TABLE 1. Clinical Characteristics of Study Population in 1988

Variables	Men		Women	
	MetS (−) (N=834)	MetS (+) (N=216)	MetS (−) (N=983)	MetS (+) (N=419)
Age, years	58±11	58±11	57±11	62±10†
Systolic blood pressure, mm Hg	132±20	145±18†	126±19	145±19†
Diastolic blood pressure, mm Hg	79±11	87±10†	74±10	81±11†
Antihypertensive medication, %	11.8	21.3†	9.0	29.6†
Hypertension, %	37.4	70.4†	24.0	67.5†
Proteinuria, %	7.0	11.6*	3.0	6.9†
Electrocardiogram abnormalities, %	18.7	19.7	12.5	14.6
Waist circumference, cm	80.3±7.6	88.7±6.9†	78.1±9.2	88.2±8.4*
Body mass index, kg/m^2	22.3±2.8	25.0±2.6†	22.1±2.9	24.9±3.1†
Fasting blood glucose, mmol/L	5.7±1.1	6.7±1.7†	5.5±1.0	6.3±1.7†
Diabetes, %	6.7	29.2†	3.0	17.7†
Serum total cholesterol, mmol/L	5.09±1.06	5.19±1.14	5.47±1.05	5.78±1.09†
Serum triglycerides, mmol/L	1.13 (0.43–2.95)	2.46 (0.83–7.32)†	0.90 (0.44–1.86)	1.58 (0.61–4.10)†
Serum high-density lipoprotein cholesterol, mmol/L	1.31±0.29	1.06±0.27†	1.42±0.28	1.14±0.22†
Smoking habits, %	51.6	45.8	6.2	7.9
Alcohol intake, %	59.5	69.4*	8.6	9.8
Regular exercise, %	12.2	8.8	9.2	9.3

Values are mean±SD or percentage.
Electrocardiogram abnormalities are defined as left ventricular hypertrophy (Minnesota code, 3–1) and/or ST depression (Minnesota code, 4–1, 2, 3).
Geometric mean values and 95% CIs of serum triglycerides are shown attributable to the skewed distribution.
*P<0.05, †P<0.01 vs MetS (−).
HDL indicates high-density lipoprotein.

significantly higher in subjects with MetS than in those without MetS for both sexes (men: 9.0 versus 4.8, P=0.03; women: 6.2 versus 3.4, P=0.01). The similar tendency was observed for hemorrhagic stroke only in men (5.1 versus 1.6, P=0.01).

Age- and multivariate-adjusted hazard ratios of MetS for the development of CVD were estimated for both sexes (Table 3). The age-adjusted analysis showed that MetS was a significant risk factor for CVD in men and women. These

TABLE 2. Age-Adjusted Incidence Rates of CVD, CHD, and Stroke According to MetS Status in 2452 Subjects During a 14-Year Follow Up by Sex

	Men				Women			
	Person-Years at Risk	No. of Events	Age-Adjusted Incidence Rate	P Value	Person-Years at Risk	No. of Events	Age-Adjusted Incidence Rate	P Value
Cardiovascular disease								
MetS (−)	9958	108	11.6		12 759	78	6.5	
MetS (+)	2416	50	21.8	<0.01	5078	71	12.9	<0.01
Coronary heart disease								
MetS (−)	10 213	53	5.7		13 010	17	1.5	
MetS (+)	2533	25	9.2	<0.01	5279	30	5.1	<0.01
Stroke								
MetS (−)	10 099	63	6.4		12 817	65	5.3	
MetS (+)	2477	31	14.1	<0.01	5122	50	8.8	0.06
Ischemic stroke								
MetS (−)	10 099	46	4.8		12 817	40	3.4	
MetS (+)	2477	20	9.0	0.03	5122	39	6.2	0.01
Hemorrhagic stroke								
MetS (−)	10 099	17	1.6		12 817	25	2.0	
MetS (+)	2477	11	5.1	0.01	5122	11	2.6	0.72

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TABLE 3. Age- or Multivariate-Adjusted HRs for Development of CVD, CHD, or Stroke According to MetS Status in 2452 Subjects During a 14-Year Follow Up by Sex

	Men						Women					
	Age-Adjusted			Multivariate-Adjusted*			Age-Adjusted			Multivariate-Adjusted*		
	HR	(95% CI)	*P* Value	HR	(95% CI)	*P* Value	HR	(95% CI)	*P* Value	HR	(95% CI)	*P* Value
Cardiovascular disease												
MetS (−)	1.00	(reference)		1.00	(reference)		1.00	(reference)		1.00	(reference)	
MetS (+)	1.93	(1.38–2.70)	<0.01	1.86	(1.32–2.62)	<0.01	1.68	(1.22–2.33)	<0.01	1.70	(1.22–2.36)	<0.01
Coronary heart disease												
MetS (−)	1.00	(reference)		1.00	(reference)		1.00	(reference)		1.00	(reference)	
MetS (+)	1.95	(1.21–3.13)	<0.01	1.94	(1.19–3.17)	<0.01	3.11	(1.71–5.65)	<0.01	2.86	(1.56–5.24)	<0.01
Stroke												
MetS (−)	1.00	(reference)		1.00	(reference)		1.00	(reference)		1.00	(reference)	
MetS (+)	2.04	(1.33–3.14)	<0.01	1.92	(1.23–2.98)	<0.01	1.43	(0.99–2.08)	0.06	1.50	(1.03–2.19)	0.03
Ischemic stroke												
MetS (−)	1.00	(reference)		1.00	(reference)		1.00	(reference)		1.00	(reference)	
MetS (+)	1.80	(1.07–3.05)	0.03	1.68	(0.98–2.89)	0.06	1.77	(1.14–2.76)	0.01	1.78	(1.13–2.79)	0.01
Hemorrhagic stroke												
MetS (−)	1.00	(reference)		1.00	(reference)		1.00	(reference)		1.00	(reference)	
MetS (+)	2.67	(1.25–5.69)	0.01	2.54	(1.18–5.49)	0.02	0.88	(0.43–1.80)	0.72	0.99	(0.48–2.05)	0.91

*Adjusted for age, proteinuria, electrocardiogram abnormalities, serum total cholesterol, smoking habits, alcohol intake, and regular exercise.

relationships remained substantially unchanged even after adjustment for the following confounding factors: age, proteinuria, electrocardiographic abnormalities, serum total cholesterol, smoking habits, alcohol intake, and regular exercise. Furthermore, MetS was found to be an independent risk factor for the development of CHD and stroke after adjustment for the confounding factors in men and women. When strokes were divided into ischemic and hemorrhagic type, multivariate-adjusted HR of MetS for ischemic stroke was marginally higher in men and significantly higher in women, whereas MetS is an independent risk factor for hemorrhagic stroke only in men.

The age- and sex-adjusted cumulative incidences of CVD, CHD, and stroke according to the number of MetS components are shown in the Figure. Because the cumulative incidence curves for one and 2 components overlapped, we combined these components. The incidences of CVD, CHD, and stroke were significantly higher among the subjects with 3 or more MetS components compared with those without any MetS component. A significant graded relationship between the number of components of MetS and the HR for developing CVD was identified from 3 MetS components and onward (Table 4). Compared with individuals with no MetS component, individuals with one, 2, 3, and 4 or more components had gradually increased HRs, respectively, for developing CVD after adjusting the confounding factors. A similar relationship was found when CVD was divided into CHD and stroke.

Because hypertension and diabetes are strong risk factors for CVD, we examined the combined as well as separate effects of MetS and hypertension or diabetes on the development of CVD. As shown in Table 5, the age- and sex-adjusted HR of CVD was significantly higher in normotensive subjects with MetS, hypertensive subjects without MetS, and hypertensive subjects with MetS compared with those without hypertension and MetS. Furthermore, there was a significant excess risk of CVD in hypertensives with MetS than in those without MetS. Similarly, the age- and sex-adjusted HR of CVD was significantly higher in nondiabetic subjects with MetS and diabetic subjects with MetS compared with those without diabetes and MetS. However, no significant difference was found in the risk of CVD in diabetic subjects without MetS. Among diabetic subjects, the risk of CVD was significantly higher in subjects with MetS than in those without MetS. These relationships remained substantially unchanged even after adjusting for the confounding factors. Furthermore, we examined the association of MetS with CVD by the multivariate analysis using hypertension and diabetes in addition to the previously mentioned risk factors as confounding factors. As a result, MetS remained a significantly independent risk factor for the development of CVD (HR, 1.38; 95% CI, 1.07 to 1.78, $P=0.01$). The risks of other risk factors were as follows: age (HR, 2.00 [per increment of 10 years]; 95% CI, 1.79 to 2.26, $P<0.01$), male sex (1.45; 1.07 to 1.97, $P=0.02$), hypertension (1.64; 1.26 to 2.12, $P<0.01$), diabetes (1.55; 1.14 to 2.13, $P<0.01$), smoking habits (1.69; 1.28 to 2.23, $P<0.01$), regular exercise (0.58; 0.39 to 0.87, $P<0.01$), proteinuria (1.64; 1.13 to 2.38, $P<0.01$), electrocardiographic abnormalities (1.29; 0.98 to 1.69, $P=0.07$), serum total cholesterol (0.99 [per increment of 1 mmol/L]; 0.89 to 1.11, $P=0.92$), and alcohol intake (0.97; 0.73 to 1.30, $P=0.84$).

