on histogram distribution. A chi-square test was used to describe the effect of genotype on biomarker levels

Table 1
All variables are presented as mean ± SEM.

Characteristic		On registration [*n* = 93]
Age, years	60–64 [*n* = 4]	80.95 ± 0.90
	65–74 [*n* = 16]	
	75–84 [*n* = 36]	
	85–94 [*n* = 36]	
	95–99 [*n* = 1]	
Total cholesterol, mg/dL		206.13 ± 4.62
LDL-C, mg/dL		120.73 ± 4.28
HDL-C, mg/dL		57.89 ± 2.05
Triglycerides, mg/dL		113.37 ± 5.60
Creatinine, mg/dL		0.84 ± 0.03
BNP, pg/mL		77.67 ± 8.17
Glucose, mg/dL		99.29 ± 3.52
TNF-α, pg/mL		3.96 ± 0.31
IL-6, pg/mL		7.08 ± 2.04
cGMP, pmol/mL		7.28 ± 0.40
NOx, μmol/L		55.38 ± 4.00
Hemoglobin, g/dL		11.89 ± 0.20

LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; BNP, B-type natriuretic peptide; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; cGMP, cyclic guanosine 5'-monophosphate; NOx, nitric oxide metabolites.

Table 2
The ten SNPs were analyzed for all subjects ($n=93$) except for PAI-1 4G/5G ($n=90$) and PPARα Leu162Val ($n=91$).

Type of SNP	Distribution of different genotypes			Total n
Factor V Leiden	93 [GG; Arg/Arg]	0 [AG; Gln/Arg]	0 [AA; Gin/Gin]	[93]
Prothrombin G20210A	93 [GG]	0 [AG]	0 [AA]	[93]
Factor XIII Val34Leu	93 [GG; Val/Val]	0 [TG; Leu/Val]	0 [TT; Leu/Leu]	[93]
Factor VII Arg353Gln	81 [GG; Arg/Arg]	11 [AG; Gln/Arg]	1 [AA; Gln/Gln]	[93]
MTHFR C677T	35 [CC; Ala/Ala]	39 [TC; Val/Ala]	19 [TT; Val/Val]	[93]
β-fibrinogen G-455A	73 [GG]	19 [AG]	1 [AA]	[93]
PAI-1 4G/5G	9 [5G/5G]	41 [4G/5G]	40 [4G/4G]	[90]
PPARα Leu162Val	91 [CC; Leu/Leu]	0 [GC; Val/Leu]	0 [GG; Val/Val]	[91]
eNOS Glu298Asp	80 [GG; Glu/Glu]	12 [TG; Asp/Glu]	1 [TT; Asp/Asp]	[93]
ERα IVS1-401	13 [CC]	44 [TC]	36 [TT]	[93]

For example, '0 [AG; Gln/Arg]' indicates that the genotype is an A and G allele heterozygous genotype coding for Gln and Arg, with $n=0$ subjects. SNP, single nucleotide polymorphism; G, guanine; A, adenine; T, thymine; C, cytosine; Arg, arginine; Gln, glutamine; Val, valine; Leu, leucine; Ala, alanine; Glu, glutamic acid; Asp, aspartic acid; IVS, intervening sequence.

dichotomized by the demarcation line. A probability value under 0.05 as determined by two-tail analyses was considered statistically significant.

Results

Study population and genotype distribution

The patient profiles are shown in Table 1. The observed population contains clinical backgrounds such as the following with some holding multiple diseases; angina ($n=3$), arteriosclerosis ($n=2$), cardiac insufficiency ($n=5$), cerebrovascular accident which contains a sequela ($n=19$), diabetes ($n=11$), hyperlipemia ($n=14$), hypertension ($n=42$), and none disease carriers ($n=28$) (supplemental Table 1b). The distribution of the different genotypes for each of the ten analyzed SNPs is shown in Table 2. As suggested in the introduction, we confirmed that neither prothrombin G20210A nor factor V Leiden was present at detectable levels in our samples. Genotype frequencies were in Hardy–Weinberg equilibrium for all the SNPs, analyzed by chi-square test on observed versus expected genotype frequencies (all $P>.05$) (supplemental Table 2b). We proceeded with dominant model analyses to investigate the relation of genotypes to clinical values, and those which appeared to have significant differences are further addressed.

Table 3
All variables are presented as mean ± SEM.

