We speculated that the transcriptional regulation of GTPCH1 mRNA is responsible for the decreased GTPCH1 mRNA by high glucose treatment. Preliminary experiment showed that the decreased activity of GTPCH1 associated decreased protein level. Gesierich et al. reported the importance of the complex formation of GTPCH1 with GTPCH1 feedback regulatory protein (GERP) in negative feedback regulation by end product BH_4, and phenylalanine upregulated GTPCH1 mRNA without changing GERP (Gesierich et al., 2003; Hattori et al., 2003). They speculated that the substrate level and transcription of the interacting protein regulation of BH_4 biosynthesis. In the present study, the amount of BH_4 was decreased by high glucose treatment, and the protein also decreased.

HMG-CoA reductase inhibitors are now generally convinced to be a potent antiatherosclerotic agent. Its pleiotropic effects include a direct stimulatory effect on eNOS or iNOS as reported (Gorren et al., 2002; Hayashi et al., 1995a; List et al., 1997). And we have for the first time revealed that HMA-CoA reductase inhibitors could upregulate GTPCH1 mRNA expression, thus stimulate the activity of GTPCH1 as well as intracellular BH_4 levels in cultured endothelial cells, directly. Statin was reported to enhance cytokine-mediated inducible nitric oxide synthesis in smooth muscle cells (Hattori et al., 2002). It has been reported that the effect of statin was abolished by exogenous mevalonate or GTPCH1 inhibitor, GGTI-298. Our data further, give a richer meaning to the pleiority of antiatherosclerotic effects of HMG–OA reductase inhibitor (Laufs et al., 1998; Tsunekawa et al., 2001). Finally, mannitol concentration was adjusted to the osmotic pressure in 50 mM high glucose. Preliminarily, we examined the effect of mannitol on high glucose treatment, and we made sure that it did not significant effect on eNOS protein and mRNA and GTPCH1 mRNA, and BH_4 concentration.

Conclusively, high glucose could lead to the dysfunction of eNOS by inhibiting the synthesis of BH_4 and activating NADPH oxidase. Statin could enhance eNOS activity through stimulating GTPCH1, thus increases BH_4 levels, directly.

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L-citrulline and L-arginine supplementation retards the progression of high-cholesterol-diet-induced atherosclerosis in rabbits

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The objective of this study was to evaluate the influence of ingested L-arginine, L-citrulline, and antioxidants (vitamins C and E) on the progression of atherosclerosis in rabbits fed a high-cholesterol diet. The fatty diet caused a marked impairment of endothelium-dependent vasorelaxation in isolated thoracic aorta and blood flow in rabbit ear artery *in vivo*, the development of atheromatous lesions and increased superoxide anion production in thoracic aorta, and increased oxidation-sensitive gene expression [Elk-1 and phosphorylated cAMP response element-binding protein]. Rabbits were treated orally for 12 weeks with L-arginine, L-citrulline, and/or antioxidants. L-arginine plus L-citrulline, either alone or in combination with antioxidants, caused a marked improvement in endothelium-dependent vasorelaxation and blood flow, dramatic regression in atheromatous lesions, and decrease in superoxide production and oxidation-sensitive gene expression. These therapeutic effects were associated with concomitant increases in aortic endothelial NO synthase expression and plasma NO_2^- + NO_3^- and cGMP levels. These observations indicate that ingestion of certain NO-boosting substances, including L-arginine, L-citrulline, and antioxidants, can abrogate the state of oxidative stress and reverse the progression of atherosclerosis. This approach may have clinical utility in the treatment of atherosclerosis in humans.

antioxidant | nitric oxide | amino acids | endothelial nitric oxide synthase

Atherosclerosis is an inflammatory disease (1) characterized by vascular endothelial cell dysfunction and diminished production of NO (2–5). Endothelial NO synthase (eNOS) gene transfer can reduce atherogenesis in hypercholesterolemic animals (6). NO is a widespread signaling molecule in the cardiovascular system, which functions in multiple ways to protect against the initiation and progression of atherosclerosis (7–9). For example, NO aids in preventing the adhesion and aggregation of blood cells and platelets along the endothelial cell lining in blood vessels (7, 8) and is a potent inhibitor of vascular smooth muscle cell proliferation (10). NO is a potent antioxidant that can elicit antiinflammatory effects by scavenging certain reactive oxygen species (11–13), and it can prevent the oxidation of low-density lipoprotein cholesterol and thereby retard the progression of atherosclerosis (5, 14). Moreover, NO deficiency is generally associated with up-regulation of oxidation-sensitive genes, whereas increased NO production leads to decreased expression of oxidation-sensitive genes (7, 15). NO is synthesized by NOS, which utilizes L-arginine as substrate and produces L-citrulline as the second reaction product. L-arginine can be synthesized from L-citrulline in endothelial and other cell types, thereby providing a recycling pathway for the conversion of L-citrulline to NO via L-arginine (16–19).

The oral administration of L-arginine to animals (7, 12, 20–26) and humans (5, 8, 27–29) has been demonstrated to slow the progression of atherosclerosis or its component processes. Likewise, antioxidants can elicit antiatherosclerotic effects (13, 30–33). Coadministration of antioxidants with L-arginine produced an enhanced antiatherosclerotic response (7, 13). The mechanism of action of L-arginine appears to be increased production of NO, whereas antioxidants likely work by protecting the newly formed NO against destruction by reactive oxygen species. The principal explanation for the therapeutic response to L-arginine has been increased substrate availability to eNOS, for example by competing with asymmetric dimethylarginine, an endogenous competitive inhibitor of eNOS that is prevalent in states of atherosclerosis (33–36). In two recent studies, however, chronic administration of L-arginine to low-density lipoprotein receptor-deficient mice produced a marked increase in expression of eNOS protein (25, 26). Thus, up-regulation of eNOS could explain the antiatherosclerotic response to L-arginine. The oral administration of L-citrulline, as a precursor to L-arginine and NO, was reported to be beneficial in sickle cell disease in humans (37). Studies indicate that the L-citrulline to L-arginine recycling pathway in endothelial cells may be the principal mechanism for sustaining localized L-arginine availability for eNOS-catalyzed NO production (17–19). The objective of the present study was to examine the actions of L-arginine, L-citrulline, and antioxidants administered orally to rabbits with atherosclerosis.

Materials and Methods

Animals, Protocols, and Metabolic Treatments. A total of 49 New Zealand White male rabbits, aged 3–4 months and weighing 2.0 to 2.4 kg, were housed individually at 20 ± 3°C with free access to water. Rabbits were divided into the following seven groups (six rabbits per group), depending on diet; amino acid, vitamin, and test agents were administered for 12 weeks: Gp1-HCD, high-cholesterol diet (HCD) (standard diet plus 0.5% cholesterol); Gp2-Arg, HCD plus L-arginine (2.5% in drinking water); Gp3-Cit, HCD plus L-citrulline (2.0% in drinking water); Gp4-Arg+Cit, HCD plus L-arginine and L-citrulline; Gp5-Vit, HCD plus vitamin C (sodium ascorbate; 0.25% in drinking water) and vitamin E (DL-α-tocopherol; 150 mg/kg per day by oral gavage); Gp6-Arg+Vit, HCD plus L-arginine and vitamins C and E; and Gp7-Mix, HCD plus L-arginine, L-citrulline and vitamins C and E. In some experiments, an additional group was studied, Gp8-C (control; standard diet; $n = 6$). Feeding was restricted per rabbit to 120 g per day. Blood was sampled 24 h after the last feeding.

Abbreviations: NO_x, NO_2^- + NO_3^-; eNOS, endothelial NO synthase; *p*-CREB, phosphorylated cAMP response element-binding protein; HCD, high-cholesterol diet; Gp*n*, group *n*.

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§L.J.I. wishes to disclose that he helped develop and has a financial interest in a commercially available dietary supplement that contains some of the amino acids and antioxidants studied in this report.

Table 1. Plasma levels of lipid, NO products, and cGMP

	Gp1-HCD	*Gp2-Arg*	*Gp3-Cit*	*Gp4-Arg + Cit*	*Gp5-Vit*	*Gp6-Vit + Arg*	*Gp7-Mix*
T.Chol., mg/dl	1,758 ± 162	1,584 ± 104	1,460 ± 210	1,350 ± 178	1,473 ± 171	1,662 ± 162	1,257 ± 172
Triglycerides, mg/dl	104.2 ± 7.1	120.7 ± 10.5	89.8 ± 4.4	88.8 ± 4.5	98.4 ± 7.1	120.5 ± 8.3	72.3 ± 6.9
HDL-C, mg/dl	29.1 ± 4.8	29.4 ± 3.3	26.0 ± 3.2	34.4 ± 4.1	33.5 ± 5.3	33.9 ± 4.5	35.8 ± 4.1
$NO_2^- + NO_3^-$, nM	25.1 ± 2.6	27.3 ± 4.1	25.2 ± 4.0	35.1 ± 5.2*	17.2 ± 3.6	26.8 ± 1.8	29.1 ± 2.1*
cGMP, pg/ml	19.1 ± 3.3	18.9 ± 2.8	32.3 ± 3.7	36.7 ± 5.2*	19.3 ± 4.5	32.9 ± 6.3	41.0 ± 6.1*
Body weight, kg	3.12 ± 0.31	2.96 ± 0.40	3.11 ± 0.31	3.02 ± 0.21	3.23 ± 0.34	2.97 ± 0.23	3.02 ± 0.32

Refer to *Materials and Methods* for the definitions of the treatment groups. Data are expressed as the mean ± SEM from six rabbits per group. T. Chol, total cholesterol; HDL-C, high-density lipoprotein-cholesterol.
*Significant difference ($P < 0.05$) vs. Gpl-HCD.

All experiments were conducted in accordance with the institutional guidelines for animal research.

Determination of Plasma Lipids, NO Metabolites, and cGMP. Total cholesterol and triglyceride levels were measured as described (38). HDL-cholesterol was determined after precipitation with phosphotungstate–$MgCl_2$ (39). Plasma NO_2^- plus NO_3^- (NO_X) was measured by using an NO detector–HPLC system (ENO10; Eicom, Kyoto, Japan), as described (40). Plasma cGMP concentration was determined by specific RIA (RPN226, Amersham Pharmacia) (41).

Isometric Tension Measurements. After 12 weeks of treatment, rabbits were killed by exsanguination after anesthesia with pentobarbital (50 mg/kg i.v.). The thoracic aorta was excised from the orifice of the left first costal artery down to the diaphragm, cleaned, and sliced into 2-mm-wide transverse rings. Aortic rings were mounted in organ chambers, and isometric tension was measured exactly as described (6). Rings were contracted submaximally with prostaglandin $F_{2\alpha}$, and endothelium-dependent relaxation elicited by acetylcholine chloride and endothelium-independent relaxation by nitroglycerin (Nihon Kayaku, Tokyo) was determined. In some experiments, indomethacin (5×10^{-6} M) was added 60 min before the experiment to rule out any influence of prostanoids on smooth muscle tone. All indicated concentrations are final concentrations in the bath medium.

Tissue Blood Flow Determined by Laser Doppler Perfusion Imaging. To investigate blood flow near the right central ear artery, we used a laser Doppler imaging system (laser Doppler perfusion imager PIMII, Perimed AB, Linköping, Sweden), as described (42, 43). This method provides a 2D mapping of blood flow in tissues and is based on the principles of laser Doppler flowmetry (42).

Histological Evaluation of Atherosclerosis in Rabbit Aorta. Cross sections of the thoracic aorta used for the evaluation of endothelium-dependent relaxation were stained with van Gieson's elastic stain to determine intimal thickness. Morphometric analysis was performed as described by Weiner *et al.* (44) and modified by this laboratory (6). Six samples from each rabbit aorta were analyzed.