Discussion

To our knowledge, our study is the first prospective cohort study of a general Japanese population with a long duration of follow up reporting the association of MetS with incident CVD using the modified NCEP definition. The sole study from Japan, which examined a similar association, was based on a diabetic population.[27] We found a clearly increased incidence of CVD during 14 years of follow up in both men

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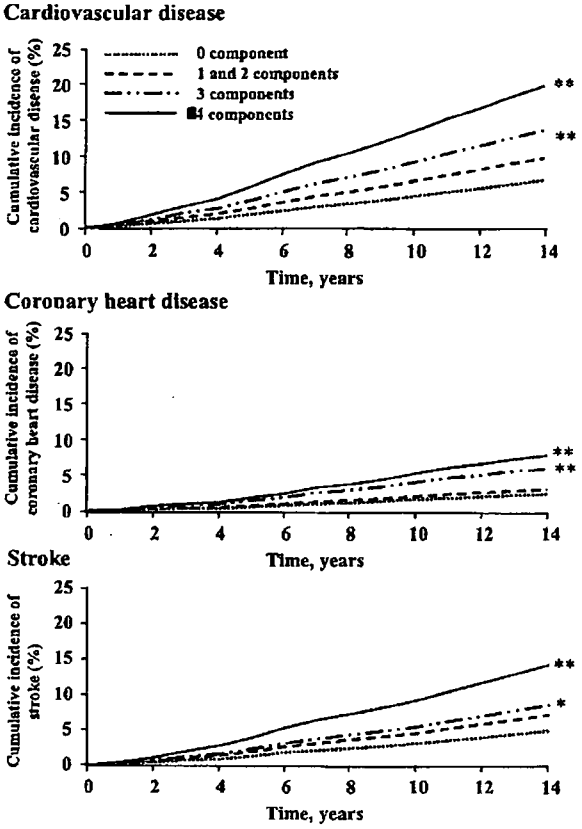

Age- and sex-adjusted cumulative incidences of CVD, CHD, and stroke according to the number of the metabolic syndrome components in 2452 subjects during a 14-year follow up. *P<0.05, **P<0.01 versus 0 component.

and women with MetS compared with those without MetS. Besides, the risk of MetS for the development of CVD remained significant even after adjustment for hypertension, diabetes, and other potentially confounding factors.

In our study, subjects with MetS had little over 70% increases in CVD risk compared with those without MetS. Similar or higher HRs (1.4- to 5.0-fold) of MetS for CVD/CHD were reported from different European and American studies.[7,13-25] Differences in the study populations, prevalence of individual components of MetS, follow-up length, and MetS definition used seem to be the main causes behind the variation in the HRs. In our study, CHD risk related to MetS is higher in women than in men, which is consistent with the studies from the Western world.[17]

Our study showed that the risk of incident combined CVD, and CHD and stroke separately, was found to increase with the number of components of MetS and increased by 3-fold or more in those with 4 or more MetS components compared with those without any component. It also revealed that the risk of CVD increased in incremental fashion with the number of components of MetS and became predictive of CVD (also CHD and stroke separately) when the number of components reached 3. This phenomenon gives credence to the requirement of ≥3 components in the NCEP definition for establishing the diagnosis of MetS. Thereby, it can be assumed that the modified NCEP definition of MetS is well predictive for CVD in the general Japanese population.

One prospective study based on a Japanese diabetic population mentioned that MetS based on the NCEP definition was predictive for CVD in men and was not in women.[27] The same authors again reported that the new International Diabetes Federation definition[34] was not predictive for CVD in either male or female patients with diabetes.[35] On the other hand, in our study, MetS based on the NCEP definition was consistently predictive of CVD not only in both men and women, but also in subjects with diabetes. We speculate that this discrepancy resulted from the difference in the cutoff point of the waist circumference between the 2 studies. The former used the waist circumference definition for abdominal obesity proposed by the Japan Society for the Study of Obesity (85 cm for men and 90 cm for women),[36] whereas in our study, we used the waist circumference definition for Asian populations (90 cm for men and 80 cm for women), which was recommend by the International Diabetes Federation to use for the Japanese population.[37] Further research is needed to refine the MetS definition, which would be applicable to various populations, including Japanese.

There was a possibility that the increased risk of MetS for CVD resulted from the influences of hypertension or diabetes, which are components of MetS and major risk factors for developing CVD. However, our stratified analysis showed that the MetS was a significant risk factor for CVD in normotensive subjects as well as in nondiabetic individuals and has a similar risk for CVD as hypertension; the risk is even higher than that of diabetes. Moreover, in the multivariate analysis, MetS was found to be a significant risk factor for CVD independent of hypertension, diabetes, and other confounding risk factors. These results imply the significant roles of MetS in the development of CVD and the need for prevention and early management of the MetS components. In addition, diabetes is not predictive of CVD in subjects without MetS in our study. This finding might suggest that good diabetic control is useful. However, because the number of our diabetic subjects without MetS is small, further studies are necessary to elucidate this issue in detail.

The strengths of our study include its longitudinal population-based study design, long duration of follow up, sufficient number of CVD events and almost perfect follow up of subjects, examining the data in men and women separately, and exclusion of patients with CVD at baseline. Moreover, it is the first study to examine prospectively CVD in relation to MetS based on a general Japanese population. One limitation of our study is that the diagnosis of MetS was based on a single measurement of its components at baseline as was the case in other epidemiological studies.[13-25,27-29] During the follow up, risk factor levels could be changed attributable to modification of lifestyle or medication, and misclassification of the MetS is possible. Thus, it would weaken the association found in this study, biasing the results toward the null hypothesis. Therefore, the true association may be stronger than that shown in our findings.

In conclusion, we have shown that the prevalence of MetS is sizeable in Japanese middle-aged men and women and it is predictive of future CVD in both sexes based on a prospective study with 14 years of follow up. Our findings suggest that early identification of MetS and appropriate behavioral and therapeutic intervention may reduce the burden of CVD in the long run.

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TABLE 4. Age- or Multivariate-Adjusted HRs for Development of CVD, CHD, and Stroke According to the Number of the MetS Components in 2452 Subjects During a 14-Year Follow Up

	Population at Risk	No. of Events	Age- and Sex-Adjusted			Multivariate-Adjusted*		
			HR	(95% CI)	*P* Value	HR	(95% CI)	*P* Value
Cardiovascular disease								
No. of MetS components								
0	436	30	1.00	(reference)		1.00	(reference)	
1	756	84	1.49	(0.98–2.26)	0.06	1.45	(0.95–2.20)	0.08
2	625	72	1.47	(0.96–2.26)	0.08	1.39	(0.91–2.15)	0.15
3	394	65	2.12	(1.37–3.28)	<0.01	1.95	(1.25–3.04)	<0.01
≥4	241	56	3.19	(2.03–5.02)	<0.01	2.99	(1.89–4.73)	<0.01
Coronary heart disease								
No. of MetS components								
0	436	13	1.00	(reference)		1.00	(reference)	
1	756	35	1.41	(0.75–2.67)	0.29	1.38	(0.72–2.62)	0.33
2	625	22	1.05	(0.53–2.09)	0.89	0.95	(0.47–1.90)	0.88
3	394	32	2.55	(1.33–4.89)	<0.01	2.29	(1.18–4.47)	0.01
≥4	241	23	3.36	(1.68–6.72)	<0.01	2.96	(1.45–6.01)	<0.01
Stroke								
No. of MetS components								
0	436	20	1.00	(reference)		1.00	(reference)	
1	756	58	1.52	(0.91–2.53)	0.11	1.48	(0.89–2.47)	0.14
2	625	50	1.50	(0.89–2.53)	0.13	1.45	(0.86–2.46)	0.16
3	394	41	1.89	(1.10–3.25)	0.02	1.78	(1.03–3.09)	0.04
≥4	241	40	3.16	(1.83–5.46)	<0.01	3.05	(1.75–5.31)	<0.01

*Adjusted for age, sex, proteinuria, electrocardiogram abnormalities, serum total cholesterol, smoking habits, alcohol intake, and regular exercise.