Characteristic	GG [$n=81$; 87.1%]	AG+AA [$n=12$; 12.9%]	P-value [GG vs. AG+AA]
Age, years	81.04 ± 1.00	80.33 ± 1.82	.795
Total cholesterol, mg/dL	207.75 ± 5.16	195.50 ± 8.24	.373
LDL-C, mg/dL	119.10 ± 6.07	131.19 ± 7.15	.337
HDL-C, mg/dL	59.44 ± 2.24	47.93 ± 3.85	.049*
Triglycerides, mg/dL	111.34 ± 6.12	125.42 ± 13.85	.380
Creatinine, mg/dL	0.84 ± 0.03	0.80 ± 0.04	.736
BNP, pg/mL	78.81 ± 9.04	69.97 ± 17.57	.681
Glucose, mg/dL	98.11 ± 3.82	108.00 ± 8.51	.370
TNF-α, pg/mL	3.91 ± 0.35	4.22 ± 0.66	.721
IL-6, pg/mL	7.32 ± 2.40	5.80 ± 1.91	.788
cGMP, pmol/mL	7.47 ± 0.45	6.30 ± 0.85	.299
NOx, μmol/L	52.56 ± 4.11	70.27 ± 12.16	.106
Hemoglobin, g/dL	11.92 ± 0.22	11.58 ± 0.65	.645

$P<.05$ is indicated by an asterisk (*). Mann–Whitney *U*-tests were performed on HDL-C, Creatinine, BNP, and cGMP values, while unpaired Student's *t*-tests were used for the other values. Of note, GG [$n=71$] and AG+AA [$n=12$] for triglycerides, and GG [$n=37$] and AG+AA [$n=5$] for glucose were analyzed. The G/A allele codes for Arg/Gln. Arg, arginine; Gln, glutamine; G, guanine; A, adenine; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; BNP, B-type natriuretic peptide; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; cGMP, cyclic guanosine 5'-monophosphate; NOx, nitric oxide metabolites.

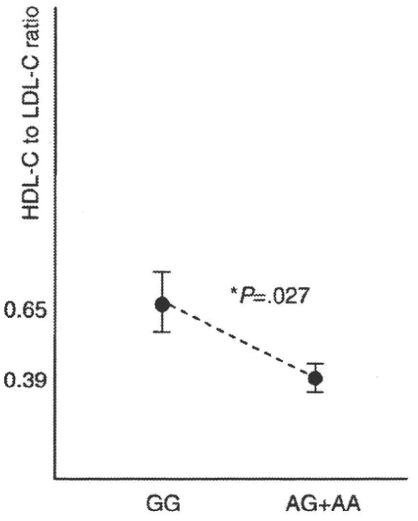

Fig. 1. Genotype affects the HDL-C to LDL-C ratio. The Mann–Whitney *U*-test was performed on factor VII Arg353Gln genotypes vs. HDL-C to LDL-C ratio. $P<.05$ is indicated by an asterisk (*). Bar indicates SEM. HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; G, guanine; A, adenine; Arg, arginine; Gln, glutamine.

Factor VII gene polymorphism and HDL-C

As shown in Table 3, the factor VII Arg353Gln polymorphism was linked to HDL-C. The GG genotype encoding for Arg/Arg was associated with higher HDL-C levels than the AG+AA genotypes ($P=.049$). This finding is supported by comparison of the HDL-C to LDL-C ratio for the GG genotype (0.65 ± 0.10) and the AG+AA genotypes (0.39 ± 0.05), which were significantly different ($P=.027$) (Fig. 1). We did not observe a significant difference among LDL-C levels between genotypes, although there was a similar trend.

Endothelial nitric oxide synthase gene polymorphism and triglycerides

As shown in Table 4, the eNOS Glu298Asp polymorphism was related to triglycerides. The GG genotype encoding for Glu/Glu was associated with higher triglyceride levels than the TG+TT genotypes

Table 4
All variables are presented as mean ± SEM.

Characteristic	GG [$n=80$; 86.0%]	TG+TT [$n=13$; 14.0%]	P-value [GG vs. TG+TT]
Age, years	80.48 ± 1.00	83.85 ± 1.61	.195
Total cholesterol, mg/dL	208.24 ± 4.82	192.25 ± 14.85	.244
LDL-C, mg/dL	122.80 ± 4.14	105.79 ± 18.66	.351
HDL-C, mg/dL	57.73 ± 2.29	59.03 ± 3.45	.837
Triglycerides, mg/dL	117.82 ± 6.00	80.90 ± 11.47	.031*
Creatinine, mg/dL	0.84 ± 0.03	0.80 ± 0.11	.767
BNP, pg/mL	73.79 ± 8.28	101.60 ± 28.74	.240
Glucose, mg/dL	99.80 ± 3.57	n.a.	n.a.
TNF-α, pg/mL	4.02 ± 0.34	3.71 ± 0.80	.702
IL-6, pg/mL	5.81 ± 1.70	12.65 ± 8.14	.195
cGMP, pmol/mL	7.19 ± 0.42	7.67 ± 1.14	.648
NOx, μmol/L	57.00 ± 4.79	48.40 ± 4.82	.405
Hemoglobin, g/dL	11.95 ± 0.22	11.43 ± 0.66	.452