Detection of Superoxide Anion ($O_2^{\bullet-}$) in Rabbit Aorta. $O_2^{\bullet-}$ in each aortic segment was assayed by measuring the intensity of chemiluminescent probes in the presence of a Cypridina luciferin analog, 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazol, 2-apyrazine-3-one (45). Briefly, the generation of $O_2^{\bullet-}$ from a 2-mm length of aorta was detected by using a luminescence reader (BLR-201, Aloka, Tokyo), in the absence and presence of superoxide dismutase to verify specificity of the assay for $O_2^{\bullet-}$.

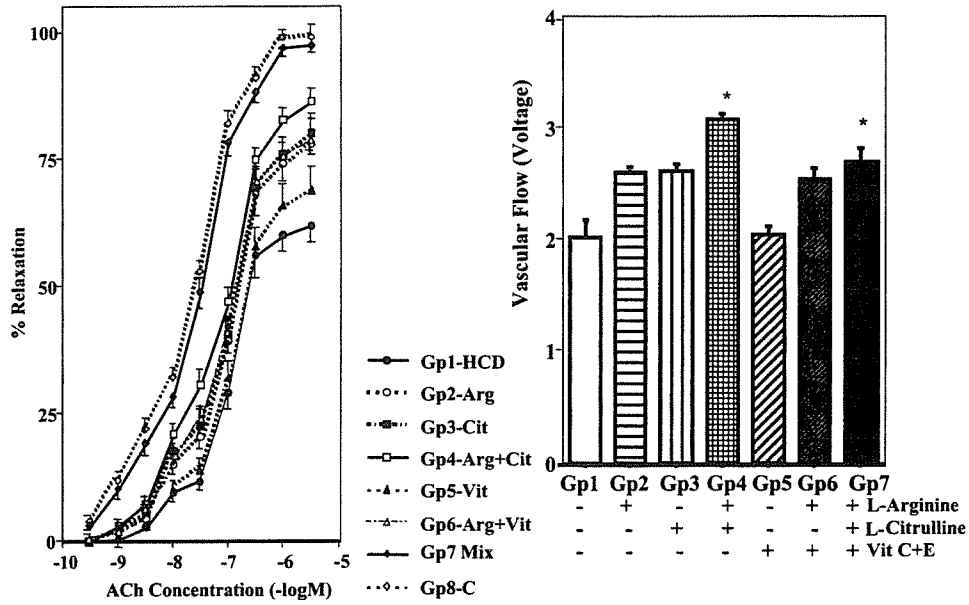

Fig. 1. Vascular responses of thoracic aortas of rabbits. (*Left*) Cumulative concentration-response curves to acetylcholine (ACh) during contraction evoked by prostaglandin $F_{2\alpha}$ (2.6×10^{-6} M) in thoracic aortas from eight groups of rabbits ($n = 6$ per group). (*Right*) Rate of blood flow near the right central ear artery in seven groups of rabbits ($n = 6$ per group). Refer to *Materials and Methods* for definitions of treatment groups. Data are illustrated as the mean ± SEM from six rabbits per group. *, Significant difference ($P < 0.05$) vs. Gp1-HCD.

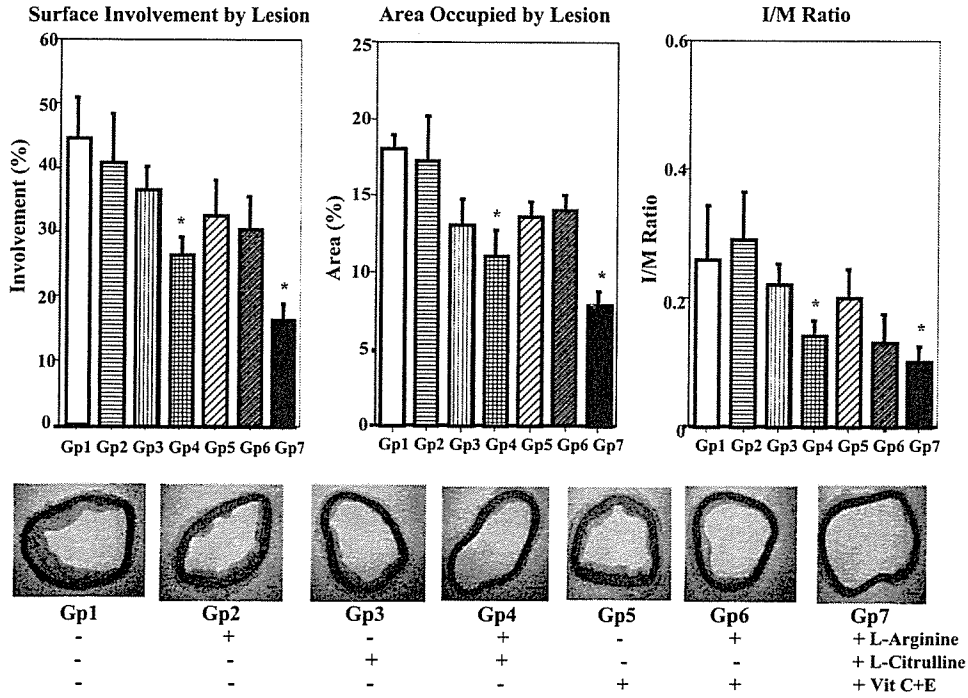

Fig. 2. Histological evaluation of atherosclerotic areas of thoracic aortas from seven groups of rabbits. (*Upper Left*) Surface involvement of atherosclerotic areas in thoracic aorta. (*Upper Center*) Area occupied by atherosclerotic lesions of the aortic arch and thoracic aorta, Gp1. (*Upper Right*) The intima/media (I/M) ratio of the aortic arch and thoracic aorta. In each of the above, data are illustrated as the mean ± SEM from six rabbits per group, and * signifies significant difference ($P < 0.05$) vs. Gp1. (*Lower*) Representative elastica van-Gieson (EVG)-staining photographic images of cross sections of thoracic aortas from seven groups of rabbits. Original magnification. The scale bar in the lower right corner of each image signifies 200 μm.

Detection of eNOS, ets-Like Gene-1 (Elk-1), and Phosphorylated cAMP Response Element-Binding Protein (*p*-CREB). Tissue sections (5 mm) from arterial segments were homogenized (46), and Western blot analysis was performed (47). Gels were transblotted onto nitrocellulose membranes and blocked with milk powder overnight, and samples were incubated with monoclonal antibodies (1:500 dilution for 1.5 h at room temperature) against Elk-1, *p*-CREB, and eNOS (epitope of NOS-III, no crossreactivity with NOS-I or -II; Santa Cruz Biotechnology) (46–48). Proteins were detected by chemiluminescence (Amersham Pharmacia Biotech enhanced chemiluminescence kit). All other details have been described (46–48).

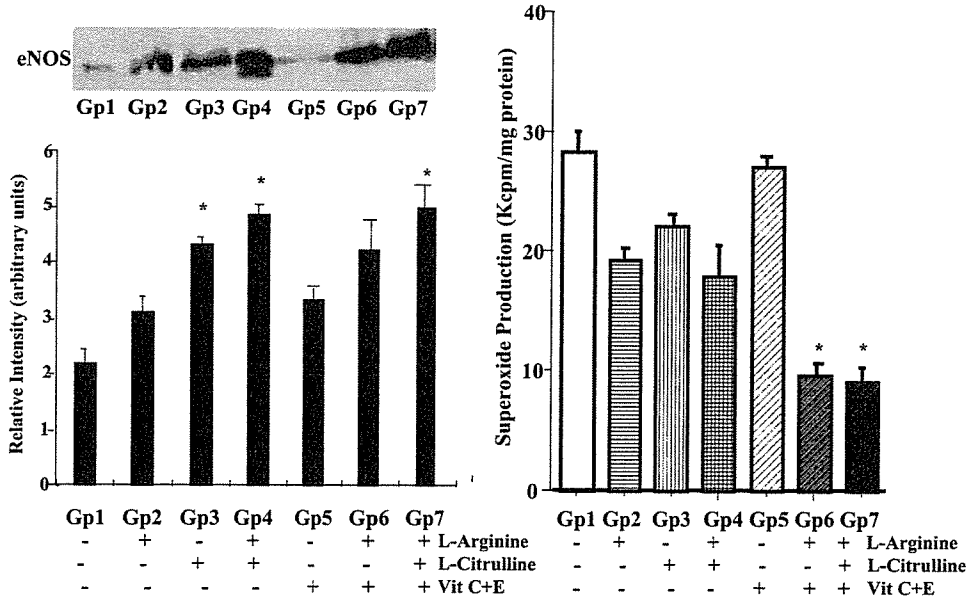

Fig. 3. NO and superoxide production in thoracic aortas from seven groups of rabbits. (*Left*) Quantification of eNOS protein in thoracic aorta using Western blotting. The Western blot represents a single typical experiment. The bar graph illustrates data from six experiments. Relative amounts of eNOS protein are shown. (*Right*) Superoxide production in thoracic aortas of seven groups of rabbits ($n = 6$ per group). *K*cpm, multiply numbers shown by 1,000. Data are illustrated as the mean ± SEM from six rabbits per group. *, Significant difference ($P < 0.05$) vs. Gp1.

Data Analysis. Results were expressed as mean ± SEM and represent unpaired data. Data were compared by analysis of variance with repeated measurements. When a significant F value was obtained, Scheffé's test for multiple comparisons was used to identify the differences among groups. P values of <0.05 were considered to be statistically significant.

Results

Influence of Various Treatments on Blood Chemistry. All rabbits appeared to be healthy throughout the study. No significant differences in body weight or plasma triglycerides were observed among groups. Treatment with L-arginine plus L-citrulline with (Gp7-Mix) or without (Gp4-Arg+Cit) antioxidants showed a significant increase in plasma NO_X and cGMP levels, as compared with those of the HCD group (Gp1-HCD). However, antioxidant or L-arginine treatment alone had no such effect. Therefore, the combination of L-arginine and L-citrulline possesses the capacity to elevate both NO and cGMP. Presumably, cGMP is elevated because the increased NO production results in activation of soluble guanylate cyclase (Table 1).

Influence of Fatty Diet and NO-Boosting Supplements on Endothelium-Dependent Vasorelaxation in Thoracic Aorta and Tissue Blood Flow. Acetylcholine elicited endothelium-dependent relaxation of aortic rings (Fig. 1), and the magnitude of relaxation in hypercholesterolemic animals (Gp1-HCD) was markedly diminished when compared with that of the regular diet group (Gp8-C). However, endothelium-dependent relaxation of the aorta from Gp7-Mix animals was remarkably greater than that from Gp1-HCD and was similar to that for Gp8-C animals. Aortic relaxation in Gp4-Arg+Cit, Gp2-Arg, Gp3-Cit, and Gp6-Arg+Vit tended to increase compared with that of the Gp1-HCD group. The endothelium-independent vasorelaxant, nitroglycerin, produced equivalent magnitudes of relaxation in all groups (data not shown). Indomethacin did not appreciably affect endothelium-dependent relaxation (data not shown). Blood flow near the right central ear artery showed a significant improvement in the Gp4-Arg+Cit and Gp7-Mix groups compared with Gp1-HCD (Fig. 1), and these findings are in agreement with the *in vitro* data from isolated aortic rings. Therefore, ingestion of NO-boosting supplements can improve endothelium-dependent relaxation in atherosclerotic rabbits.

Influence of Fatty Diet and NO-Boosting Supplements on Atheromatous Lesions in Thoracic Aorta. Histological examination of the thoracic aorta revealed markedly smaller atheromatous lesions in the L-arginine plus L-citrulline treatment groups (Gp4-Arg-Cit and Gp7-Mix) than in the hypercholesterolemic group (Gp1) (Fig. 2). The atherosclerotic area was reduced by >50% in animals that received L-arginine, L-citrulline, and antioxidants. Note that the effective treatments also reduced the surface involvement of atherosclerosis and intima/media ratios (Fig. 2). Thus, the initial stages of atherosclerosis and plaque formation were both inhibited by treatment with L-arginine, L-citrulline, and antioxidants.