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TABLE 5. Age- and Sex-Adjusted or Multivariate-Adjusted HRs of the MetS for Development of CVD According to the Presence or Absence of Hypertension or Diabetes in 2452 Subjects During a 14-Year Follow Up

	Population at Risk	No. of Events	Age- and Sex-Adjusted		Multivariate-Adjusted*	
			HR	(95% CI)	HR	(95% CI)
Hypertension						
HT (−)+MetS (−)	1269	89	1.00	(reference)	1.00	(reference)
HT (−)+MetS (+)	200	25	1.79	(1.14–2.79)*	1.75	(1.12–2.75)*
HT (+)+MetS (−)	548	97	1.81	(1.35–2.43)†	1.75	(1.29–2.37)†
HT (+)+MetS (+)	435	96	2.59	(1.93–3.48)†‡	2.45	(1.81–3.32)†‡
Diabetes						
DM (−)+MetS (−)	1732	171	1.00	(reference)	1.00	(reference)
DM (−)+MetS (+)	498	84	1.60	(1.23–2.09)†	1.54	(1.17–2.02)†
DM (+)+MetS (−)	85	15	1.35	(0.80–2.30)	1.38	(0.81–2.34)
DM (+)+MetS (+)	137	37	2.75	(1.93–3.93)†‡	2.60	(1.81–3.74)†‡

*Adjusted for age, sex, proteinuria, electrocardiogram abnormalities, serum total cholesterol, smoking habits, alcohol intake, and regular exercise.

*$P<0.05$, †$P<0.01$ vs reference.

‡$P<0.05$ vs HT(+)+MetS (−) or DM (+)+MetS (−).

HT indicates hypertension; DM, diabetes mellitus.

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Disclosures

None.

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

Toshio Hayashi*†, Packiasamy A. R. Juliet*, Hisako Matsui-Hirai*, Asaka Miyazaki*, Akiko Fukatsu*, Jun Funami*, Akihisa Iguchi*, and Louis J. Ignarro†‡§

*Department of Geriatrics, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8550, Japan; and †Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California, 650 Charles E. Young Drive South, Los Angeles, CA 90095

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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*.

†To whom correspondence may be addressed. E-mail: hayashi@med.nagoya-u.ac.jp or lignarro@mednet.ucla.edu.

§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.

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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. Gp1-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 $O_2^{\bullet-}$.

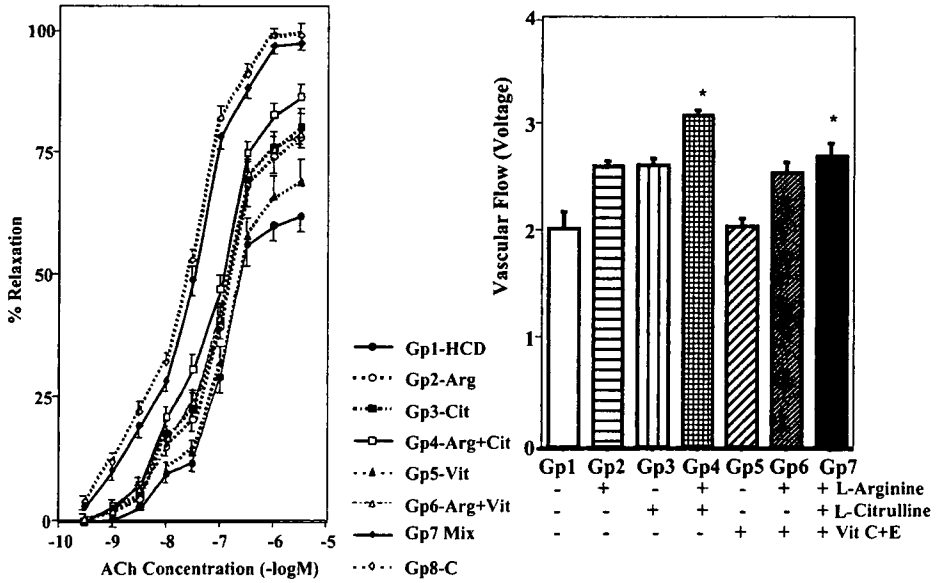

Fig. 1. Vascular responses of thoracic aortas of rabbits. (*Left*) Cumulative concentration-response curves to acetylcholine (ACh) during contraction evoked by prostaglandin $F_{2\alpha}$ (2.6×10^{-6} M) in thoracic aortas from eight groups of rabbits ($n = 6$ per group). (*Right*) Rate of blood flow 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.

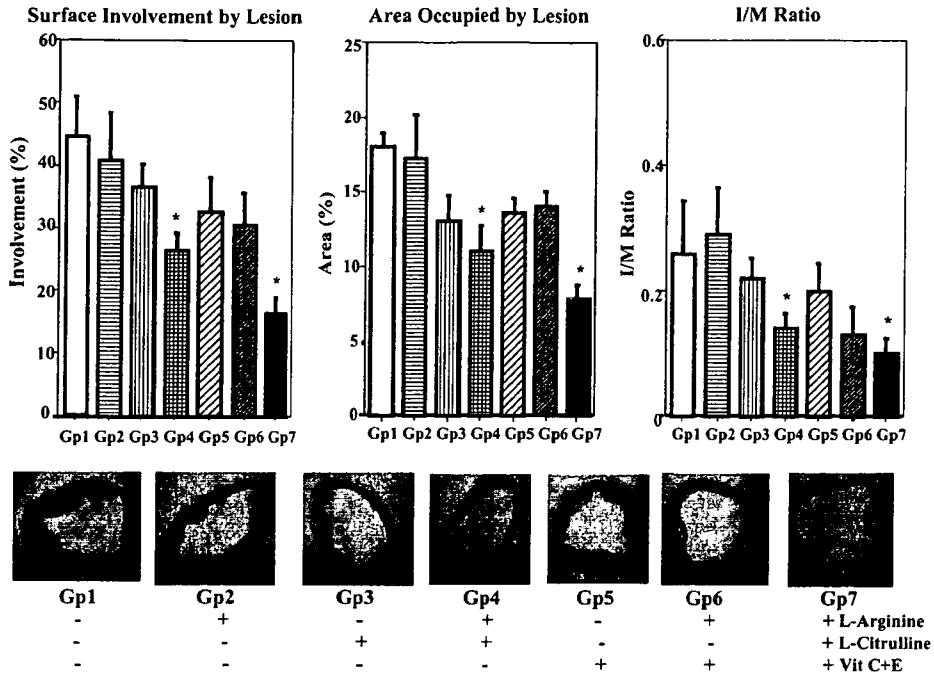

Fig. 2. Histological evaluation of atherosclerotic areas of thoracic aortas from seven groups of rabbits. (*Upper Left*) Surface involvement of atherosclerotic areas in thoracic aorta. (*Upper Center*) Area occupied by atherosclerotic lesions of the aortic arch and thoracic aorta, Gp1. (*Upper Right*) 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).

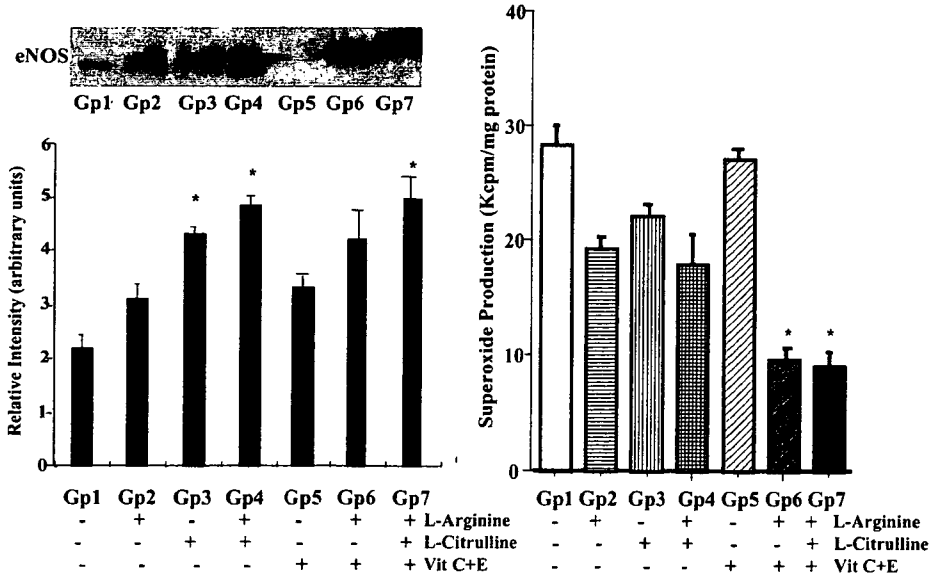

Fig. 3. NO and superoxide production in thoracic aortas from seven groups of rabbits. (*Left*) Quantification of eNOS protein in thoracic aorta using Western blotting. The Western blot represents a single typical experiment. The bar graph illustrates data from six experiments. Relative amounts of eNOS protein are shown. (*Right*) Superoxide production in thoracic aortas of seven groups of rabbits ($n = 6$ per group). Kcpm, 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.