$P<.05$ is indicated by an asterisk (*). Mann–Whitney *U*-tests were performed on LDL-C and creatinine values, while unpaired Student's *t*-tests were used for the other values. Of note, GG [$n=73$] and TG+TT [$n=10$] for triglycerides, and GG [$n=41$] and TG+TT [$n=1$] for glucose were analyzed. Since the latter separated as TG+TT [$n=1$], analyses were not performed (n.a.). The G/T allele codes for Glu/Asp. Glu, glutamic acid; Asp, aspartic acid; G, guanine; T, thymine; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; BNP, B-type natriuretic peptide; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; cGMP, cyclic guanosine 5'-monophosphate; NOx, nitric oxide metabolites.

($P=.031$). Since triglycerides is a clinical marker with an accepted demarcation line, that is 100 mg/dL, we used the chi-square test to assess whether the GG genotype and TG+TT genotypes has or has not had an effect on triglyceride levels dichotomized by this line. As shown in Fig. 2, genotype does not influence the demarcation line but has an effect on the triglyceride levels ($P=.021$).

Estrogen receptor alpha gene polymorphism and BNP

As shown in Table 5, the ERα IVS1-401 polymorphism was related to the plasma BNP concentration, with the CC genotype being associated with a relatively lower plasma BNP concentration when compared to the TC+TT genotypes ($P=.031$). Since BNP is a standard clinical marker for heart failure with a well-known demarcation line, that is 80 pg/mL, we used the chi-square test to assess whether the CC genotype and TC+TT genotypes has or has not had an effect on BNP levels dichotomized by this line. As shown in Fig. 2, it was revealed that genotype does not influence the demarcation line but has an effect on the BNP levels ($P=.031$).

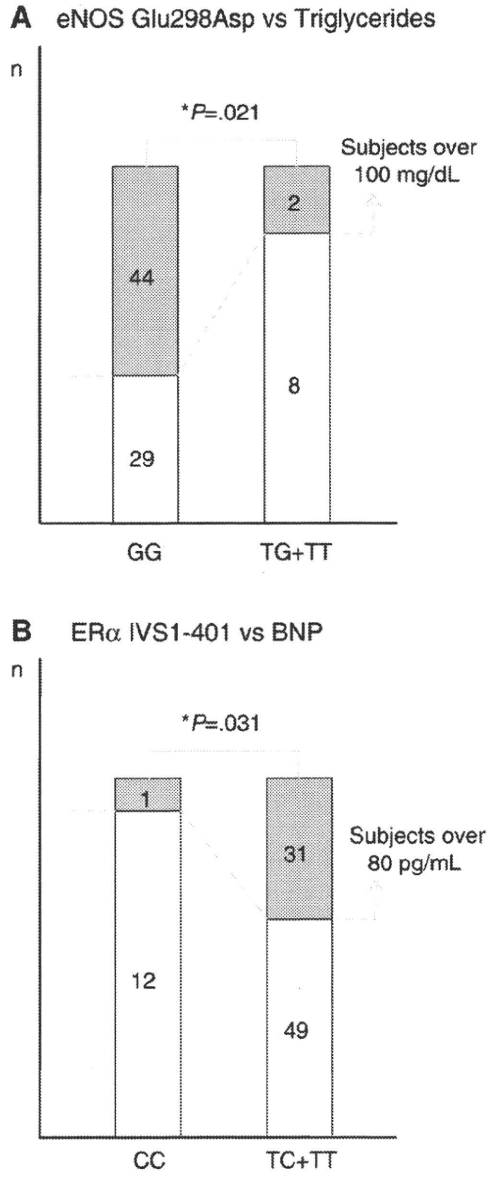

Fig. 2. The eNOS and ERα genotypes influence the concentration of triglycerides and BNP. Chi-square tests were done on eNOS Glu298Asp genotypes vs. triglyceride concentration separated by 100 mg/dL (A), and on ERα IVS1-401 genotypes vs. BNP concentration separated by 80 pg/mL (B). The gray box above the dotted line indicates the number of subjects who were over the demarcation line. $P<.05$ is indicated by an asterisk (*). Each box was sized to reflect the percentage for visual purposes. Glu, glutamic acid; Asp, aspartic acid; G, guanine; T, thymine; IVS, intervening sequence; BNP, B-type natriuretic peptide; C, cytosine.