Influence of Fatty Diet and NO-Boosting Supplements on Protein Expression of eNOS in Thoracic Aorta. Treatment of rabbits with L-citrulline alone (Gp3) and with the combination of L-arginine and L-citrulline (Gp4) and the combination of L-arginine, L-citrulline, and antioxidants (Gp7) caused a significant increase in eNOS protein expression as compared with Gp1 (Fig. 3). Interestingly, L-citrulline was more effective than L-arginine, and antioxidants tended to increase the effect of L-arginine (Gp6). These data indicate that NO-boosting supplements can up-regulate eNOS gene expression.

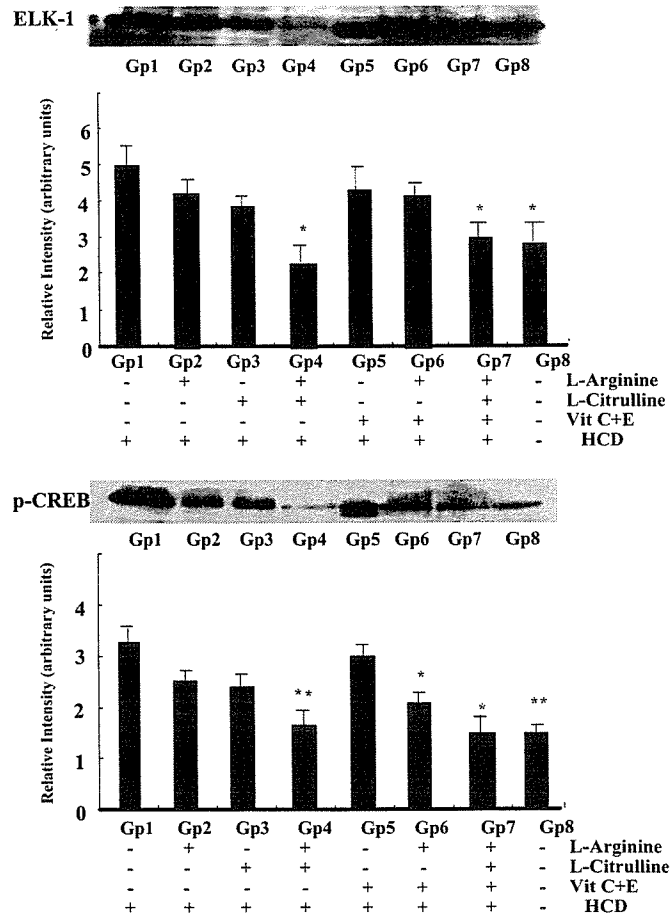

Fig. 4. Quantification of Elk-1 and *p*-CREB protein in thoracic aortas from seven groups of rabbits using Western blotting. Western blots represent a single typical experiment. The bar graphs illustrate data from six experiments. Data are illustrated as the mean ± SEM from six rabbits per group. *, Significant difference ($P < 0.05$) vs. Gp1.

Inhibitory Action of NO-Boosting Supplements on the Increased Production of Superoxide Anion and Elk-1 and *p*-CREB Protein Expression in Aorta from Hypercholesterolemic Rabbits. $O_2^{\bullet-}$ production was ≈3-fold greater in aorta from hypercholesterolemic (Gp1-HCD) rabbits than from control (Gp8-C) rabbits (data not shown). Ingestion of L-arginine and/or L-citrulline tended to decrease $O_2^{\bullet-}$ production, although the differences were not statistically significant (Fig. 3). However, the combination of antioxidants with L-arginine (Gp6-Arg+Vit) or L-arginine plus L-citrulline (Gp7-Mix) produced a marked reduction in $O_2^{\bullet-}$ production (Fig. 3). In accord with the well known association between hypercholesterolemia and oxidative stress in tissues, a cholesterol-rich diet caused an increase in oxidation-sensitive Elk-1 and *p*-CREB expression (compare Gp1 and Gp8; Fig. 4). L-arginine plus L-citrulline, with (Gp7) or without (Gp4) antioxidants completely prevented cholesterol diet-induced increase in Elk-1 and *p*-CREB expression (Fig. 4). The combination of L-arginine plus antioxidants (Gp6) produced the same effect on *p*-CREB.

Discussion

The objective of the present study was to evaluate the influence of ingested L-arginine, L-citrulline, and antioxidants (vitamins C and E) on the progression of atherosclerosis in rabbits fed a HCD. The hallmark of diet-induced atherosclerosis in rabbits is vascular endothelial cell dysfunction, which is characterized by

marked impairment of endothelium-dependent vasorelaxation in isolated arteries as well as blood flow *in vivo* and atherosclerosis with distinct atheromatous lesions (3, 6, 30, 40, 45). Moreover, atherosclerosis is characterized by the progressive development of oxidative stress, as evidenced by the increased production of $O_2^{\bullet-}$ in arteries and increased expression of oxidation-sensitive genes such as Elk-1 and *p*-CREB (12, 13, 15, 25). The systemic administration of L-arginine and antioxidants to atherosclerotic animals has been demonstrated to slow the progression of disease (7, 12, 20–26). However, the effects of L-citrulline alone or in combination with L-arginine or L-arginine plus antioxidants have not been reported.

L-arginine plus L-citrulline, alone or with antioxidants, caused a marked improvement in endothelium-dependent vasorelaxation and rabbit ear blood flow, dramatic regression in atheromatous lesions, and decrease in $O_2^{\bullet-}$ production. These therapeutic effects were associated with concomitant increases in aortic eNOS expression and plasma levels of nitrite plus nitrate (NO_X) and cGMP. The data reveal that chronic ingestion of the dietary supplements used in this study promotes an increase in NO production and action. NO_X are stable oxidation products of NO and represent markers of NO production. cGMP is the intracellular second messenger that mediates many physiological actions of NO, and its formation is stimulated by NO. In view of the evidence that NO improves endothelial dysfunction, causes vasorelaxation *in vitro* and vasodilation *in vivo*, and slows the progression of atherosclerosis (1, 7, 8), it is reasonable to conclude that the pharmacological effects observed in rabbits after dietary supplementation with L-arginine, L-citrulline, and antioxidants are attributed to increased production and action of NO.

The chronic oral administration of L-arginine with or without antioxidants to mice was shown to increase the protein expression of eNOS in the aorta (25, 26). Similarly, in the present study, NO-boosting supplements caused a marked up-regulation of eNOS in rabbit aorta. In addition, L-citrulline was tested and found to increase eNOS protein expression. The combined administration of L-citrulline plus L-arginine, with or without antioxidants produced an even greater up-regulation of eNOS. eNOS up-regulation was accompanied by elevated plasma NO_X and cGMP, thereby indicating indirectly that the up-regulated eNOS was functionally active. There are at least two possible mechanisms by which L-arginine could have increased NO production. One is up-regulation of eNOS, and a second is increased availability of substrate (L-arginine) to eNOS. The latter mechanism might appear to be less likely than the first, because K_m for L-arginine as a substrate for eNOS is 2–15 μM, whereas plasma L-arginine levels in mammals are 100–200 μM, thereby suggesting that eNOS may already be saturated with substrate. This enigma has been termed the "arginine paradox." However, current evidence suggests that the bulk of intracellular endothelial L-arginine may not be available for NO production. Plasmalemmal caveolae may be the principal source of L-arginine available to eNOS (18, 19). Moreover, the L-citrulline to L-arginine recycling pathway is localized to caveolae and may be the principal source of available L-arginine (17–19). Cytosolic L-arginine availability for eNOS may be limited by uptake into plasmalemmal caveolae (49), and administration of excess L-arginine may create a sufficient concentration gradient to make more L-arginine available to eNOS. Alternatively, the "arginine paradox" has been explained by the presence during atherosclerosis of elevated levels of asymmetric dimethylarginine (ADMA), a competitive inhibitor of eNOS (5, 33–36, 50). Excess L-arginine could effectively compete with ADMA for binding sites on eNOS. Our observations both here and previously in mice (25, 26) that L-arginine can up-regulate eNOS offers a previously undescribed explanation of the "arginine paradox."

L-citrulline, the second product of the NOS reaction, was reported to elicit endothelium-dependent relaxation of rat aorta accompanied by increases in tissue nitrite and cGMP (51). This is consistent with the knowledge that L-citrulline is converted to L-arginine by mammalian cells, including endothelial cells (16–19, 52, 53). This recycling pathway might be important in sustaining the production of NO in endothelial cells, especially when L-arginine becomes limiting, as is possible in atherosclerosis. In the present study, L-citrulline produced pharmacological effects that closely resembled those of L-arginine administration and NO action. L-citrulline caused a marked improvement in endothelium-dependent vasorelaxation in response to acetylcholine, and the combination of L-citrulline and L-arginine produced a synergistic response in elevating plasma NO_X and cGMP, improving rabbit ear artery blood flow and slowing the progression of atherosclerosis. A key observation was the marked up-regulation of eNOS on chronic administration of L-citrulline, and this response might explain, in part or entirely, the NO-like pharmacological effects of L-citrulline.

Atherosclerosis is an inflammatory disease characterized by endothelial dysfunction and impairment of NO production (1, 2, 8). Herein lies a plausible explanation for atherogenesis, because it is well appreciated that NO elicits a multifaceted array of pharmacological actions, all of which are protective against the progression of atherosclerosis (7, 8). A common feature of inflammation and atherosclerosis is oxidative stress (15), which can lead not only to cell membrane injury but also the destruction of NO. Thus, the natural antioxidant properties of NO are lost, and oxidative stress continues unabated. In the present study, fatty diet-induced atherosclerosis and oxidative stress were reversed upon oral administration of L-arginine, L-citrulline, and antioxidants. These observations suggest that NO is the active species in reducing both the markers for oxidative stress and the progression of atherosclerosis.

Cardiovascular disease is the leading cause of morbidity and untimely death both in men and women in the U.S. and may be largely avoidable and even reversible by adopting more sensible programs involving a healthy diet and moderate exercise. A diet low in saturated fat and rich in antioxidants could counter the development of oxidative stress and boost NO production and action (11, 13, 15, 31, 33). Likewise, moderate exercise would boost NO production and decrease the expression of oxidation-sensitive genes (26). The present study demonstrates, at least in rabbits, that chronic ingestion of L-arginine, L-citrulline, and antioxidants can reverse the progression of atherosclerosis. Similar observations were made in humans with L-arginine and antioxidants (5, 8, 27–29). Therefore, taken together, embarking on a low-fat and high-antioxidant-diet moderate exercise program and regimen of NO-boosting dietary supplements might result in a lower incidence of deaths attributed to cardiovascular disease.