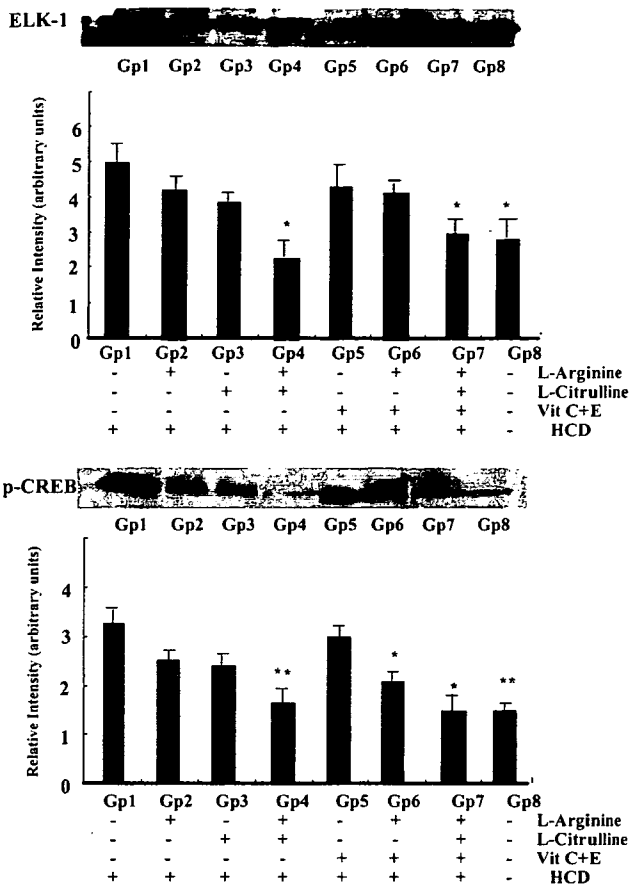

Fig. 4. Quantification of Elk-1 and p-CREB protein in thoracic aortas from seven groups of rabbits using Western blotting. Western blots represent a single typical experiment. The bar graphs illustrate data from six experiments. Data are illustrated as the mean ± SEM from six rabbits per group. *, Significant 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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Modulating role of estradiol on arginase II expression in hyperlipidemic rabbits as an atheroprotective mechanism

Toshio Hayashi*†, Teiji Esaki*‡, Daigo Sumi§, Tapan Mukherjee§, Akihisa Iguchi*, and Gautam Chaudhuri§

*Department of Geriatrics, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8550, Japan; ‡Department of Geriatrics, Kainan Hospital Aichi Prefectural Welfare Federation of Agricultural Cooperatives, 396 Yatomi-town, Ama-gun, Aichi Prefecture 498-8502, Japan; and §Department of Obstetrics and Gynecology and Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, 650 Charles E. Young Drive South, University of California, Los Angeles, CA 90095-1735

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We evaluated the effects of a 0.5% cholesterol-enriched diet (HCD) on nitric-oxide synthase (NOS) and arginase expression and the modulating role of 17β-estradiol (E_2) on this phenomenon. Thirty oopherectomized rabbits were divided into three groups and treated for 15 weeks. Group I received normal chow; group II, HCD; and group III, HCD plus E_2 pellets. Animals in group II showed an increase in plasma lipids, and they demonstrated atheromatous lesions as well as expression of arginase I and II accompanied by a significant number of BrdU-positive cells in endothelial cells and intimal muscle cells, suggestive of an increase in cellular proliferation. There was significant expression of inducible NOS and increased staining of nitrotyrosine-positive areas. These were not observed in group I animals. In both groups, E_2 levels were low. In group III animals, E_2 supplementation led to a decrease in atheromatous lesions and BrdU-positive cells and reduced expression of both inducible NOS and arginase I and II accompanied by a decrease in nitrotyrosine staining. E_2 levels were increased. Our results suggest that E_2 was responsible for these effects, despite the animals being hyperlipidemic, similar to those in group II. Because arginase is responsible for cell proliferation by converting L-arginine to polyamines, our results indicate that expression of arginase may play an important role in cellular proliferation in atherosclerosis, and inhibition of arginase expression by E_2 may be another potential mechanism in attenuating atherogenesis.

arteriosclerosis | L-arginine | nitric oxide | endothelium | nitric-oxide synthase

Estrogens retard the development of atherosclerosis by attenuating the adhesion of circulating monocytes to endothelial cells and their subsequent migration to the subendothelial layer (1). Decreased expression of vascular cell adhesion molecule 1 (VCAM-1) and monocyte chemoattractant protein 1 (MCP-1) by estrogens may at least in part account for this effect (1–3). We (4, 5) and others (6) have demonstrated that estrogens increase nitric oxide (NO) production by endothelial cells, and it is now known that NO, either on its own (7) or produced after stimulation by estrogens (8), can attenuate the cytokine-induced expression of VCAM-1 (1) as well as MCP-1 (9, 10). Furthermore, the inhibition of NO production in animals administered an inhibitor of nitric-oxide synthase (NOS) results in potentiation of atherosclerotic lesions in high-cholesterol diet (HCD)-induced atherosclerosis (11) and also causes atherosclerotic coronary lesions, especially at microvascular levels in experimental animals (12). L-Arginine is a substrate for NOS, which catalyzes formation of N^{ω}-hydroxy-L-arginine as an intermediate that subsequently forms NO (13).

L-Arginine can be a substrate for NOS, which catalyzes its breakdown to release NO in endothelial cells. L-Arginine is also converted by arginase to ornithine, the only source of synthesis in mammalian cells of the polyamines putrescine, spermidine, and spermine, which are essential for cell proliferation and regulation of the cell cycle (14, 15) and, which are, therefore, proatherosclerotic. The exact mechanism by which polyamines increase cell proliferation is not known. In vertebrates there are two isoforms of arginase, both of which catalyze the conversion of arginine to ornithine and urea. They differ with regard to subcellular localization, tissue distribution, and certain enzymatic properties, reflecting the fact that different genes encode them (14, 15). Arginase I is expressed almost exclusively in the cytosol of liver cells, whereas arginase II is located within the mitochondrial matrix and is expressed at low levels in many tissues (15). Furthermore, citrulline, the end product of the NOS-mediated reaction, is converted to L-arginine by arginosuccinate synthetase and arginosuccinate lyase (16). The present work was, therefore, undertaken to assess whether arginase expression is increased in atherosclerotic lesions and to determine the modulating role of estrogen, if any, on this phenomenon.

Results

Blood Chemistry. All of the rabbits appeared to be healthy throughout the study. No significant differences in serum high-density lipoprotein (HDL)-cholesterol, total serum protein, or body weight existed among the three groups over the course of the study. In animals fed normal chow (group I), there was no difference in total cholesterol levels compared with basal values. The addition of 0.5% cholesterol to the diet (groups II and III) increased the total cholesterol levels significantly compared with the baseline value (Table 1).The treatment with E_2 did not significantly affect the plasma lipid levels in this study; however, it increased the plasma E_2 concentration up to physiological levels similar to that observed in ovary-intact rabbits (Table 1), as reported in ref. 2.

Histological Examination of Atherosclerosis. In animals fed normal chow (group I), no atheromatous lesions were observed. On the other hand, histological examination of the thoracic aortas of animals fed a HCD and who received a placebo pellet (group II) revealed more atheromatous lesions, as indicated by the mean percentage of luminal encroachment and the mean lesion area in the hypercholesterolemic than in the animals fed a HCD but who received E_2 pellets (group III). The area of atherosclerosis in the thoracic aorta was reduced by 70% in the E_2-treated group (group III) compared with the HCD group receiving placebo pellets (group II) (Fig. 1 *Left* and *Center*). The intima:media (I:M) ratios also decreased after E_2 treatment (group III vs. group II) (Fig. 1 *Right*).

Conflict of interest statement: No conflicts declared.

Abbreviations: E_2, 17β-estradiol; EDNO, endothelium-derived nitric oxide; eNOS, endothelial nitric-oxide synthase; HCD, high-cholesterol diet; I:M ratio, intima:media ratio; iNOS, inducible nitric-oxide synthase; MCP-1, monocyte chemoattractant protein 1; NOS, nitric-oxide synthase; NZW, New Zealand White; VCAM-1, vascular cell adhesion molecule 1.

†To whom correspondence should be addressed. E-mail: hayashi@med.nagoya-u.ac.jp.

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Table 1. Plasma lipid (total cholesterol, triglycerides, and HDL-cholesterol), total protein, and E_2 concentration in rabbits fed a standard diet (group I), HCD (group II), and HCD plus E_2 (group III)

Group	Total cholesterol, mg/dl	Triglycerides, mg/dl	HDL-cholesterol, mg/dl	Total protein, g/dl	E_2, pg/ml
I	80.2 ± 8.2	36.9 ± 5.1	30.4 ± 4.1	8.1 ± 1.0	15.8 ± 4.1
II	1,451.5 ± 120.5*	82.4 ± 15.6*	30.9 ± 5.2	7.8 ± 1.1	12.9 ± 2.5
III	1,298.8 ± 140.9*	74.5 ± 11.5*	35.2 ± 8.2	8.2 ± 1.1	40.0 ± 2†

Rabbits were treated with each condition for 15 weeks. Results are the mean ± SEM of 10 rabbits. *P* was measured by an unpaired Student *t* test. *, $P < 0.05$ versus group I (control); †, $P < 0.05$ versus group I (control) and group II (HCD).

BrdU-Positive Neointima Lesion. BrdU-positive cells, mainly composed of endothelial cells and intimal smooth muscle cells, were significantly decreased in group III compared with group II (Fig. 2).

