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Original Article

Low Testosterone Level Is an Independent Determinant of Endothelial Dysfunction in Men

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We investigated whether a low plasma testosterone level is related to endothelial dysfunction in men with coronary risk factors. One hundred and eighty-seven consecutive male outpatients (mean age \pm SD: 47 \pm 15 years) who underwent measurement of flow-mediated vasodilation (FMD) of the brachial artery using ultrasonography were enrolled. The relationship between plasma hormones and FMD was analyzed. Total and free testosterone and dehydroepiandrosterone-sulfate (DHEA-S) were significantly correlated with %FMD ($r=0.261$, 0.354 and 0.295 , respectively; $p<0.001$), while estradiol and cortisol were not. %FMD in the highest quartile of free testosterone was 1.7-fold higher than that in the lowest quartile. Multiple regression analysis revealed that total and free testosterone were related to %FMD independent of age, body mass index, hypertension, hyperlipidemia, diabetes mellitus and smoking ($\beta=0.198$ and 0.247 , respectively; $p<0.01$), and were independent of age, body mass index, systolic blood pressure, total cholesterol, high-density lipoprotein cholesterol, fasting plasma glucose, smoking and nitroglycerin-induced dilation ($\beta=0.196$ and 0.227 , respectively; $p<0.01$). DHEA-S was not significantly related to %FMD in multivariate analysis. In conclusion, a low plasma testosterone level was associated with endothelial dysfunction in men independent of other risk factors, suggesting a protective effect of endogenous testosterone on the endothelium. (*Hypertens Res* 2007; 30: 1029–1034)

Key Words: androgen, sex hormone, vasodilation, endothelium, risk factor

Introduction

Androgen levels decline with advancing age in men (1, 2). Decreases in hormonal activity have been considered physiologic, but are often associated with the pathological process of aging, which includes such effects as erectile dysfunction, osteopenia, sarcopenia, depressed mood and cognitive impairment (1, 3). Also, not all but many recent observational studies have shown that a low plasma testosterone level is associated with advanced atherosclerosis (4, 5), and a higher incidence of cardiovascular disease (6), suggesting that

endogenous testosterone may protect against the development of cardiovascular disease in men. The inverse correlations between testosterone and coronary risk factors such as obesity (4, 7) and high blood pressure (8, 9), plasma lipids (4, 7, 8), and plasma glucose (7, 10) may provide insight into the mechanism of the effect of testosterone on cardiovascular disease. Furthermore, anti-ischemic (11, 12) and endothelium-dependent vasodilating (13, 14) effects of testosterone supplementation have been reported. These findings led us to hypothesize that men with a low plasma testosterone level would have impaired vasomotor function.

To test this hypothesis, we conducted a cross-sectional sur-

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Table 1. Characteristics of Study Subjects (N=187)

Age (years)	47±15	[20–79]
Body mass index (kg/m ²)	25.7±4.6	[19.0–47.2]
Risk factors		
Hypertension (n (%))	69 (37)	
Hyperlipidemia (n (%))	82 (44)	
Diabetes mellitus (n (%))	37 (20)	
Current smoker (n (%))	87 (46)	
Hemodynamic and vascular measurements		
Systolic blood pressure (mmHg)	126±16	[98–185]
Diastolic blood pressure (mmHg)	76±13	[53–128]
%FMD	5.4±3.7	[0.0–20.2]
%NTG	13.6±5.0	[1.6–27.2]
Carotid IMT (mm)	0.96±0.36	[0.3–1.4]
Blood chemistry and hormones		
Total cholesterol (mmol/L)	5.23±1.00	[3.06–8.70]
HDL cholesterol (mmol/L)	1.28±0.42	[0.67–3.42]
Triglycerides (mmol/L)	1.73±1.40	[0.36–9.94]
Fasting plasma glucose (mmol/L)	5.78±1.10	[4.21–12.54]
Hemoglobin A1c (%)	5.5±1.3	[3.9–10.4]
Total testosterone (nmol/L)	17.4±5.7	[4.6–33.6]
Free testosterone (pmol/L)	61.0±22.5	[18.7–166.8]
DHEA-S (µmol/L)	4.78±2.51	[0.56–11.96]
Estradiol (pmol/L)	120±31	[50–216]
Cortisol (nmol/L)	375±133	[83–742]

Values except risk factors are expressed as the mean±SD [range]. %FMD, percent flow-mediated dilation of brachial artery; %NTG, percent nitroglycerin-induced dilation of brachial artery; IMT, intima-media thickness of common carotid artery; HDL, high-density lipoprotein; DHEA-S, dehydroepiandrosterone-sulfate.

vey of 187 men by examining flow-mediated dilation of the brachial artery (%FMD) and plasma sex hormones, and showed that a low testosterone level was associated with endothelial dysfunction.