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Endothelial cellular senescence is inhibited by nitric oxide: Implications in atherosclerosis associated with menopause and diabetes

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Senescence may contribute to the pathogenesis of atherosclerosis. Although the bioavailability of nitric oxide (NO) is limited in senescence, the effect of NO on senescence and its relationship to cardiovascular risk factors have not been investigated fully. We studied these factors by investigating senescence-associated β-galactosidase (SA-β-gal) and human telomerase activity in human umbilical venous endothelial cells (HUVECs). Treatment with NO donor (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-aminoethyl)amino]diazen-1-ium-1,2-diolate (DETA-NO) and transfection with endothelial NO synthase (eNOS) into HUVECs each decreased the number of SA-β-gal positive cells and increased telomerase activity. The NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) abolished the effect of eNOS transfection. The physiological concentration of 17β-estradiol activated hTERT, decreased SA-β-gal-positive cells, and caused cell proliferation. However, ICI 182780, an estrogen receptor-specific antagonist, and L-NAME each inhibited these effects. Finally, we investigated the effect of NO bioavailability on high glucose-promoted cellular senescence of HUVECs. Inhibition by eNOS transfection of this cellular senescence under high glucose conditions was less pronounced. Treatment with L-arginine or L-citrulline of eNOS-transfected cells partially inhibited, and combination of L-arginine and L-citrulline with antioxidants strongly prevented, high glucose-induced cellular senescence. These data demonstrate that NO can prevent endothelial senescence, thereby contributing to the anti-senile action of estrogen. The ingestion of NO-boosting substances, including L-arginine, L-citrulline, and antioxidants, can delay endothelial senescence under high glucose. We suggest that the delay in endothelial senescence through NO and/or eNOS activation may have clinical utility in the treatment of atherosclerosis in the elderly.

diabetes mellitus | endothelial nitric oxide synthase | estrogen | aging

Aging is known to be a major cardiovascular risk factor (1). Cellular senescence is the limited ability of human cells to divide when cultured *in vitro* and is usually accompanied by phenotypic changes in morphology, gene expression, and function (2). These changes have been implicated in human aging. Until recently, little attention has been paid to the potential impact of vascular cellular senescence on age-related vascular disorders. Senescent cells from aged animals express increased levels of proinflammatory molecules, suggesting that cellular senescence *in vivo* contributes to the pathogenesis of human atherosclerosis (3).

The telomere hypothesis is a widely accepted explanation of the occurrence of senescence (4). Telomeres, repetitive DNA sequences at the ends of eukaryotic chromosomes, shorten as a linear function of increasing cellular division, and according to the hypothesis, short telomere length triggers the onset of senescence (5, 6). Telomerase, a ribonucleoprotein, can synthesize new telomeric repeats and restore telomere length. Cellular senescence usually accompanies telomere shortening and increases in senescence-associated β-galactosidase (SA-β-gal) (assayed at pH 6), which is distinguishable from endogenous lysosomal β-gal activity, is considered to be a marker of cellular senescence (7).

According to a free-radical theory, reactive oxygen species (ROS) may be potential candidates responsible for senescence, and oxidative stress may promote senescence by shortening telomere through inactivation of the Src kinase family (8–10). Therefore, not only atherosclerosis but also senescence has been shown to progress via ROS (11). NO is a widespread signaling molecule in the cardiovascular system, which functions in multiple ways to protect against the initiation and progression of atherosclerosis (12, 13). NO prevents the adhesion and aggregation of blood cells and inhibits vascular smooth muscle cell proliferation (14). However, neither the role nor the effect of endothelial NO on senescence is fully known. As NO can abrogate the state of oxidative stress, NO may thus have the potential to affect cellular senescence by scavenging senescent stimuli such as ROS.

Accordingly, the present study was performed to examine whether or not NO and the activation of eNOS can delay endothelial senescence. We also considered estrogen depletion and diabetes mellitus among various cardiovascular risk factors as applied models of the combined effects of NO on both atherosclerosis and cell longevity.

The morbidity of cardiovascular disease dramatically increases after menopause (15). In such cases, estrogen depletion has been speculated as a cause of the disease, and estrogen plays an antiatherogenic role both *in vivo* and *in vitro* (16, 17). Although hormone replacement therapy was reported not to prevent cardiovascular disease in a clinical trial, this ineffectiveness was due to the increased frequency of thrombosis produced by estrogen in advanced atherosclerosis and to the adverse effect of coprescribed progesterone (18). The fact that females are known to live several years longer than males world-wide strongly supports the antiatherogenic effect of estrogen.

Diabetes mellitus is also a major cardiovascular risk factor, and the etiology of diabetic atherosclerosis is suggested to include the increase of ROS and the decrease of NO bioavailability as a result

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Abbreviations: eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; SA-β-gal, senescence-associated β-galactosidase; hTERT, human telomerase reverse transcriptase; HUVECs, human umbilical vein endothelial cells; L-NAME, N^G-nitro-L-arginine methyl ester; DETA-NO, (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-aminoethyl) amino] diazen-1-ium-1,2-diolate; PDL, population-doubling level.

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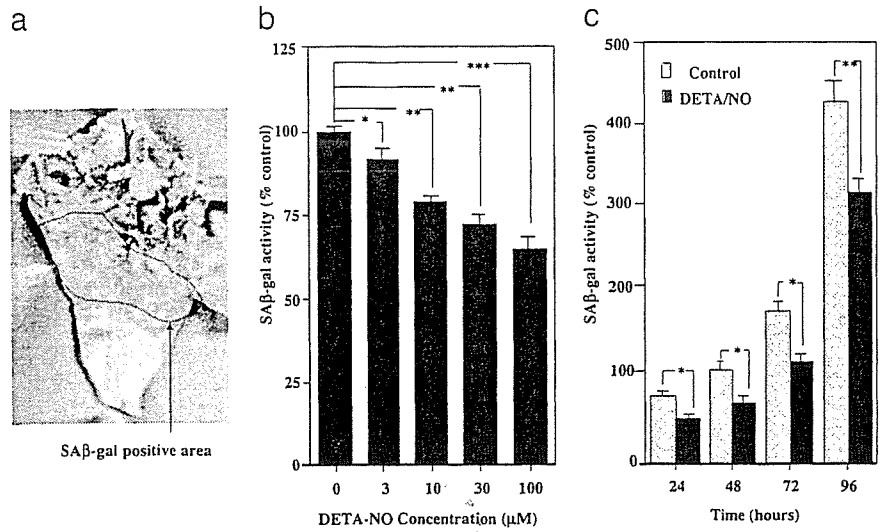

Fig. 1. SA-β-gal activity as cellular senescence. (*a*) SA-β-gal-positive staining was observed in atherosclerotic lesions of the intimal side of human thoracic aorta, which was obtained by autopsy. No staining was detected in the nonatherosclerotic area and advanced atherosclerotic area, including the necrotic core and ulcer complicated lesion. (*b*) Concentration-dependent decrease in SA-β-gal activity in HUVECs by DETA-NO. HUVECs were treated with DETA-NO for 24 h. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$ vs. DETA-NO-untreated control. (*c*) Time-dependent decrease in SA-β-gal activity in HUVECs by DETA-NO. HUVECs were treated with 10 μM DETA-NO for 24–96 h. *, $P < 0.05$; **, $P < 0.01$ vs. the corresponding control. Control sample, which is treated for 48 h, is expressed as 100%.

of high glucose levels (19). The incidence of cardiovascular diseases is increased in elderly diabetic patients, but the relationship between senescence and diabetes on endothelial function has yet to be elucidated (20). NO is synthesized by NOS, which utilizes L-arginine as a substrate and produces L-citrulline as the second reaction product. L-arginine can be synthesized from L-citrulline in endothelial cells through a recycling pathway (21). This pathway may be the principal mechanism for sustaining localized L-arginine availability for endothelial nitric oxide synthase (eNOS)-catalyzed NO production (21, 22). In the present study, we examined the effect of NO boosting on high glucose and/or senescence by the regulation of eNOS.

Results

NO Delays Cellular Senescence. The effect of NO on endothelial cellular senescence was investigated by evaluating SA-β-gal used as a cellular senescence marker and human telomerase activity used as an indicator of elongation of telomere length in human umbilical vein endothelial cells (HUVECs). We also examined SA-β-gal activity in the thoracic aorta and coronary arteries obtained from 3 autopsied elderly individuals. Fig. 1*a* shows that SA-β-gal activity was observed in the mild atherosclerotic area in human thoracic aorta. Treatment with the NO donor, (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-aminoethyl) amino] diazen-1-ium-1,2-diolate (DETA-NO), for 24 h significantly decreased the SA-β-gal activity in HUVECs (Fig. 1 *b* and *c*). The effect of DETA-NO was found to be both concentration-dependent (3–100 μM, Fig. 1*b*) and time-dependent (24–96 h treatment, Fig. 1*c*). Coincubation with N^G-nitro-L-arginine methyl ester (L-NAME) (300 μM) did not affect the action of DETA-NO (data not shown). DETA-NO also increased telomerase activity in HUVECs (data not shown).

Transfection with eNOS into HEK 293 cells or HUVECs for 48 h increased the NO metabolite, $NO2^-$ (Fig. 2*a*), and also significantly increased telomerase activity (Fig. 2*b*). On the other hand, the number of SA-β-gal-stained cells was reduced by eNOS transfection (data not shown). Fig. 2*c* shows the effects of eNOS-related substrate and products on SA-β-gal staining in HUVECs. Coincubation with the NOS inhibitor L-NAME (300 μM) tended to decrease the number of SA-β-gal-stained cells by inhibiting NO release from HUVECs. SA-β-gal-positive staining also tended to decrease in the presence of L-arginine and/or L-citrulline. However, their effects on SA-β-gal-stained cells are not statistically significant even though they were given together.

Estrogen Delays Cellular Senescence via an NO-Dependent Mechanism. We next investigated the effect of E2 on cellular senescence in HUVECs. At physiological concentrations (10 nM), E2 treatment reduced the number of SA-β-gal-positive cells, especially in large population-doubling level (PDL) cells (Fig. 3*a*). Fig. 3*b* shows representative photographs of SA-β-gal-stained cells in HUVECs of PDL 22. E2 decreased the number of SA-β-gal-stained cells, whereas this effect was prevented by coincubation with L-NAME (Fig. 3*b*). E2 markedly activated telomerase, and this activation was inhibited by ICI 182780, an estrogen receptor-specific antagonist, and by L-NAME (Fig. 3*c*). These results suggest that the counteracting effect of E2 on senescence involves an eNOS-dependent mechanism by means of activation of estrogen receptors.

The physiological concentration of E2 also enhanced proliferation of HUVECs (Fig. 4). As the HUVEC proliferating activity tended to slow down in senescent cells, this basal mechanism seems to be different from that underlying the effect of E2 on telomerase and SA-β-gal. On the other hand, L-NAME treatment decreased proliferation of HUVECs in all PDL (Fig. 4*a*). The peak effect on cell proliferation was achieved with physiological concentrations of E2, whereas higher E2 concentration produced a lesser effect (Fig. 4*b*).

