Dose-Dependent Modulatory Effects of Insulin on Glucose-induced Endothelial Senescence in vitro and in vivo: A Relationship between Telomeres and Nitric Oxide


Department of Geriatrics, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan (H.M.-H., T.H., K.I., M.M., H.K., A.I.); Department of Molecular and Medical Pharmacology (S.Y., Y.H.), Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama 930-0194, Japan; and Department of Molecular and Medical Pharmacology, David Geffen School of Medicine at University of California Los Angeles, Los Angeles, CA 90095 (L.J.I.)

Copyright 2011 by the American Society for Pharmacology and Experimental Therapeutics.
A running title: Glucose, insulin, and endothelial senescence

Corresponding author:
Toshio Hayashi, M.D., Ph.D.
Department of Geriatrics, Nagoya University Graduate School of Medicine, Nagoya
466-8550, Japan
Tel: 81-52-744-2364; Fax: 81-52-744-2371; E-mail: hayashi@med.nagoya-u.ac.jp

The number of text pages: 31
The number of tables: 0
The number of figures: 7
The number of references: 36
The number of words in the Abstract: 237
The number of words in the Introduction: 511
The number of words in the Discussion: 1316

ABBREVIATIONS:
ROS, reactive oxygen species; NO, nitric oxide; IGF, insulin-like growth factor; PI3-K, phosphatidylinositol 3-kinase; L-Arg, L-arginine; NOS, nitric oxide synthase; L-NAME, N^{\text{G}}-nitro-L-arginine methyl ester; AICAR, 5'-aminoimidazole-4-carboxamide ribonucleoside; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; HUVECs, human umbilical venous endothelial cells; HAECs, human aortic endothelial cells; SA-\beta-gal: senescence-associated-\beta-galactosidase; eNOS, endothelial nitric oxide
synthase; NOx, nitrite and nitrate; siRNA, small-interfering RNA; VCAM-1, vascular cell adhesion molecule-1; STZ, streptozotocin

SECTION: Cardiovascular Pharmacology
ABSTRACT

The elderly is 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 NO. Small-interfering RNA targeting endothelial nitric oxide (NO) synthase reduced the anti-senescence effects of insulin. Physiological concentrations of insulin also reversed high glucose-induced increases in p53 and vascular cell adhesion molecule-1 and decreases in SMP30. On the other hand, given at any concentrations under normal glucose or at high concentrations under high glucose, the ability of insulin 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 to 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 these numbers are still increasing. Diabetic patients have a 2.5~4-fold increased risk of cardiovascular events and their life spans can be shortened by as much 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, like 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 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 C. 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 ACCORD, 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, 2008).

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

Senescence ensuing from cell replication is termed “replicative senescence”, which implicates an intrinsic mechanism responsible for the life span of somatic cells (Hayashi et al., 2008). Mitosis-related telomere shortening is critical. A decrease in telomerase activity precedes telomere shortening (Bosnar et al., 1998). The senescence response is elicited by many stressful stimuli, such as DNA damage (McLaren et al., 2004) and ROS (Parrinello et al., 2003). Human cells exposed to these stressors display features of “stress-induced premature senescence (SIPS)” within several hours or a few days that are likely 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.
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-4H-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 (HUVEC) and human aortic endothelial cells (HAEC) were purchased from Lonza Walkersville (Walkersville, MD) and cultured in EGM-2 until the start of the experiment. The cells were cultured in modified EGM-2 that lacked IGF-1 but contained 2% FBS during the experimental term. It contained only less than \(10^{-12}\) M insulin, which was considered to have no effect 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 C12FDG (fluorogenic substrate 5-dodecanoyl-aminofluorescein di-β-D-galactopyranoside, 33 mM) at 37°C for 30 min. Cells were trypsinized and analyzed using a FACSCaliber flow cytometer (Becton Dickinson, 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 GmbH, 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, Hercules, CA).

**Human Telomere Length Assay**

Telomere length was measured by FISH 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 condition.