Immunocytochemical Analysis. ***Smooth muscle cell α-actin, monocytes/macrophages, inducible NOS (iNOS), and nitrotyrosine (one of the reaction products of $ONOO^-$).*** The atheroma in the aorta was composed of many macrophages derived from foam cells and intimal smooth muscle cell proliferation (Fig. 3). A significant reduction in the atherosclerotic area, as well as a decrease in the relative number of macrophages, was observed in animals in the E_2-treated group in this study (Fig. 3 *Left*). The number of smooth muscle cell α-actin-positive cells was not changed between groups II and III (Fig. 3 *Right*). The iNOS and nitrotyrosine-positive areas were decreased in the E_2-treated group (group III) compared with the placebo group (group II) (Fig. 4). We observed iNOS expression in the T cells and macrophages in the advanced atherosclerotic plaque of the thoracic aortas of group II, consistent with previous data (17).

Arginase I, arginase II, and arginosuccinate synthetase. The atheroma in the aorta expressed a large amount of arginase I and II, as well as arginosuccinate synthase in animals in group II; however, the expression of arginase I and II, but not arginosuccinate synthase, decreased in the aortas from group III (Fig. 5).

Discussion

Endothelial dysfunction leading to expression of adhesion molecules plays an integral part in the initiation of atherosclerosis. Expression of adhesion molecules on the endothelial surface leads to adhesion of monocytes to endothelial cells, which is one of the early steps in the development of atherosclerosis (18). Endothelial dysfunction is also characterized by impaired endothelium-derived nitric oxide (EDNO) production and impaired endothelium-dependent vasodilation (19). Only low concentrations of NO are normally produced by the endothelial cells physiologically, which protect the endothelial cell and, therefore, play an important role in attenuating the onset of atherosclerosis (20, 21). On the other hand, once atherosclerosis develops, activated macrophages are present in the lesion area, and these macrophages express iNOS (17). This process leads to relatively high concentrations of NO as well as superoxide (O_2^-), leading to formation of peroxynitrite

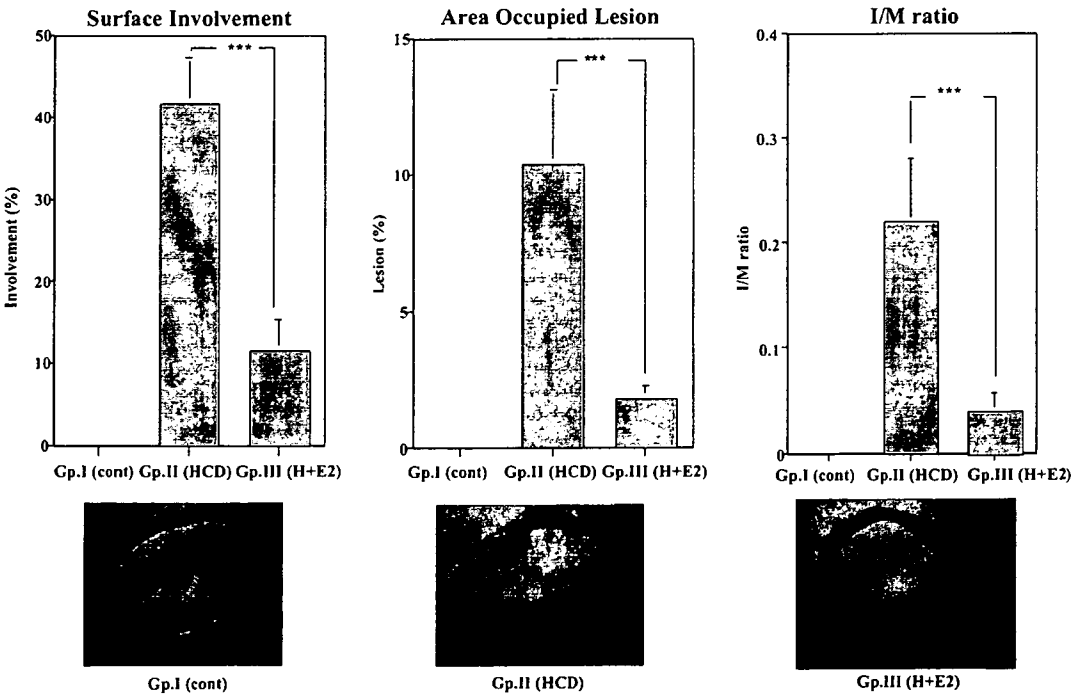

Fig. 1. Histological evaluation of the atherosclerotic area of thoracic aortas as indicated by the surface involvement, mean lesion area (percent of area occupied by lesion), and I:M ratio (*Upper*) and representative photographs (*Lower*). (*Upper Left*) Surface involvement of the atherosclerotic area of thoracic aortas from rabbits (group I, normal chow; group II, 0.5% HCD and placebo pellet; group III, 0.5% HCD and E_2 pellet). ***, $P < 0.001$. (*Upper Center*) Area occupied by the atherosclerotic area of thoracic aortas from the three groups of rabbits. ***, $P < 0.001$. (*Upper Right*) I:M ratio of thoracic aortas from three groups of rabbits. ***, $P < 0.001$. (*Lower*) Representative photographs of thoracic aortas from rabbits. (*Lower Left*) Group I. (*Lower Center*) Group II. (*Lower Right*) Group III. (Original magnification, ×40.)

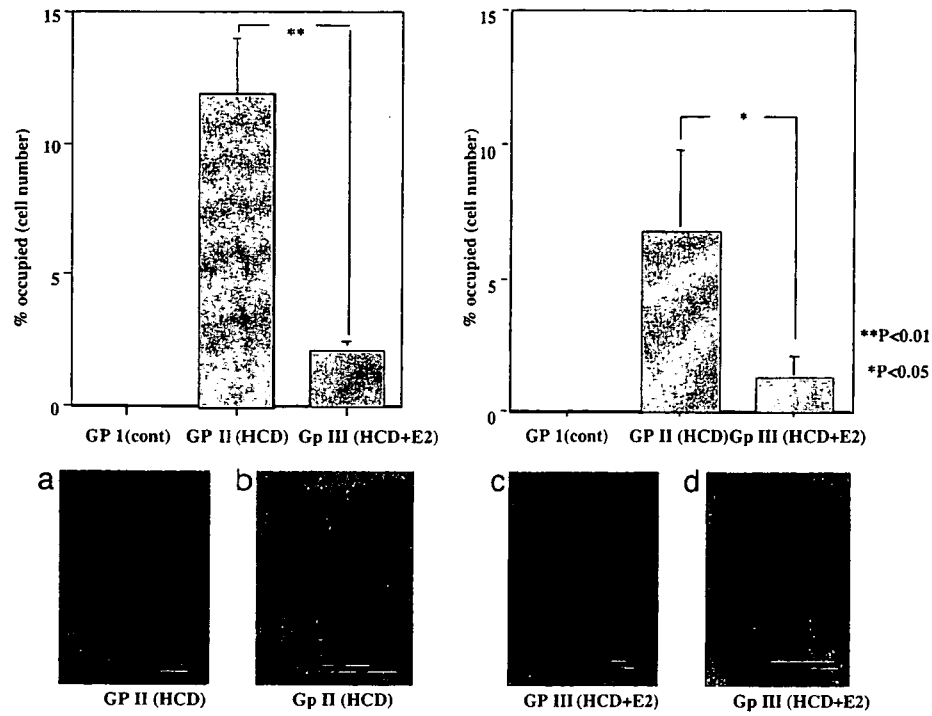

Fig. 2. Immunohistochemical analysis with the anti-BrdU-positive, neointimal area of the thoracic aortas of New Zealand White (NZW) rabbits from the atherosclerotic group. (*Upper Left*) Atherosclerotic area. (*Upper Right*) Nonatherosclerotic area. In group III, the number of positive cells was significantly decreased compared with group II. *, $P < 0.05$; **, $P < 0.01$. (*Lower*) Representative photographs of the thoracic aortas from rabbits. (*Lower Left a* and *b*) Group II. (*Lower Right c* and *d*) Group III. [Original magnification, ×100 (*a* and *c*); ×400 (*b* and *d*). Scale bars, 100 μm.] The arrows point to anti-BrdU-positive cells.

($ONOO^-$) in the lesion area, which is toxic to the endothelial cells (22, 23). L-Arginine is the precursor to NO (13, 22), and therefore, L-arginine should either have a protective or deleterious role with regard to atherosclerosis depending on whether endothelial NOS (eNOS) or iNOS is expressed.