The Effect of NO Bioavailability on High-Glucose-Induced Cellular Senescence. Finally, the effects of NO bioavailability on cellular senescence under high glucose conditions were investigated. Exposure to high glucose for 24 h decreased the expression level of eNOS protein in a manner dependent on the concentration of glucose, resulting in decreases of 19% at 11 mM and 33% at 22 mM glucose compared with the control (5.5 mM glucose) level (Fig. 5*a*). Mannitol, used as an osmolarity control, had no influence on eNOS protein level. In HUVECs cultured under high glucose conditions (11, 22, and 31 mM) for 3 days, nitrite ($NO2^-$) production was decreased (Fig. 5*b*) and intracellular ROS production was increased (data not shown) in a manner dependent on the concentration of glucose. Treatment with L-arginine, L-citrulline, and antioxidants (vitamin C and E) alone or in combination showed a significant recovery of the decreased nitrite level under high glucose condi-

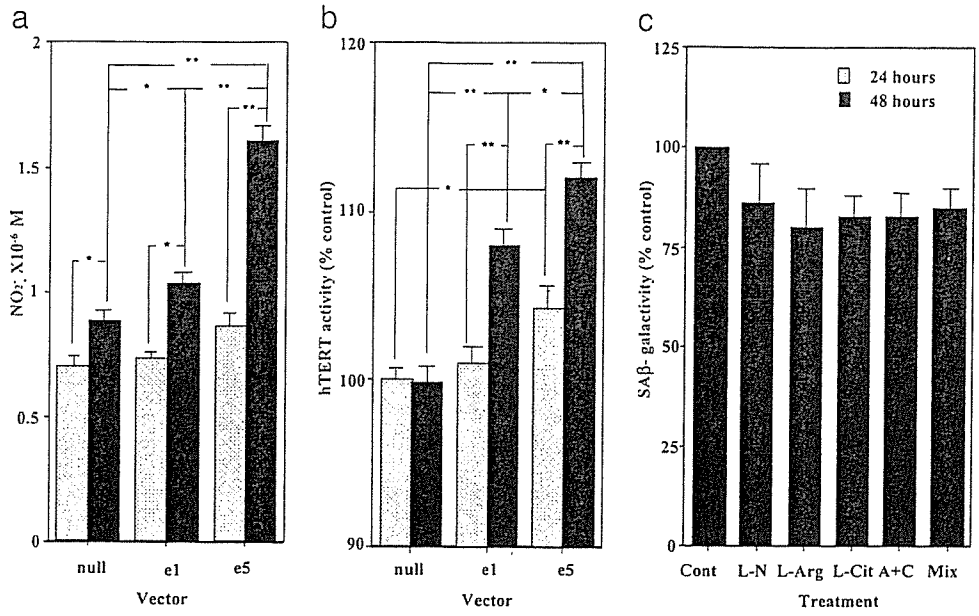

Fig. 2. Influence of eNOS modulation on cellular senescence. (*a*) The effect of transfection with eNOS on nitrite production by HEK 293 cells. Transfection with eNOS into cells was performed; e5 included five times the amount of eNOS vector compared with e1. The nitrite concentrations in the medium 24 and 48 h after transfection are shown. *, $P < 0.05$; **, $P < 0.01$. (*b*) The effect of transfection with eNOS on telomerase activity in HEK 293 cells. The activity of hTERT in cells 24 and 48 h after transfection are shown. *, $P < 0.05$; **, $P < 0.01$. (*c*) The effects of treatment with L-NAME (L-N, 300 μM), L-arginine (L-arg, 1 mM), and L-citrulline (L-cit, 300 μM) alone or in combination (A+C) on SA-β-gal activity in HUVECs. The treatment time was 24 h. Mix = L-arginine, L-citrulline and vitamin E plus vitamin C (each, 100 μM).

tions (Fig. 5*c*). When L-arginine, L-citrulline and antioxidants were given together, the recovery of nitrite production was more marked.

High glucose exposure for 72 h promoted cellular senescence as indicated by increases in SA-β-gal-positive staining (Fig. 6) and decrease in telomerase activity (data not shown). The number of SA-β-gal-positive staining cells under high glucose conditions tended to decrease slightly after incubation with L-arginine, L-citrulline, and antioxidants alone, and was significantly decreased when they were given together (Fig. 6 *a* and *b*). Moreover, transfection with eNOS tended to prevent cellular senescence slightly, and the combined presence of L-arginine, L-citrulline, and antioxidants very effectively prevented it under high glucose conditions (Fig. 6*c*).

Discussion

The free-radical theory of aging proposes that degenerative senescence is largely the result of the cumulative effect of ROS (9, 10). It is possible that some association exists between increased oxidative stress and reduced telomerase activity. Interestingly, individuals with shorter white blood cell telomeres tend to show a >2.8-fold higher coronary risk than the highest quartile for telomere length, after adjusting for age (23).

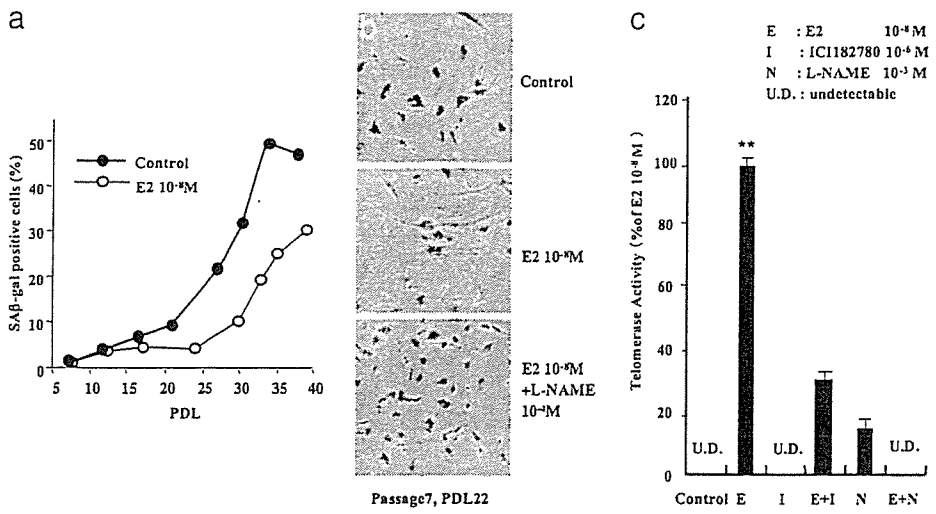

Fig. 3. Effect of estrogen on cellular senescence. (*a*) The relative levels of SA-β-gal-positive staining cells in different PDL when HUVECs were untreated and treated with 10^{-8} M E2 for 24 h. Positive staining cells were evaluated by FACscan. (*b*) Representative photographs of SA-β-gal staining in control, 10^{-8} M E2-treated, and 10^{-8} M E2- and 10^{-4} M L-NAME-treated cells. Note that treatment with E2 decreased the number of SA-β-gal-positive cells, which was prevented by further treatment with L-NAME. Cells were used in PDL 22 at passage 7. (*c*) The effects of E2 (E, 10^{-8} M), ICI 182780 (I, 1 μM), and L-NAME (N, 1 mM) on telomerase activity in HUVECs. UD, undetectable. **, $P < 0.01$ vs. control.

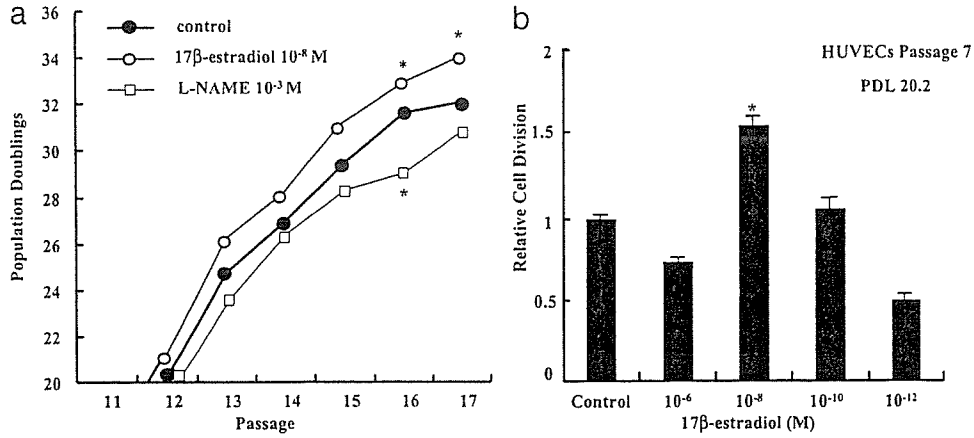

Fig. 4. Effects of E2 on endothelial cell proliferation. (*a*) The effects of E2 (10^{-8} M) and L-NAME (1 mM) on population doublings in each passage of HUVECs. The treatment time with E2 or L-NAME was 24 h. *, $P < 0.05$ vs. control. (*b*) The effects of different concentrations of E2 on relative cell division of HUVECs. Cells were used in PDL 20.2 at passage 7. *, $P < 0.05$: cell division vs. control.

Telomerase counteracts the shortening of telomeres and contains a catalytic subunit, the hTERT (4, 5). The introduction of hTERT into human cells extends both their lifespan and their telomeres to lengths typical of those of young cells (5, 6). The regulation of hTERT involves both transcriptional and posttranscriptional mechanisms. Transcriptional regulation is believed to be the main regulatory mechanism in cancer cells (24). Telomerase activity can be posttranscriptionally regulated by kinases such as protein kinase C (PKC), extracellular signal-regulated kinase 1/2 (ERK1/2), and Akt [Akt/PKB (protein kinase B)] in endothelial cells (8–10). ROS formation leads to an increase in Src-family kinase activation and a reduction of Akt expression in aging endothelial cells. It is speculated that phosphorylation by Akt keeps hTERT in an active status in the nucleus, whereas increasing the activation of Src-family kinases induces the nuclear export of hTERT, thereby reducing the ability to lengthen telomeres and protect from aging. Along with the enhanced ROS formation, we found that a decrease in telomerase activity preceded the onset of replicative senescence. Thus, ROS such as the superoxide radical and H_2O_2, which are formed during aerobic metabolism, are generally considered to be important regulators of the aging processes, and their production may be mainly due to the actions of NADPH oxidase and the mitochondria (9, 10, 24–26). In the present study, we showed that DETA-NO, an NO donor, and eNOS transfection activate hTERT and increase scavenging of ROS. L-NAME inhibited the effect of eNOS transfection. These results mean that telomerase activity was likely regulated by NO bioavailability. Our data indicated that eNOS transfection has comparable effects to hTERT transfection on both cellular aging and telomerase activity. In addition, these findings might also indicate that endothelial cell aging is linked to the balance between ROS formation and NO bioavailability, which in turn affects telomerase activity.

eNOS transfection has an antiatherosclerotic effect even in cases of advanced atherosclerosis, and the administration of L-arginine with the gene transfer of eNOS enhances the effect of eNOS transfection (27, 28). We showed that the coadministration of antioxidants with L-arginine and L-citrulline produces an enhanced antiatherosclerotic response in advanced atherosclerosis (29). L-arginine seems to increase the production of NO, whereas antioxidants most likely protect the newly formed NO against destruction by ROS. Recent evidence indicates that the bulk of intracellular endothelial L-arginine may not be available for NO production, because intracellular L-arginine for eNOS may be limited by uptake into plasmalemmal caveolae (30). The pathway by which L-citrulline is recycled to L-arginine is localized to the caveolae and it may be the main source of available L-arginine (21, 22, 29, 31). L-citrulline is converted to L-arginine by mammalian cells, including endothelial cells. This recycling pathway might, therefore, play an important role in sustaining the production of NO in endothelial cells by providing available L-arginine, especially in advanced atherosclerosis or diabetes mellitus, when plasma L-arginine levels are depleted.

Physiological concentrations of E2 activate telomerase activity and decrease the number of SA-β-gal-stained cells through the

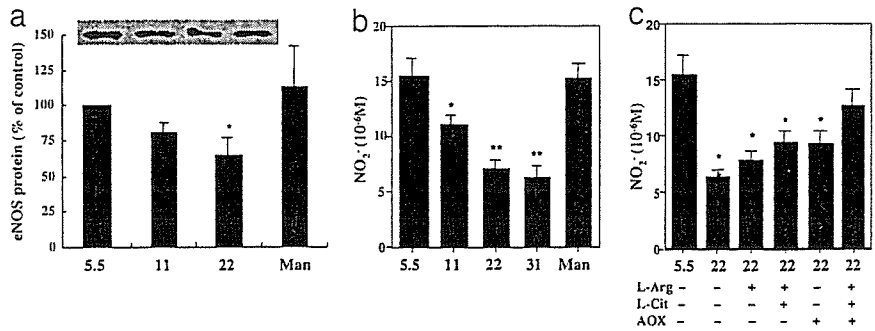

Fig. 5. Influence of high glucose on eNOS expression and nitrite production. (*a*) The effect of exposure to different concentrations of glucose on the level of eNOS protein expression in HUVECs. Mannitol (Man) was given as an osmolarity control. Cells were kept under different glucose conditions for 72 h. *, $P < 0.05$ vs. normal (5.5 mM) glucose. (*b*) The effect of exposure to different concentrations of glucose on nitrite levels in culture medium of HUVECs. *, $P < 0.05$; **, $P < 0.01$ vs. normal glucose. (*c*) The effects of L-arginine (L-arg, 1 mM), L-citrulline (L-cit, 300 μM), and antioxidants (AOX, 100 μM vitamin E plus 100 μM vitamin C) alone or in combination on nitrite levels in culture medium in HUVECs, which were reduced by 22 mM glucose. *, $P < 0.05$; **, $P < 0.01$ vs. normal glucose.