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-H2DCFDA) (Invitrogen, Carlsbad, CA) (Chandra et al., 2003). Cells were incubated with CM-H2DCFDA (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, Santa Cruz, CA) or an anti-VE-cadherin antibody (Alexis Biochemicals, San Diego, CA). Endothelial cells were treated with an ROS detection reagent (CM-H2DCFDA, Invitrogen). The nucleus was counterstained with Hoechst 33258 (Nacalai Tesque, Kyoto, Japan). Images were observed using a Leica TCS-SP5 confocal system.
Transfection of eNOS siRNAs

siRNAs targeting human eNOS were developed in our laboratory (Miyazaki-Akita et al., 2007). Non-silencing 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 utilized:

5’-CGAGGAGACUUCGAAUCUUU-3’ (sense) and
5’-PAGAUUCGGAAGUCUCCUCGUU-3’ (antisense) for eNOS siRNA;
5’-UUCUUCGAACGUGUCACGUdTdT-3’ (sense) and
5’-ACGUGACACGUUCCGAGAdTdT-3’ (antisense) for control siRNA. siRNA (1 nM) was transfected using Lipofectamine TM RNAiMAX (Invitrogen). After incubation for 72 h, the down-regulation of eNOS expression was confirmed by western blotting and NOx levels.

Generation of Streptozotocin (STZ) Diabetic Animal Model

We generated young (8 weeks old) and aged (82 weeks old) diabetic rats (Sprague-Dawley (SD) rats) using 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 I.U./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 ANOVA 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- (Figs. 1A and B) and time-dependent manner. Under normal glucose, all concentrations of insulin increased SA-β-gal activity in HUVECs and HAECs (Figs. 1A 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 ($10^{-7} - 10^{-6}$ M) enhanced the high glucose (22 mM)-induced increase in SA-β-gal activity (Figs. 1A and 2A), although insulin at $10^{-6}$ M did not cause further increase in SA-β-gal activity beyond that of extremely high glucose (31 mM) alone (Fig. 1A). To rule out an osmotic effect, we added 25 mM mannitol to 5.5 mM glucose and 9.5 mM mannitol to 21 mM glucose. Mannitol was without effect on cellular senescence (data not shown).

Replicative and Stress-Induced Senescence

Telomerase activity decreased significantly after 3 days of exposure to high glucose in HUVECs, and subsequently, telomere length was significantly shortened by 4 weeks, which indicated replicative senescence (Figs. 2B and C). Physiological concentrations ($10^{-10} - 10^{-9}$ M) of insulin prevented this decrease in telomerase activity and telomere shortening induced by high glucose (Figs. 2B and C). However, such effects were not observed at high concentrations of insulin ($10^{-8} - 10^{-6}$ M) (Figs. 2B and C). The endothelial expression levels of p53, a canonical inducer of cellular senescence (Kletsas et al., 2004), and 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 (Figs. 3A 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 to 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, and they were prevented by insulin at both physiological and supra-physiological 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.

**ROS Generation and VCAM-1 Expression**

The exposure of HUVECs to high glucose (22 mM) for 3 days increased ROS generation (Figs. 4A 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 (Figs. 4A 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, was without 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, 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 to the negative control under normal and high glucose conditions (Figs. 5D and E). The increases in eNOS protein (Fig. 5D) and NOx (Fig. 6) observed under high glucose in the presence of physiological insulin were significantly reduced by eNOS siRNA (Fig. 5D).

Under normal glucose, transfection with eNOS siRNA alone marginally
affected SA-β-gal activity (Fig. 5F), and physiological insulin significantly increased SA-β-gal activity with eNOS siRNA. High concentrations of insulin significantly increased SA-β-gal activity regardless of whether eNOS siRNA was applied. However, under high glucose, treatment with eNOS siRNA further significantly enhanced SA-β-gal activity and blunted the decreased activity induced by physiological concentrations of insulin. Similarly, 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, 429 ± 117 mg/dl in the diabetic, and 153 ± 39 mg/dl in the insulin-treated diabetic groups. 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 stained cells were observed in the endothelial cells of aortas in each group (Figs. 7A 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 (Figs. 7C 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 over-expression 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 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 et al., 2007). Oxidized low-density lipoproteins inhibit endothelial telomerase activity (Breitschopf et al., 2001). Similarly, long-term exposure of HUVECs to mild oxidative stress caused by perturbation of the glutathione redox-cycle results in accelerated telomere erosion (Perrinello et al., 2003; Polyarchou and Papadimitriou, 2005). Oxidative stress may also stimulate replicative- and stress-induced senescence. Interestingly, 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). Life-style 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 [Akt/PKB (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, interestingly, 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 the PI3-K pathway eliminated the anti-senescence 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 (~10^{-8} M) due to eating and other stimuli, including chemical injections. High concentrations of insulin (~2\times10^{-7} M) are observed temporarily after an injection of large insulin doses in some diabetic patients (Epel et al., 2004). These plasma concentrations may be similar to the concentrations in the endothelial cell environment in our study.