Chronic administration of L-arginine to HCD rabbits enhanced the synthesis of EDNO and reduced or reversed the progression of intimal lesions (24, 25). In these studies, the animals initially received 0.5% a HCD for only 10 weeks. Subsequently, L-arginine or vehicle was administered for an additional 13 weeks while the HCD was continued. We demonstrated that administration of L-arginine led to regression of the preexisting intimal lesions. More recently, it has been demonstrated in humans that oral supplementation with large amounts (6–21 g/day) of L-arginine, the precursor to EDNO (26), as well as intake of a nutrient bar enriched with L-arginine (26), designed to enhance EDNO, improved flow-

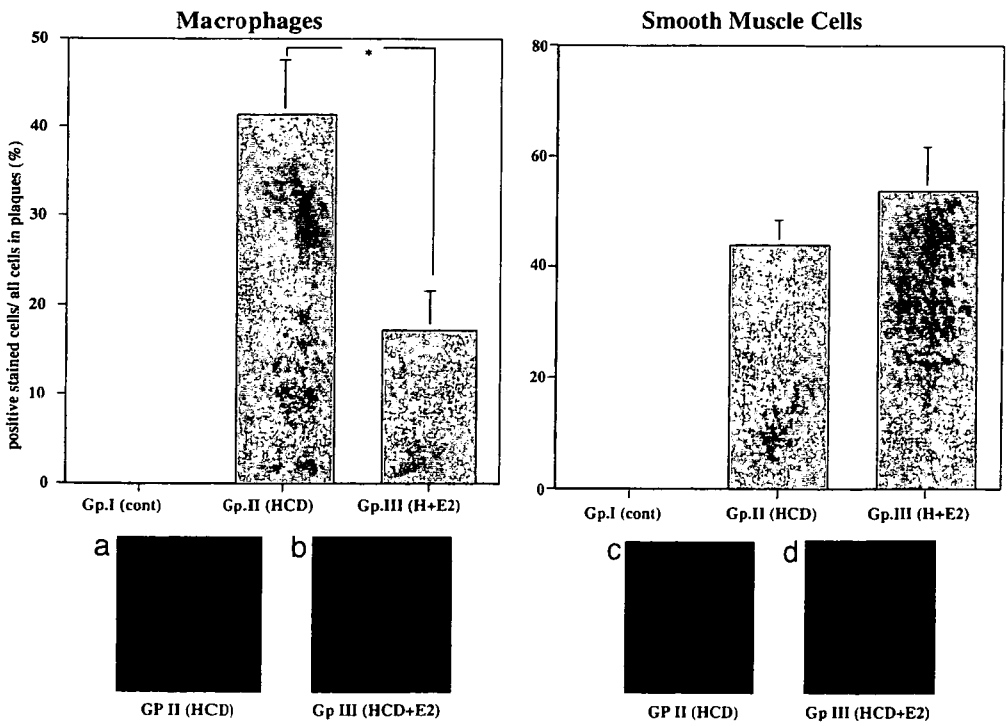

Fig. 3. Immunohistochemical analysis with anti-macrophage and anti-smooth muscle cell α-actin monoclonal antibody of thoracic aortas of NZW rabbits from the atherosclerotic group. (*Upper Left*) Area occupied by macrophages in subintimal atherosclerotic plaque of thoracic aortas of groups I, II, and III rabbits. *, $P < 0.05$. (*Upper Right*) Area occupied by smooth muscle cell α-actin in subintimal atherosclerotic plaque of thoracic aortas of groups I, II, and III rabbits. (*Lower*) Representative photographs of thoracic aortas from rabbits. (*Lower Left a*) Group II. (*Lower Left b*) Group III. Macrophages were detected in both the core and fibrous cap in a section stained with a monoclonal antibody against rabbit macrophages. (Original magnification, ×100.) (*Lower Right c*) Group II. (*Lower Right d*) Group III. Smooth muscle cell α-actins were detected in the media and subintimal atherosclerotic plaque area of thoracic aortas of groups II and III rabbits. No significant difference between groups II and III was observed. (Original magnification, ×100. Scale bars, 25 μm.)

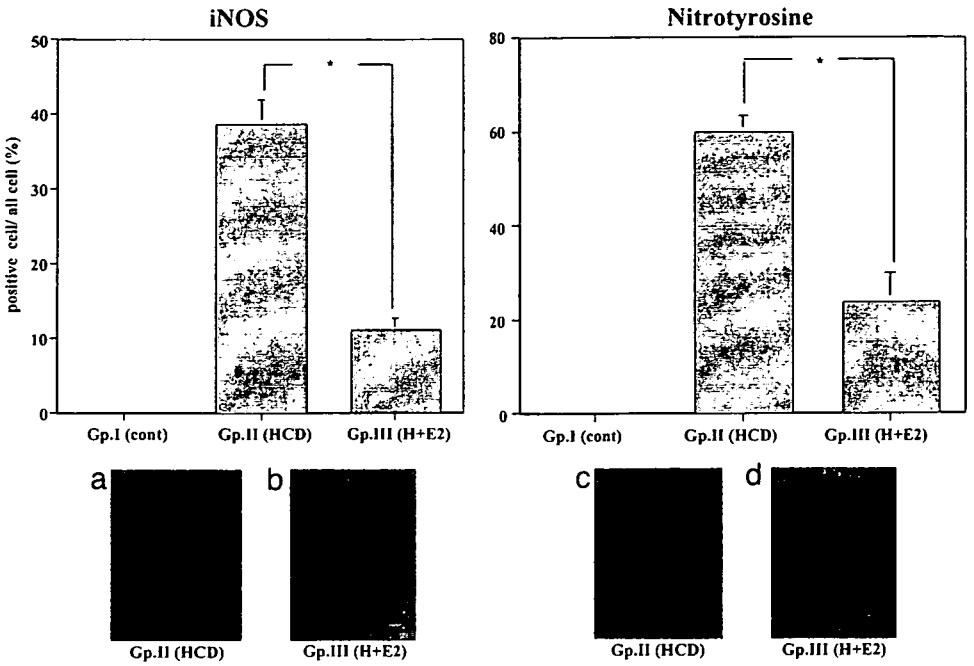

Fig. 4. Immunohistochemical analysis with anti-iNOS and anti-nitrotyrosine monoclonal antibody of thoracic aortas of NZW rabbits from atherosclerotic group. (*Upper*) Area occupied by iNOS-positive cells (*Left*) and nitrotyrosine-positive cells (*Right*) in subintimal atherosclerotic plaque of thoracic aortas of rabbits. *, $P < 0.05$. (*Lower*) Representative photographs of thoracic aortas from rabbits. (*Lower Left a*) Group II. (*Lower Left b*) Group III. iNOS was detected adjacent to the necrotic core. (Original magnification, ×100. Scale bars, 25 μm.) (*Lower Right c*) Group II. (*Lower Right d*) Group III. Nitrotyrosine was detected in the subintimal atherosclerotic plaque area of the thoracic aortas. (Original magnification, ×100. Scale bars, 25 μm.)

mediated endothelium-dependent vasodilation in hypercholesterolemic individuals. Although L-arginine is the precursor to EDNO, there are other actions of L-arginine independent of NO production that may also have been the cause of improved flow-mediated endothelium-dependent vasodilation in hypercholesterolemic individuals.

In our studies, when the animals were fed a HCD for a much longer period, i.e., 15 weeks, there was an 8-fold increase in arginase I and II expression along with the expression of iNOS compared with animals fed normal chow. Arginase II expression is also up-regulated in rheumatoid arthritis (27, 28) in a way similar to that seen in our studies on atherosclerosis. It has been suggested that in cells where both arginase II and iNOS activity occurs, there is a reciprocal regulation, suggesting that agents that induce arginase II could down-regulate the levels of NO and divert L-arginine metabolism toward cell proliferation and/or tissue regeneration (28). Similarly, the concomitant expression of iNOS as well as arginase can markedly reduce basal NO synthesis in endothelial cells because of a decrease in the intracellular arginine content in these cells (28). In our study, increased arginase expression most likely led to increased cellular proliferation and reduced endothelial cell NO formation, and thus, the protective effects that the endothelial-derived NO has on the atherosclerotic process were absent.

Similar to our results in rabbits, an increase in arginase II enzyme activity in apolipoprotein $E^{-/-}$ mice has been found by another group of investigators (29). Furthermore, in these mice, L-arginine induced vasoconstriction in segments of mouse aorta. The contraction induced by L-arginine was much more pronounced in atherosclerotic apolipoprotein $E^{-/-}$ mice compared with control animals. These contractions were converted to relaxations in the presence of an arginase inhibitor (29). On the basis of our results and those of others (29), it is possible to speculate that after expression of arginase in atherosclerotic lesions, L-arginine administration may have a deleterious effect on the atherosclerotic process rather than the beneficial effect obtained in the absence of arginase expression.

We (1) and others (30, 31) have previously demonstrated that estrogens may attenuate atherogenesis by increasing NO production by endothelial cells (4–6). It has also been shown that estrogens can decrease the expression of TNFα-stimulated VCAM-1 expression by a NO-mediated mechanism (8, 32). This action of estrogens in increasing eNOS would allow L-arginine to be directed to the L-arginine–NO pathway, leading to attenuation of initiation of atherosclerosis. Results from this study further indicate that estrogens attenuate the expression of arginase II, which would blunt the L-arginine–polyamine pathway and prevent cell proliferation. This action would then allow L-arginine to be directed to the L-arginine–NO pathway, thereby offering another potential mechanism by which estrogen attenuates atherogenesis. E_2 treatment did not affect the expression of arginosuccinate synthetase, indicating that estrogens do not affect the availability of L-arginine from citrulline.