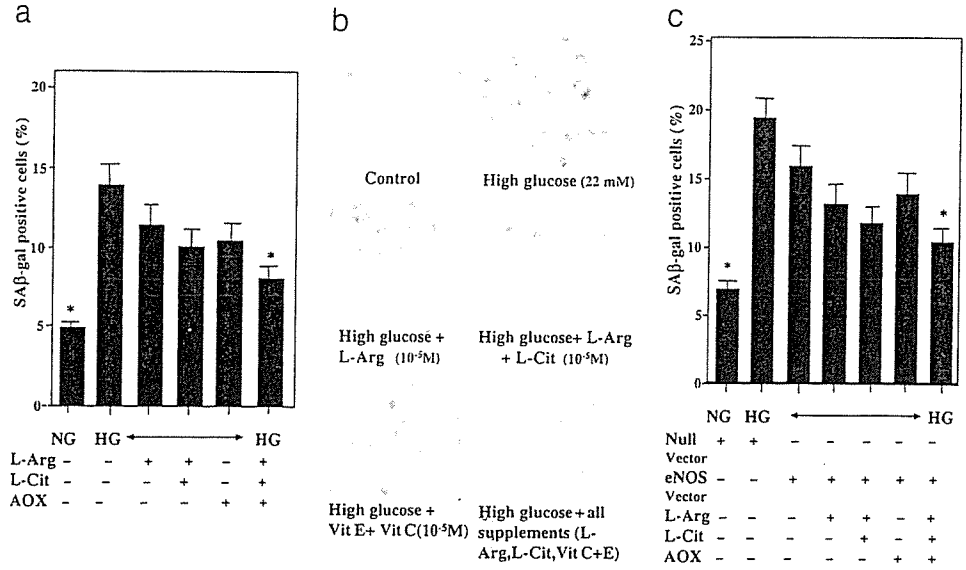

Fig. 6. Influence of high glucose for 72 h on cellular senescence of HUVECs. (a) The effects of L-arginine (L-arg, 1 mM), L-citrulline (L-cit, 300 μM), and antioxidants (AOX, 100 μM vitamin E plus 100 μM vitamin C) on the increase in β-gal-positive stained cells when exposed to high (22 mM) glucose. *, $P < 0.05$ vs. high glucose without any treatment. (b) Representative photographs showing cellular senescence by staining cells with SA-β- gal. (c) Modulation by transfection with eNOS of the effects of L-arginine, L-citrulline, and antioxidants on the increase in SA-β-gal-positive-stained cells when exposed to high glucose. Null vector is control vector of eNOS Vector. *, $P < 0.05$ vs. high glucose without any treatment. NG, normal glucose; HG, high glucose (22 mM).

estrogen receptor and NO-dependent mechanisms. E2 treatment also stimulated the proliferation of HUVECs through the estrogen receptor and NO-dependent mechanisms. We reported the possibility that such effects of estrogen were mediated by the direct effect on eNOS and the scavenging effect on ROS-producing enzymes such as NADPH oxidase, especially the p22phox subunit (32, 33). It is, therefore, proposed that estrogen exerts its effect on endothelial cell senescence by increasing NO bioavailability, which may then reduce ROS generation and subsequently prevent the nuclear export of TERT.

Atherosclerosis is an inflammatory disease characterized by endothelial dysfunction, impairment of NO production (1, 12 13), and oxidative stress (11), which can lead not only to cell membrane injury but also to the destruction of NO. Diabetic macroangiopathy occurs under almost the same conditions, with increased levels of superoxide from NADPH oxidase and impairment of NO production (34, 35). In the present study, high-glucose-induced endothelial dysfunction, oxidative stress, and cellular senescence were reversed with the administration of L-arginine, L-citrulline, and antioxidants. A lack of GTP cyclohydrolase I, which is the rate-limiting enzyme of tetrahydrobiopterin (BH4) synthesis, a cofactor of eNOS, also reduces NO production (36). We speculate that not only BH4 but also L-arginine, L-citrulline, and antioxidants are important in diabetic macroangiopathy. Although NO is known to be involved in reducing both oxidative stress and the progression of atherosclerosis, the present study also assessed the consequence of the NO-mediated delay of cellular senescence on the progression of atherosclerosis. The aforesaid notwithstanding, the local expression (bioavailability) of NO remains an important factor in the maintenance of normal tissue function. We also cannot exclude the possibility that other factors than NO is involved in the progressive cellular senescence in diabetes.

Taken together, the present data provide evidence demonstrating an NO-dependent mechanism in the delay of endothelial cell senescence. Consequently, the antiatherosclerotic action of NO is particularly profound under conditions of aging, estrogen depletion, and diabetes mellitus. NO could, therefore, scavenge the age-associated increase in ROS and thereby reduce the coronary risk factor-induced increase in ROS. Moreover, our data indicate that NO may also prevent endothelial cell senescence, possibly by interfering with the redox balance of endothelial cells.

Methods

Materials. We used 17β-estradiol (Sigma, St. Louis, MO), D-glucose, D-mannitol (Wako, Osaka, Japan), Takara One Step RNA PCR Kit (Takara, Kyoto, Japan), and eNOS monoclonal antibody (BD Biosciences, San Jose, CA). ICI 182780 was kindly provided by Zeneca Pharmaceuticals. L-NAME and DETA-NO were obtained from Sigma-Aldrich (St. Louis, MO). Monoclonal antibodies to β-galactosidase (Chemicon International, Lexington, NY) were used (7, 37).

Cell Culture. HUVECs were purchased from Clonetics (San Diego, CA) and cultured in low-glucose EBM-2 supplemented with 10% calf serum, EBM-2 including EGM-2 SingleQuots (Clonetics), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2, 95% air. These cells were positive for the endothelial cell-specific von Willebrand factor and angiotensin-1-converting enzyme activity. The cells were seeded into six-well plates, and subconfluent cell monolayers were studied within six to eight passages. Before starting the experimental procedures, the medium was removed and replaced with phenol red-free low-glucose D-MEM supplemented with 1% calf serum, 0.06% glutamine, and 1% penicillin–streptomycin. In some experiments accompanying eNOS transfection, HEK 293 cells were treated instead of HUVECs because of relative ease of transfection. The rate of PDL was calculated at each passage until growth arrest based on the following formula: $PDL = (\log_{10}Y - \log_{10}X)/\log_{10}2$ (Y indicates the number of cells counted at the end of the passage; X is the number of cells seeded). Cumulative population doubling was calculated as the sum of all of the changes in population doubling.

Measurement of Nitrite. The methods for measuring nitrite (NO_2^-) production by HUVECs have been previously described by our laboratory. In brief, samples of the incubation culture medium were recovered after centrifugation to remove any precipitated materials. The nitrite concentrations of the supernatants were determined by high-performance liquid chromatography (ENO10; EICOM,

Kyoto, Japan) as described (29, 38). The incubated medium was not completely free of nitrite; therefore, an aliquot of medium was assayed by the same process as the medium obtained from the cultured cells. We used the nitrite value obtained in the medium alone as a blank, and it was subtracted from all of the samples.

Flow Cytometric Analysis of ROS Generation. The determination of intracellular oxidant production in HUVECs was based on the oxidation of 2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (H_2DCFDA) resulting in the formation of the fluorescent compound 2′,7′-dichlorofluorescein (DCF) (Molecular Probes, Eugene, OR) (29, 38). Carboxy-H2DCFDA freely diffuses across the cell membrane, is diacylated, and incorporates into hydrophobic lipid regions of the cell. HUVECs were incubated at 37°C for 30 min in PBS in which 2 μl of 5 mM H2DCFDA was added. After incubation, the dye was aspirated and the cells were trypsinized and washed once by centrifugation at 1,670 × *g* for 5 min to remove trypsin and extracellular H_2DCFDA. HUVECs were resuspended in PBS and transferred into 5 ml of polystyrene round-bottom tubes with cell-strainer caps (Becton Dickinson, Franklin Lakes, NJ). They were protected from light and kept cold until ready for analysis on a FACS caliber flow cytometer (Becton Dickinson) set at ≈515- to 545-nm excitation. The emission filters used a 530/30-nm bandpass.

SA-β-Gal. HUVECs and tissues were fixed and stained for SA-β-gal activity as described (37). In brief, the cells were fixed for 10 min in 2% formaldehyde, 0.2% glutaraldehyde in PBS, and incubated for 12 h at 37°C without CO_2 with fresh β-gal staining solution: 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM $MgCl_2$, pH 6.0. The cells were counterstained with 4′6-diamidinophenylindole (DAPI; 0.2 mg/ml in 10 mM NaCl) for 10 min to count the total cell number. The percentage of SA-β-gal-positive cells was determined by counting the number of blue cells within a sample of 1,000 cells. We also used the Flow Cytometric Analysis.

Human Telomerase Activity. The quantitative determination of telomerase activity was performed according to the manufacturer's protocol for the TeloTAGGG telomerase PCR ELISAPLUS Kit (Roche Diagnostics, Mannheim, Germany) based on the telomeric repeat application protocol (TRAP) assay. To measure telomerase activity, 2 μg of protein was used in the PCR.

Western Blot Analysis of eNOS. Total protein was extracted from the endothelial cells and then analyzed by Western blotting (38, 39). Briefly, the protein concentration was determined with a Dc protein assay kit (Bio-Rad Laboratories, Hercules, CA). Samples of cell homogenate (5 μg) were subjected to electrophoresis on polyacrylamide gels, and proteins were transferred to poly(vinylidine difluoride) filter membranes. To reduce any nonspecific binding, the membrane was preincubated for 30 min at room temperature in TTBS (150 mM NaCl/10 mM Tris, pH 8.0/0.05% Tween 20) containing 5% nonfat milk. The membrane was then incubated overnight with the primary antibody at 3:10,000 dilutions in PBS (0.075 μg/ml). The membrane was incubated with the horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution) for 60 min at room temperature. The blots were washed in TTBS and subsequently visualized with the aid of a SuperSignal West Dura Trial Kit (Pierce Biotechnology, Rockford, IL), exposed to x-ray film, and analyzed by the NIH Image Software program produced by Wayne Rasband (National Institutes of Health, Bethesda, MD). Loading of equal amounts of protein was confirmed by Coomassie brilliant blue and Amido black staining of protein in each lane of the same blot.

Construction of an Adenovirus Vector Carrying eNOS and Transfer into Cultured ECs. Recombinant adenoviruses containing eNOS cDNA were constructed by using the ADENO-QUEST Kit (Quantum, Quebec City, Canada) (27). Briefly, bovine eNOS cDNA (provided by T. Michel, Harvard University, Cambridge, MA) was cloned into the AdBM5pAG vector. The resulting plasmid was then cotransfected with viral DNA into HEK 293 cells. We incubated 5×10^5 HUVECs in a six-well plate for 24 h, then incubated cells with adenoviruses at a multiplicity of infection of 20 for 24 h. For all of the studies, the viral titers were adjusted to 2×10^9 pfu/ml. Adenoviruses carrying an *Escherichia coli lacZ* gene encoding a nucleus-localized variant of β-gal (Ad. β-gal) or no cDNA (Ad.-null) were also used. We also used eNOS/pcDNA3.1(+) and Qiagen Effectane Transferase Reagent.

Statistics. All data are given as means ± SEM from at least three independent experiments. Comparisons between the two groups were made based on the nonparametric Mann–Whitney *U* test. Statistical significance was evaluated with repeated-measures ANOVA by using a least-significant difference (LSD) post hoc test or ANOVA for multiple comparisons (SPSS Software 11.0). Differences were considered to be significant at a value of $P < 0.05$.