Another finding of this study is that high concentrations of insulin promoted senescence independently of glucose concentrations. The mechanism(s) 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 on 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 anti-atherogenic effect and to 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 due to 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 aortae 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 aortae 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 in 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

The authors thank Kengo Tomita for his excellent help in creating the figures in this paper.

Authorship Contributions

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


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

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


Footnotes

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (195910403).
Legends for Figures

**Figure 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, high and extremely high-glucose concentrations (NG, HG, EHG) in HUVECs; n = 6. *, P<0.05 vs. NG, †, P<0.05 vs. HG, #, P<0.05 vs. EHG.  
B: Effects of insulin on SA-β-gal activity under normal and high glucose (NG, HG) in HAECs; n = 6. *, P<0.05 vs. NG, †, P<0.05 vs. HG.  
C: Cytochemical staining for SA-β-gal activity. NG, 5.5 mM; HG, 22 mM; EHG, 31 mM.

**Figure 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 vs. 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 vs. NG, †, P<0.05 vs. HG.

**Figure 3.** Effects of insulin on p53 expression, SMP30 expression, and eNOS and Akt phosphorylation in HUVECs. A: Effects of low and high concentrations of insulin on p53 expression under normal and high glucose (3 days of exposure). The top trace is a representative Western blot of p53 and actin; n = 6. **, P<0.01 vs. NG without insulin. NG, 5.5 mM; HG, 22 mM.  
B: Effects of low and high concentrations of insulin on SMP30 expression under normal and high glucose (3 days of exposure). The top trace is
a representative Western blot of SMP30; n = 6. *, P<0.05 vs. NG without insulin. †, P<0.05 vs. HG without insulin. NG, 5.5 mM; HG, 22 mM. C: Effects of low and high concentrations of insulin on the phosphorylation of eNOS and Akt under normal and high glucose (3 days of exposure). The top trace is a representative Western blot; n = 6. †, P<0.05 vs. HG without insulin. NG, 5.5 mM; HG, 22 mM.

**Figure 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 vs. NG without insulin. NG, 5.5 mM; HG, 22 mM. B: Images of intracellular ROS visualization using CM-H2DCFDA. 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 bottom panels, nuclei were counterstained with Hoechst.

**Figure 5.** Analysis of the possible mechanism(s) 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 high glucose for 72 h. n = 6. *, P<0.05; **, P<0.01 vs. HG. NG, 5.5 mM; HG, 22 mM. D and E: 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. Non-silencing control siRNA was used as a negative control and scrambled siRNA was used as a control. Top trace of each panel: representative Western blots of eNOS and β-actin; n = 5. *, P<0.05; **, P<0.01 vs. NG. †, P<0.05; ††, P<0.01 vs. HG. 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 vs. NG. †, P<0.05 vs. HG. NG, 5.5 mM; HG, 22 mM.

**Figure 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 (HPLC) system. Cells were incubated for 3 days under normal or high glucose. *, P<0.05. NG, 5.5 mM; HG, 22 mM.

**Figure 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 vs. control.
Figure 1

A HUVECs  

B HAECs  

C

NG  

HG  

HG Insulin 10^{-10} M  

HG Insulin 10^{-6} M
Figure 3

A

p53

Actin

Relative densities of p53/actin

Insulin (log M)

-10 -6 -10 -6

NG HG (22 mM)

B

SMP 30

SMP-30 expression (% control: NG)

Insulin (log M)

-10 -6 -10 -6

NG HG (22 mM)

C

pAkt/Akt

peNOS/eNOS

pAkt+Akt

peNOS+eNOS

(relative intensities of phosphorylation)

(log M) 0 -10 -6 0 -10 -6

NG HG

NG HG

NG HG

NG HG
Figure 6

[Diagram showing NOx production (µM) in response to different conditions.

**Insulin (log M):**
- -
- -
- -
- -12
- -10
- -10
- -8
- -6
- -6

**eNOS siRNA:**
- -
- +
- +
- -
- -
- -
- -
- -
- +

**Conditions:**
- NG
- HG

*Significant differences indicated by asterisks.*

Downloaded from Jpet.aspetjournals.org on May 9, 2017.
Figure 7

A  Young control  Young diabetic  Young diabetic-insulin

B  Young

SA-β-gal activity (% of total area)

Control  Diabetic  Diabetic-Insulin

C  Aged control  Aged diabetic  Aged diabetic-insulin

D  Aged

SA-β-gal activity (% of total area)

Control  Diabetic  Diabetic-Insulin

*