The precise mechanism(s) by which E_2 attenuates the expression of arginase is not known, and it was not assessed in our study. E_2 can have varied effects on arginase activity. E_2 benzoate evoked a 3-fold elevation in the arginase activity of the dorsal prostate, in contrast to the decreased arginase activity in the ventral prostate after E_2 administration (33). However, the type of arginase was not indicated in the study. Further studies are needed to assess the mechanism(s) by which estrogen modulates arginase II activity.

In conclusion, the results from our studies indicate that after prolonged feeding of a HCD, arginase expression is increased in hyperlipidemic rabbits in the atherosclerotic lesion area, whereas the expression of arginase II is significantly reduced by simultaneous administration of E_2. The increase in arginase II activity may account for the associated cellular proliferation by diverting L-arginine to form polyamines, whereas E_2, by inhibiting arginase II expression, attenuates atherosclerosis by providing a substrate for eNOS to synthesize NO, which is atheroprotective. It is also possible that L-arginine may be beneficial in the early stages of atherosclerosis before the expression of arginase II, whereas it may have deleterious effects if administered later on when significant lesions have already developed, and arginase II, expressed as L-arginine is administered, would then lead to cell proliferation (34). Further studies are needed to assess whether the timing of L-arginine administration may be the key determining factor as to whether it would be atheroprotective or lead to deleterious effects.

Materials and Methods

Chemicals and Solutions. Monoclonal antibodies against smooth muscle cell α-actin (HHF35) and monocytes/macrophages

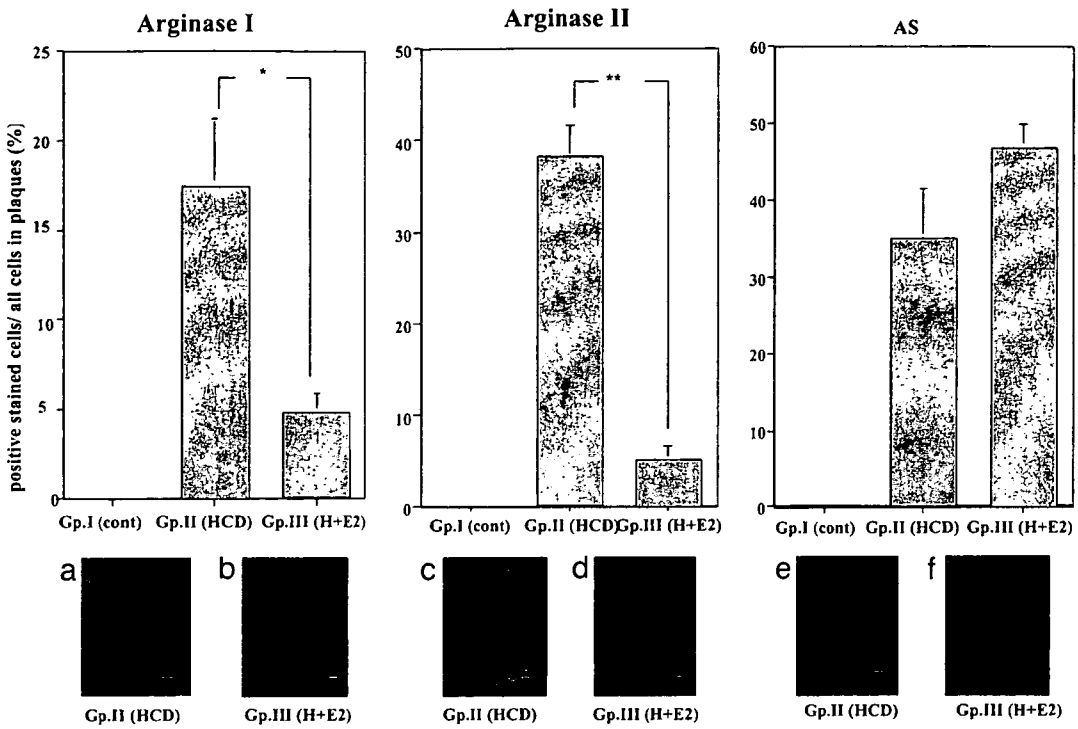

Fig. 5. Distribution of arginase I and II and arginosuccinate synthase in atherosclerotic aortas. (*Upper*) Immunohistochemical analysis with anti-arginase I and II and arginosuccinate synthetase (AS) antibody of thoracic aortas of NZW rabbits from the different groups. *, $P < 0.05$ and **, $P < 0.01$. (*Lower*) Immunohistochemical analysis with anti-arginase I [(*a*) group II and (*b*) group III)] and anti-arginase II [(*c*) group II and (*d*) group III] monoclonal antibodies and the anti-arginosuccinate synthetase monoclonal antibody [(*e*) group II and (*f*) group III] of the thoracic aortas of NZW rabbits from the atherosclerotic group. (Original magnification, ×100. Scale bars, 25 μm.)

(RAM11) were purchased from Enzo Diagnostics and DAKO, respectively. Antibodies against iNOS, nitrotyrosine, arginase I, and arginosuccinate synthase were all purchased from Transduction Laboratories (Lexington, KY). Antibodies against arginase II were gifts from M. Gotoh and M. Mori (Kumamoto University School of Medicine) (35). BrdU, a thymidine analog that labels newly synthesized DNA, was purchased from Sigma. Peroxidase-conjugated anti-BrdU was purchased from DAKO.

Animals. Female NZW rabbits weighing 3–3.5 kg were used. All animals were fed regular chow for 2 weeks. They were housed in individual cages and underwent oopherectomy with placement of either placebo or E_2 pellets (10 mg, 60-day release). Eight weeks after the placement of pellets, new E_2 or placebo pellets were placed, and the animals were killed at 15 weeks.

The method for oopherectomy was similar to that described in ref. 1. For diets, the animals were divided into three groups. Group I received normal chow. Group II received a 0.5% HCD for 15 weeks and a placebo pellet. Group III received a 0.5% HCD and an E_2 pellet. The pellets were replaced by new pellets at the end of 8 weeks to ensure that the animals had E_2 released from the pellets for the full duration of the study. At the end of the 15-week feeding period, animals were anesthetized. Anesthesia was initiated by with acepromazine (0.3 mg/kg i.v.) and ketamine (10 mg/kg i.v.) followed by isoflurane [2% (vol/vol) by inhalation]. For postoperative analgesia, the animals were also administered buprenorphine (0.03 mg/kg i.m.) twice each day for 2–3 days.

The animal protocol was formally approved by the Animal Research Committee of the University of California at Los Angeles and by the Nagoya University Graduate School of Medicine.

Determinations of Plasma Lipids and Estradiol Concentration. To assess lipid and E_2 levels, an aliquot of blood from each animal was collected into tubes containing EDTA. The total cholesterol and triglyceride levels were measured by enzymatic assays as described in refs. 36 and 37. The HDL-cholesterol was determined after precipitation with phosphotungstate/$MgCl_2$ (37). The plasma concentration of E_2 was examined as described in ref. 38.

Histological Evaluation of Aortic Atherosclerosis. After 15 weeks of feeding, the animals were euthanized with pentobarbital (100 mg/kg i.v.). The descending thoracic aorta was quickly dissected out, and adjoining segments were either snap frozen in liquid nitrogen or preserved in formaldehyde. Cross sections of the descending thoracic aorta were stained with hematoxylin/eosin (SRL, Tokyo) to examine the endothelial lining and with van Gieson's elastic stain (SRL) to determine the thickness of the intima. Morphometric analysis was performed as described by Weiner *et al.* (39). Briefly, the complete section of each block was projected onto a vertical surface with a projecting microscope. Six samples from each rabbit aorta were analyzed with the objective lens. The contours of the lumen and the internal elastic lamina were traced, and the tracings were digitized with a graphics tablet. The surface involvement by atherosclerotic lesion was calculated by dividing the lesion circumference by the circumference of the internal elastic lamina. The circumferences of the lesion area and normal area were defined as circumferences of each part of the internal elastic lamina. The area occupied by atherosclerotic lesions was defined as the percent area bounded by the lumen and the internal elastic lamina. The control luminal area was calculated from the perimeter of the internal elastic lamina as described in ref. 40. The I:M ratio was calculated (41). Data were transferred to a minicomputer (Macintosh iMac; Apple, San Jose, CA) for further analysis.

BrdU Incorporation and Immunohistochemistry. BrdU was administered at 18 h (100 mg/kg s.c. and 30 mg/kg i.v.) and 12 h (30 mg/kg

i.v.) before harvest. BrdU labeling was carried out on 5-μm frozen sections (42). Background staining was blocked by incubation with 5% normal goat serum for 30 min, and then the sections were incubated with a monoclonal antibody to BrdU (1:200; DAKO) at 4°C overnight followed by an alkaline phosphatase-conjugated goat anti-mouse IgG (1:200; Jackson ImmunoResearch) at room temperature for 1 h (42). The BrdU-labeled endothelial and smooth muscle cell nuclei, identified as elongated oval regions of immunoreactivity, were counted in five sequential sections from the thoracic artery of each rabbit. The percentage of BrdU-labeled endothelial cells was expressed as the ratio of vessels having BrdU-labeled endothelial and intimal smooth muscle cells to the total number of endothelial cells and intimal smooth muscle cell profiles per cross section (43).