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Nitric oxide (NO) is a new clinical biomarker of survival in the elderly patients and its efficacy might be nearly equal to albumin

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Abstract

Background: For elderly patients, the consideration of prognostic factors is very important, but there have been few reports about the potential use of vasoactive substances as prognostic markers in the elderly.

Objective: We assessed endocrinological substances, such as plasma NO_x (metabolites of NO), as the prognostic marker in elderly. We compared their efficacy with that of such well-known markers as albumin and pro-inflammatory cytokines such as IL-6.

Methods: The patients were recruited consequently from the clinics of Nagoya University Hospital or related home care services facilities. One hundred and twenty seven elderly aged 65 and older were registered. Biochemical analyses such as albumin, total cholesterol, BNP, and NO_x were measured upon enrollment. The main outcome was the survival rate.

Results: Forty-six patients died during the follow-up period. Mann–Whitney's *U*-test showed that the levels of age, hemoglobin, total protein, serum albumin, serum creatinine, total cholesterol, HDL-cholesterol, LDL-cholesterol, high sensitive CRP, NO_x, IL-6, and TNF-α were significantly different between the living and deceased subjects. Among the dependent variables in the logistic regression analyses, only albumin and NO_x were significantly different. In the Kaplan–Meier analyses of mortality, the prognosis of patients in 3rd and 4th quartile of NO_x was significantly worse than that in 1st or 2nd quartile.

Conclusion: NO_x has potential both as a vascular marker and as a marker for predicting survival in elderly. In the latter role, it may be as effective as albumin.

Keywords: Nitric oxide; cGMP; Albumin; Biomarker; Elderly; Prognostic marker; Vascular functional marker

Many nations, including Japan, are experiencing rapid growth in their elderly populations. The main causes of death in Japanese elderly are heart disease, cerebro-vascular disease, and cancer. Several biochemical markers, such as albumin and cholesterol, have been identified as having prognostic value for mortality and hospitalization [1–3]. Recent studies also have indicated the potential role of the immune system in the pathophysiology of congestive heart failure (CHF) and malignancy [4,5]. Plasma levels of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) also have been reported to be significant prognostic predictors in patients with CHF or malignancy) [6–8]. TNF-α induces adhesion molecule expression such as ICAM-1 on endothelial cells, which promotes the progression of atherosclerosis [9]. In other words, in older populations, peripheral blood markers of nutrition or inflammation (albumin, cholesterol, IL-6, and TNF-α) have been individually shown to be increased risk for mortality [2,10,11].

In elderly people, the rate of CHF is important for predicting mortality and hospitalization rates. Brain natriuretic peptide (BNP) is a good marker of CHF, because the plasma BNP concentration is elevated according to the severity of CHF [12–15]. Binding of BNP to its receptors

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initiates natriuretic and vasorelaxant activities through an elevation in intracellular cyclic guanosine monophosphate (cGMP) [16,17]. Nitric oxide (NO) is also an important vasoactive substance, because it exerts anti-atherogenic effects by inhibiting the migration or proliferation of monocytes or smooth muscle cells and vasodilation mainly by cGMP dependent mechanism [18]. We reported that NO regulates cGMP in patients with renal insufficiency [19]. NO may be a useful prognostic marker for patients suffering from atherosclerotic diseases such as cerebral strokes or myocardial infarction, although as yet there have been no reports investigating the use of NO in this capacity. The source of NO is not only endothelial cells (endothelial NO synthase; eNOS) but also macrophages or T cells (inducible NO synthase; iNOS) and some neuronal cells (neuronal NO synthase; nNOS). The plasma level of NO_x (nitrite plus nitrate, metabolites of NO) may reflect the status of eNOS and, to some extent, the status of iNOS. Because iNOS is activated in patients with inflammations such as sepsis, advanced stages of malignancy, or progressed atherosclerotic lesions, the NO_x level may have potential as a marker of malignancy as well as atherosclerotic diseases [20,21].

For elderly patients, the consideration of prognostic factors is very important, but there have been few reports about the potential use of vasoactive substances. Therefore, in this study, we evaluated whether measurements of plasma levels of vasoactive factors such as NO_x, cytokines such as IL-6, and well-known markers such as albumin were useful as prognostic factors in the elderly.

Methods

Study sample

One hundred and twenty seven elderly subjects (48 males and 79 females; mean age, 81.3 ± 7.5 years; range, 65–101 years) were enrolled on August on 2002. The study was approved by the Ethics Committee of Nagoya University Graduate School of Medicine and written informed consent was obtained from all patients. Patients were selected consecutively among our geriatric clinics and related home care services. In brief, 91 participants were presented at Department of Geriatrics, Nagoya University Hospital and the related hospital as outpatients (31 from their homes, 31 from geriatric nursing care units, and 29 from other facilities such as private homes for the aged) and 36 were in home care services facility. At the baseline examination, participants underwent a review of their medical history, a physical examination, and assessment of cardiovascular disease risk factors. On registration, they were not suffering with acute or evident heart failure or acute inflammation whose serum CRP is larger than 2 mg/dl. They were also not suffering with acute myocardial infarction or cerebral infarction within 3 months. We followed patients up to 2.8 years. All participants had a clinical visit each year of the study period, and their laboratory data were determined at each of these visits. We had telephone contact with the patients who could not have clinical visit, or their physicians.

Measurement

We measured fasting serum or plasma levels of biochemical products including lipids and plasma levels of neurohumoral factors and cytokines. Levels of general biochemical products were measured at SRL Laboratories, Tokyo, on an automated sequential multiple analyzer. Samples for the assay of plasma norepinephrine (NE), angiotensin-II, BNP, NO_x, cGMP, IL-6, and TNF-α levels were transferred to chilled disposable tubes containing EDTA-2Na. The blood samples were immediately placed on ice and centrifuged at −4 °C, and aliquots of plasma were immediately stored at −80 °C until assay. BNP levels were measured with a specific radioimmunoassay. NE levels were measured by HPLC. NO_x levels were measured using an NO detector-HPLC system (ENO10; Eicom Co., Kyoto, Japan) [22]. cGMP concentration was determined using a specific radioimmunoassay method (RPN226; Amersham, Buckinghamshire, England) [23]. Angiotensin-II levels were measured by radioimmunoassay. Both IL-6 and TNF-α measurements were performed using a commercially available radioimmunoassay kit (Quantikine HS; R&D Systems, Minneapolis, MN). Hypertension was defined as systolic BP ⩾ 140 mmHg, or diastolic BP ⩾ 90 mmHG or antihypertensive drugs were prescribed. Hyperlipidemia was defined as follows. Total cholesterol ⩾ 220 mg/dl or LDL cholesterol (total cholesterol − HDL cholesterol − triglyceride/5) ⩾ 140 mg/dl) or anti-hyperlipidemic drugs were prescribed. Diabetes mellitus was defined as in American Diabetes Society Guidelines [24] (in brief, fasting blood glucose ⩾ 126 mg/dl or hemoglobin A1C ⩾ 6.5 g/dl). Previously diagnosed hypertension, hyperlipidemia or diabetes were also included.

Statistical analysis

The results are presented as means ± SD. Values of $P < .05$ were considered to indicate statistical significance in all analyses. All statistical analyses were performed using Stat View software (SAS Institute Inc., Cary, NC). Characteristics of the survivors and the deceased subjects were compared using Mann–Whitney's *U*-test. Characteristics that were significantly different between the survivors and deceased by Mann–Whitney's *U*-test were further subjected to inherent multiple logistic regression analysis. As a result, adjusted odds ratios were calculated. Survival curves were calculated by the Kaplan–Meier method.

Results

Clinical characteristics

Table 1 shows the baseline characteristics of patients. There were no significant differences in age or coronary risk factors among the situations where the patients were

Table 1
Baseline characteristic of patients on registration

Characteristics $n = 127$			
Profiles		Chronic medications	
Age (years old)	81.31 ± 7.48	Angiotensin converting enzyme inhibitor	24
Male	48	Angiotensin II receptor antagonist	18
Female	79	β-Blocker	9
		Ca antagonist	37
Medical history			
Hypertension	57	Diuretics	29
Hyperlipidemia	27		
Diabetes mellitus	36	Nitrate	29
		Digitalis	16
Prior congestive heart failure	24		
Ischemic heart disease	21	Antiplatelet agents	49
Arrhythmia	23	Warfarin	8
Stroke	51	Statins	16
Arteriosclerosis obliterans	6		

enrolled. Their complicating diseases were hypertension (44.9%), hyperlipidemia (21.3%), diabetes mellitus (28.3%), congestive heart failure (18.9%), ischemic heart disease (16.5%) and cerebral vascular disease (40.2%, including patients with only lacuna). Table 1 also shows the chronic medications of patients. Anti-hypertension drugs ⟨angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blocking agents (ARBs), β-adrenergic receptor blocking agents and calcium antagonists⟩, diuretics, antiplatelet agents, and nitrates [isosorbide dinitrate 20 mg/day, $n = 14$, 40 mg/day $n = 3$, nitroglycerin patch 25 mg/day $n = 12$] were prescribed frequently.

Survival

During the 2.8-year follow-up period, 46 of the 127 patients died (Table 2). Seventeen, seventeen, and twelve patients died during the 1st, 2nd, and 3rd year of the follow-up period, respectively. The causes of death were as follows: cardiovascular disease ($n = 15$ patients: 6 cases of heart failure, 3 of acute myocardial infarction, 4 of renal failure related to cardiovascular disease, and 2 of cerebro-vascular disease), infection ($n = 13$), malignancy ($n = 2$), cardiopulmonary arrest without evident cause ($n = 6$), liver cirrhosis ($n = 1$), intestinal twist ($n = 1$), dehydration ($n = 1$), senility ($n = 1$), burned to death ($n = 1$), and unknown ($n = 5$). Four patients cannot be followed by medical record or telephone contacting. There are no significant differences in gender ratio (male/female) between participants being alive after 2.8 years and those dead during 2.8 years. There are also no significant differences between them in the drugs or the suffered diseases including ischemic heart disease, congestive heart failure, and smoking.

Table 2
Biochemical characteristic of patients on registration

	On registration ($n = 127$)	Participants being alive after 2.8 years ($n = 77$)	Participants dead during 2.8 years ($n = 46$)	*P* value
Hemoglobulin (g/dl)	11.6 ± 1.9	12.2 ± 1.7	10.7 ± 1.8	.0001
Total protein (g/dl)	7.04 ± .71	7.15 ± .69	6.84 ± .69	.0129
Albumin (g/dl)	3.83 ± .52	3.98 ± .42	3.56 ± .55	.0001
Total cholesterol (mg/dl)	197.8 ± 46.0	210.6 ± 45.3	178.1 ± 41.4	.0003
HDL-cholesterol (mg/dl)	50.1 ± 20.5	53.2 ± 20.1	44.6 ± 19.7	.0078
LDL-cholesterol (mg/dl)	123.8 ± 35.2	131.9 ± 35.9	111.7 ± 31.7	.0038
Triglyceride (mg/dl)	113.3 ± 62.3	114.6 ± 55.7	111.9 ± 74.4	.2577
Creatinine (mg/dl)	.88 ± .6	.80 ± .38	.94 ± .69	.4518
cGMP (pmol/ml)	4.89 ± 3.2	4.74 ± 3.2	5.40 ± 3.0	.1114
Hs-CRP (ng/ml)	8224.9 ± 17224	6493.1 ± 15669	11123.7 ± 19391	.0150
HANP (ng/ml)	43.1 ± 56.3	38.1 ± 36.7	52.5 ± 79.4	.2673
BNP (ng/ml)	79.1 ± 81.1	71.3 ± 81.6	92.6 ± 77.9	.0565
NO_x (μmol/l)	28.3 ± 19.3	25.1 ± 18.4	33.7 ± 19.8	.0059
Norepinephrine	541.0 ± 439.7	493.0 ± 431.7	619.2 ± 478.4	.3103
Angiotensin II	9.03 ± 8.90	8.74 ± 7.78	9.50 ± 10.57	.7047
Il-6 (pg/ml)	7.81 ± 10.0	7.17 ± 9.6	8.99 ± 11.0	.0174
TNF-α (pg/ml)	1.94 ± 6.021	2.177 ± 7.7	1.58 ± .89	.0001

Values are expressed as means ± SD. $P < .05$: significant difference between alive and dead. Hs-CRP: high sensitive C reactive protein. HANP: human atrial natriuretic peptide. BNP: brain natriuretic peptide. NO_x: NO metabolites.