Table 5
All variables are presented as mean ± SEM.

Characteristic	CC [$n=13$; 14.0%]	TC+TT [$n=80$; 86.0%]	*P*-value [CC vs. TC+TT]
Age, years	79.31 ± 3.10	81.21 ± 0.92	.466
Total cholesterol, mg/dL	211.62 ± 11.37	205.22 ± 5.07	.631
LDL-C, mg/dL	128.81 ± 9.81	119.62 ± 4.68	.486
HDL-C, mg/dL	62.96 ± 7.57	57.19 ± 2.10	.362
Triglycerides, mg/dL	115.64 ± 11.67	113.03 ± 6.23	.876
Creatinine, mg/dL	0.82 ± 0.06	0.84 ± 0.03	.804
BNP, pg/mL	52.17 ± 19.69	81.82 ± 8.90	.031*
Glucose, mg/dL	102.80 ± 10.57	98.81 ± 3.78	.719
TNF-α, pg/mL	4.90 ± 1.02	3.74 ± 0.31	.149
IL-6, pg/mL	5.02 ± 1.56	7.55 ± 2.48	.633
cGMP, pmol/mL	6.53 ± 0.90	7.44 ± 0.45	.384
NOx, μmol/L	54.14 ± 6.48	55.64 ± 4.67	.889
Hemoglobin, g/dL	12.27 ± 0.74	11.82 ± 0.20	.411

$P<.05$ is indicated by an asterisk (*). Mann–Whitney *U*-tests were done on HDL-C, creatinine, BNP and cGMP values, while unpaired Student's *t*-tests were used for the other values. Of note, CC [$n=11$] and TC+TT [$n=72$] for triglycerides, and CC [$n=5$] and TC+TT [$n=37$] for glucose were analyzed. IVS, intervening sequence; C, cytosine; T, thymine; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; BNP, B-type natriuretic peptide; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; cGMP, cyclic guanosine 5'-monophosphate; NOx, nitric oxide metabolites.

Discussion

We restricted our subjects to postmenopausal elderly women (with a mean age of 80.9 years) who were free of factor V Leiden and prothrombin G20210A. In doing so, we were able to identify new correlations between the factor VII Arg353Gln polymorphism and HDL-C levels, and between the eNOS Glu298Asp polymorphism and triglyceride levels. We have demonstrated, for the first time, a statistically significant association between the ERα IVS1-401 polymorphism and plasma BNP concentration, suggesting that CC genotype carriers have relatively lower plasma BNP levels than TC+TT genotypes ($P=.031$). To our knowledge, there are no other reports linking the heart failure marker BNP with an SNP in the estrogen system.

Recently, an important meta-analysis of HRT ruled out the possibility that HRT increases HDL-C and reduces either LDL-C or the LDL-C to HDL-C ratio (Salpeter et al. 2006). Concerning SNPs, a recent meta-analysis implied that the ERα IVS1-397 (which is synonymous with -401) polymorphism does not influence the HDL-C response to HRT (Kjaergaard et al. 2007). Our focus is on elderly women, and we did not identify a relationship between ERα IVS1-401 and HDL-C levels, as shown in Table 5. We did, however, observe a relationship between HDL-C and the factor VII Arg353Gln polymorphism, with the GG genotype being associated with higher HDL-C levels than the AG+AA genotypes ($P=.049$). This finding was supported by comparison of the HDL-C to LDL-C ratio in each genotype in the same manner ($P=.027$). This ratio was not significant for eNOS Glu298Asp (GG; 0.63 ± 0.03, TG+TT 0.55 ± 0.07), and ERα IVS1-401 (CC; 0.51 ± 0.06, TC+TT; 0.63 ± 0.10). It is interesting to consider that such an SNP could partially impact the circulating levels of HDL-C in elderly individuals, but not in younger individuals; however, there is currently no scientific evidence to support the influence of SNPs on clinical biomarker levels that are only present in the elderly.

We have provided new evidence that the eNOS Glu298Asp polymorphism is related to triglyceride levels, with the GG genotype encoding for Glu/Glu being associated with higher triglyceride levels