Immunohistochemical Analysis. Cross sections of the descending thoracic aorta were deparaffinized with xylene and dehydrated with graded alcohol (17). The specimens were preincubated for 30 min with methanol containing 0.3% hydrogen peroxide and washed for 10 min with PBS. The specimens were permeabilized with 0.1% Triton X-100 in PBS for 20 min and washed with PBS. They were then blocked with normal horse serum for 1 h and incubated with primary monoclonal antibody (for smooth muscle cell α-actin, monocytes/macrophages, iNOS, nitrotyrosine, arginase I, arginase II, and arginosuccinate synthetase) diluted in PBS for 60 min, and washed again with PBS. Negative controls included substitution of irrelevant antibodies for the primary antiserum/antibody. A biotinylated rabbit anti-mouse IgG (1:500 dilution) was incubated for 30 min and washed with PBS followed by avidin–biotin peroxidase complex reagent (ABC kit; Vector Laboratories) incubation for 30 min. The result was a brown peroxidase reaction product of diaminobenzidine. The cell nuclei were counterstained with methyl green (17). In the negative controls, either PBS or irrelevant antibodies replaced the primary antiserum. Each field was scored for the number of positive stained cells against each antibody in plaques on slides, and all cells in the plaques were calculated and analyzed statistically as described in ref. 17. From each section, five digital images were obtained with a 3CCD color camera (JVC; Victor Company of Japan, Tokyo) and Leitz microscope. The intensity and distribution patterns of the staining reaction were evaluated by two blinded, independent observers (T.E. and T.M.) using a semiquantitative staining score (graded as 0 = none, 1 = weak, 2 = moderate, and 3 = strong staining). The cells whose mean scores were higher than 2 were recognized as positive staining cells.

Data Analysis. The results were expressed as the mean ± SEM. The SPSS/PC 6.01 software package (SPSS, Chicago) was used for collection, processing, and statistical analysis of all data. Statistical analysis was performed with the nonparametrical Wilcoxon signed-rank test for comparison of the means. The Spearman ρ coefficient was used to assess any significant correlations between the analyzed substances within the distinct groups. $P < 0.05$ was considered statistically significant.

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

Toshio Hayashi*†, Hisako Matsui-Hirai*, Asaka Miyazaki-Akita*, Akiko Fukatsu*, Jun Funami*, Qun-Fang Ding*, Sumitra Kamalanathan*, Yuichi Hattori‡, Louis J. Ignarro†§, and Akihisa Iguchi*

*Department of Geriatrics, Nagoya University Graduate School of Medicine, Tsuruma-cho 65, Showa-ku, Nagoya 466-8550, Japan; ‡Department of Pharmacology, School of Medicine, University of Toyama, Sugitani 2630, Toyama 930-0194, Japan; and §Department of Molecular and Medical Pharmacology, Center for the Health Sciences, David Geffen School of Medicine, University of California, 650 Charles E. Young Drive-South, Los Angeles, CA 90095-1735

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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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The authors declare no conflict of interest.

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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.

†To whom correspondence may be addressed. E-mail: hayashi@med.nagoya-u.ac.jp or lignarro@mednet.ucla.edu.

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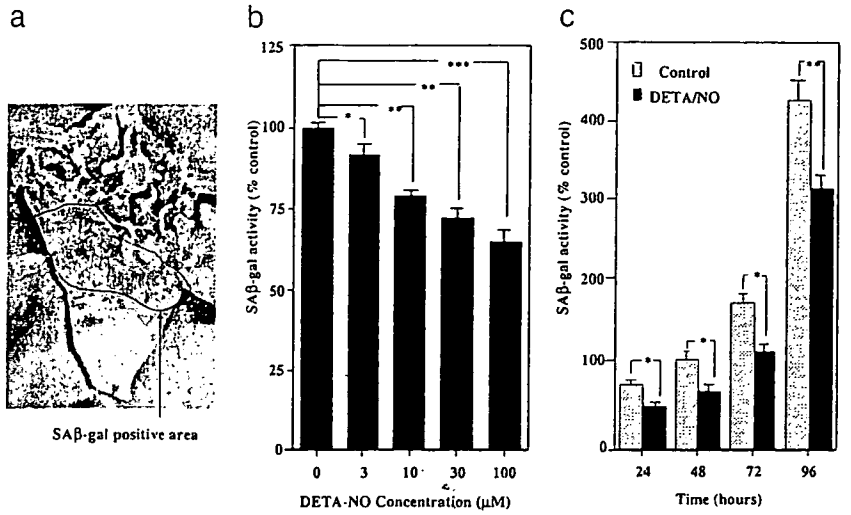

Fig. 1. SA-β-gal activity as cellular senescence. (*a*) SA-β-gal-positive staining was observed in atherosclerotic lesions of the intimal side of human thoracic aorta, which was obtained by autopsy. No staining was detected in the nonatherosclerotic area and advanced atherosclerotic area, including the 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-

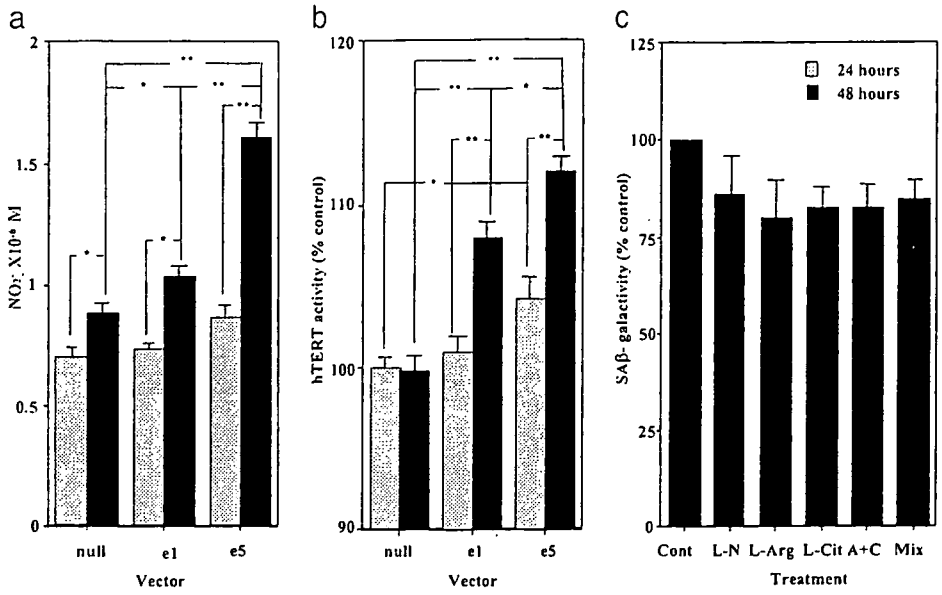

Fig. 2. Influence of eNOS modulation on cellular senescence. (*a*) The effect of transfection with eNOS on nitrite production by HEK 293 cells. Transfection with eNOS into cells was performed; e5 included five times the amount of eNOS vector compared with e1. The nitrite concentrations in the medium 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).

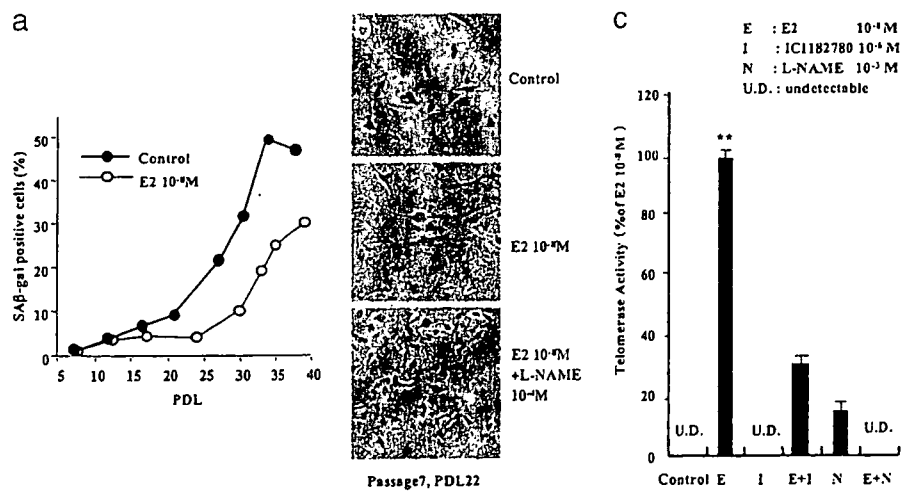

Fig. 3. Effect of estrogen on cellular senescence. (*a*) The relative levels of SA-β-gal-positive staining cells in different PDL when HUVECs were untreated and treated with 10^{-8} M E2 for 24 h. Positive staining cells were evaluated by FACscan. (*b*) Representative photographs of SA-β-gal staining 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.