Biochemical analyses

The biochemical characteristics of all participants are shown in Table 2. The age, hemoglobin, albumin, total cholesterol, HDL cholesterol, and LDL cholesterol levels on registration were significantly higher in those who survived than in those who died during the study period. Gender or creatinine was not significant in this study.

Markers profile

The plasma concentrations of the various makers are also depicted in Table 2. Plasma NO_x and IL-6 were significantly lower in those who were alive after the study period than in those who died during the period. Plasma TNF-α was also higher in the survivors than in the patients who died. BNP level on registration was not different significantly ($P = .0565$).

Multivariate survival analyses

Logistic regression was applied to analyze significant characteristics: age, hemoglobin total protein, albumin, total cholesterol, HDL-cholesterol, LDL-cholesterol, IL-6, TNF-α, and NO_x, which showed significant differences between the survivors and the deceased by using Mann–Whitney's *U*-test, which was described as above. They were subjected to logistic regression analysis. The results are depicted in Table 3. The adjusted odds ratios of albumin and NO_x were .236 and 1.027, respectively. Based on this result, 1SD decrease of albumin level from a certain level is estimated to be 2.11 in odds ratio. 1SD increase of NO_x level from a certain levels is to be 1.67 in odds ratio. As the effect of albumin and that of NO_x are independent, the odds ratio increases synergistically. NO_x was shown to correlate the serum hemoglobin, HDL-cholesterol, creatinine, and HS-CRP on baseline level, and NO_x level was known to be affected by age, gender, and creatinine (renal function). So, we thought creatinine and gender as independent risk factor, and re-applied logistic regression including creatinine and gender as well as the conditions above. The result is almost same as before that albumin and NO_x are significant, and that the adjusted odds ratios of albumin and NO_x were .195 ($P = .045$) and 1.031 ($P = .034$) respectively.

Table 3
Multivariate logistic regression analysis

	Adjusted odds ratio	95% CI	*P* value
Age (year)	1.047	.977–1.122	.194
Hemoglobulin (g/dl)	.766	.562–1.044	.0917
Total protein (g/dl)	.997	.458–2.170	.9932
Albumin * (g/dl)	.236	.058–.955	.0429
Total Cholesterol(mg/dl)	.994	.949–1.042	.8083
HDL-Cholesterol (mg/dl)	1.000	.948–1.055	.9972
LDL-Cholesterol (mg/dl)	.993	.943–1.046	.7988
Hs-CRP (ng/ml)	1.001	1.000–1.002	.0924
NO_x * (μ mol/l)	1.027	1.010–1.541	.0394
Il-6 (pg/ml)	.975	.904–1.051	.5072
TNF-α (pg/ml)	.967	.727–1.288	.8197

Values are expressed as means ± SD. $P < .05$: significant difference between alive and dead. Hs-CRP: high sensitive C reactive protein. HANP: human atrial natriuretic peptide. BNP: brain natriuretic peptide. NO_x: NO metabolites.
CI: confidence interval. * means the significant marker ($P < .05$).

These data showed that NO is a useful prognostic marker with efficacy almost equal to that of albumin, the best-known prognostic marker to date. BNP and other cytokines such as IL-6 or TNF-α were not significant prognostic markers in the present study. Further, in the patient who died by cardiovascular diseases, multivariate survival analyses showed that NO_x and the history of ischemic heart disease were significant prognostic markers. In patients who died by other causes than cardiovascular diseases, NO_x and albumin were significant markers.

Survival rate

The survival rates are shown in Fig. 1. Panel (A) shows the relation between survival rate and NO_x levels on entry, and panel (B) shows the relation between survival rate and albumin levels on entry. The survival rate goes up in proportion to NO_x levels and goes down in proportion to albumin levels. Kaplan–Meier analyses of mortality show that the prognosis of patients was dependent on NO_x levels. The prognosis of patients in 3rd and 4th quartile of NO_x was significantly worse than that in 1st or 2nd quartile of NO_x. The prognosis of patients in first quartile of albumin is worse than that of other patients, which there is no difference in prognosis between 2nd, 3rd or 4th quartile of albumin. In other words, NO_x levels reflect the viability of all patients; however,in albumin level, the patients in 4th quartile only have worst prognosis.

Discussion

The present data demonstrate that NO is a new clinical biomarker of survival in elderly patients and that its efficacy might be nearly equal to that of albumin, the best known prognostic marker to date. This is the first report of the use of a vascular functional marker such as NO as a prognostic marker in the elderly.

We assessed for around 3 years prognosis to expect short and mid term prognosis. Because elderly patients might have many illnesses—e.g., occult congestive heart failure, arteriosclerosis, latent malignancy, etc.—we evaluated nutrition markers, pro-inflammatory cytokines, natriuretic peptide and vascular endocrinological substances.

Serum albumin and cholesterol levels were reported as prognostic marker and it was reported recently that elderly individuals with low cholesterol constitute a heterogeneous group with regard to health characteristics and mortality risk [1–3]. It is needless to say that inflammatory markers or cytokines are valuable, but these substances are often

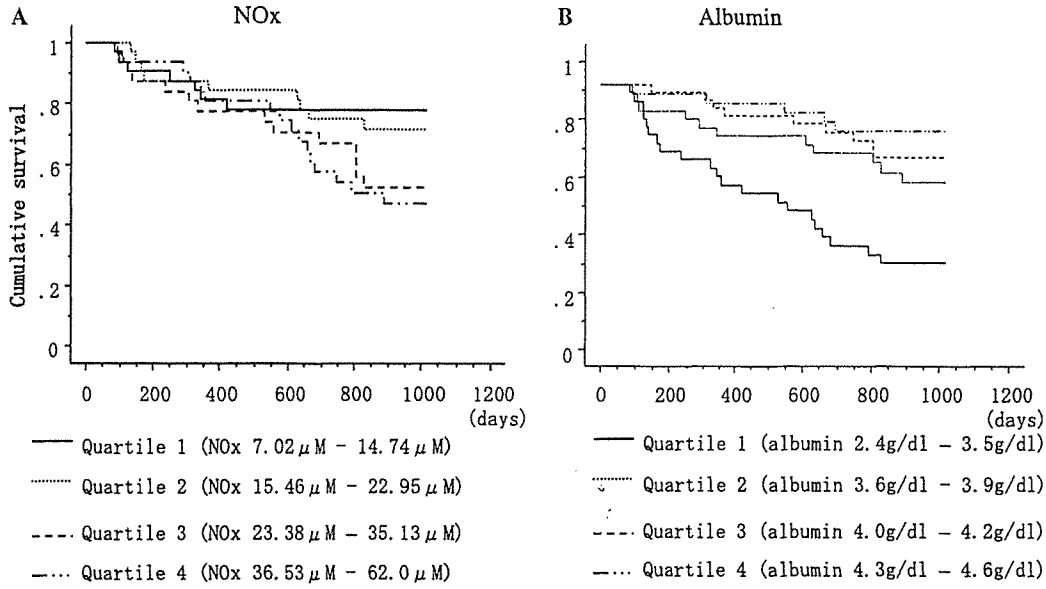

Fig. 1. Kaplan–Meier survival analysis. Circulating levels of NO_x (A) and albumin (B) were examined in relation to patient survival during follow-up. For this analysis, circulating levels of NO_x and albumin were arbitrarily divided into quartiles on registration.

affected by acute infections or chronic collagen disease such as rheumarthritis, which is very common in elderly people.

The present study revealed that low albumin, low cholesterol, high IL-6 and high sensitive-CRP indicate poor prognosis. However, logistic regression analysis indicated that only albumin and NO_x were predictive.

NO is produced by L-arginine and NO synthases (eNOS in endothelial cells, iNOS mainly in inflammatory cells, and nNOS in the nervous system). Atherosclerosis is an inflammatory disease [25] characterized by vascular endothelial cell dysfunction and diminished production of NO [26]. eNOS gene transfer can reduce atherogenesis in hypercholesterolemic animals [27]. NO is a widespread signaling molecule in the cardiovascular system, and functions in multiple ways to protect against the progression of atherosclerosis [28–30]. Plasma NO_x was difficult to assay and estimate, because protein of plasma affects the NO_x value. HPLC developed for measurement of NO_x specially is convenient and seemed to be accurate and reliable in those points. Plasma concentrations of NO_x [31] are higher in patients with CHF, and NO_x concentrations may vary according to the severity of heart failure [32]. Both the natriuretic peptide family and NO mediate their physiological action through a second messenger, cGMP [33–35]. NO increases production of cGMP by activation of soluble guanylate cyclase [33–35], while the natriuretic peptide family increases production of cGMP by activation of particle guanylate cyclase. In the present study, NO_x increased not only in the patients with heart failure but also in the patients with other atherosclerotic diseases.

In animal experiments including ours, the mRNA and protein of eNOS were increased in atherosclerotic vessels [36]. Further, coronary risk factors such as hyperlipidemia were also shown to increase mRNA of eNOS of vessels. In plasma drawn from vein, it is possible that NO_x amount increased because of high eNOS in vessels, although arterial endothelial dysfunction occurred because of intimal thickening and increased reactive oxygen species intra arteries. Taken together, we speculate NO_x levels reflects pre-clinical and clinical situation of both the vessels (atherosclerosis induced ischemia etc.) and heart (heart failure etc.) and thus NO_x was most sensitive in this study.

There were no patients with sepsis or advanced malignancy at registration. No patients died within the 3 months after registration, and the maximum levels of NO_x were at most 3 times the mean, indicating the less contribution of iNOS in this study and NO_x levels principally reflect eNOS derived one. In fact, we observed iNOS only in some macrophage derived foam cells and T lymphocytes in the peripheral of necrotic core of advanced atherosclerosis, not in mild or moderate atherosclerosis or vein [37]. Although the detailed mechanisms should be elucidated, a continuous increase in NO was suggested to have a deteriorating effect on the prognosis and might be used to predict the prognosis.

Study limitations

We measured the plasma NO_x level in order to assess basal NO production. We were afraid that plasma NO_x concentration might be affected by exogenous NO sources such as diet or drugs (NO donors or eNOS activator like angiotensin converting enzyme inhibitors; ACE-I) [38,39]. We collected fasting blood samples. We evaluated the effect of NO donors (nitroglycerin etc.), eNOS activators (ACE-Is etc.) and patients' diseases on plasma NO_x level. The Mann–Whitney's *U*-test showed no significant effects (ACEI .219, HMG-CoA reductase inhibitor .391, NO donors .291, ischemic heart diseases .307 etc). The effect of drugs may be masked between elderly patients because of

individual difference of plasma NO_x levels by other factors such as severity of diseases [40,41]. These data are consistent with previous data, however, we should suppose the effect of aging on renal function, which increase NO_x levels. The number of participants is small and it should be elucidated more for larger participants in future.

Conclusively, this study first suggests the importance of the NO related responses in the prognosis of elderly, which is as strong as that of albumin, past well-known marker. Vascular factor might be important as much as nutritional factor in elderly.

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