Methods

Subjects

One hundred and eighty-seven consecutive male outpatients of our department, who underwent examination of vasomotor function of the brachial artery and intima-media thickness (IMT) of the carotid artery in our department, were enrolled. The subjects were referred to our department to check for cardiovascular disease or risks. All of them were in chronic stable condition. A history was taken, and physical examination and laboratory tests were performed in all subjects. Subjects with a history of cardiovascular disease, including stroke, coronary heart disease, congestive heart failure or peripheral arterial disease, malignancy, overt endocrine disease or use of

Table 2. Pearson's Correlation Coefficients between Age, Vascular Measurements and Plasma Hormones

	Age	%FMD	Carotid IMT
Total testosterone	0.057	0.261 [†]	0.003
Free testosterone	-0.288 [†]	0.354 [†]	-0.259 [†]
DHEA-S	-0.604 [†]	0.295 [†]	-0.356 [†]
Estradiol	0.155*	-0.062	0.234*
Cortisol	-0.047	0.081	-0.082

%FMD, percent flow-mediated dilation of brachial artery; IMT, intima-media thickness of common carotid artery; DHEA, dehydroepiandrosterone-sulfate. [†]*p*<0.001, **p*<0.05

steroid hormones were excluded, because these conditions may have a serious influence on both plasma sex hormones and endothelial function. Subjects who showed a carotid IMT >1.5 mm were also excluded, because such subjects might have significant subclinical atherosclerosis. The characteristics of the study subjects are shown in Table 1.

Seventy-six percent of the subjects had one or more of the classical coronary risk factors, such as hypertension, hyperlipidemia, diabetes mellitus or current smoking. Hypertension, hyperlipidemia and diabetes mellitus were defined according to the diagnostic criteria (15–17) or if the subjects were taking any medications for these diseases. Ninety-five percent of the hypertensive subjects were treated: 77% with calcium antagonists, 18% with angiotensin-converting enzyme inhibitors, 12% with diuretics and 7% with β-blockers. Seventy-seven percent of the hyperlipidemic subjects were treated with statins, and 81% of the diabetic subjects were treated with oral hypoglycemic agents. None of the study subjects were taking nitrates. Each subject gave written informed consent before enrollment in this study. The study protocol was approved by the ethics committee of the Graduate School of Medicine, The University of Tokyo.

Vascular Measurement

Vasomotor function of the brachial artery was evaluated using an ultrasound machine according to the method described previously (18). Briefly, endothelium-dependent %FMD was measured as the maximal percent change of the vessel diameter after reactive hyperemia. Subsequently, endothelium-independent nitroglycerin-induced vasodilation (%NTG) was measured as the maximal percent change of the vessel diameter after sublingual administration of nitroglycerin spray (0.3 mg; Toa Eiyo Co., Tokyo, Japan). Carotid IMT was evaluated using an ultrasound machine as described previously (18). The same examiner performed the measurements of FMD throughout this study. The subjects were examined in the morning after a 14-h overnight fast, and reclined on the bed for 15 min in a quiet, temperature-controlled (22–24°C) room before measurements.

Table 3. Age-Adjusted Regression Coefficients between Vascular Measurements and Plasma Hormones

	%FMD	Carotid IMT
Total testosterone	0.282 [†]	-0.050
Free testosterone	0.324 [†]	-0.090
DHEA-S	0.262 [†]	0.036
Estradiol	-0.005	0.139
Cortisol	0.071	-0.053

Standardized regression coefficients by multiple regression analyses with %FMD or carotid IMT as a dependent variable and age and each of the hormones as independent variables are shown. [†] $p < 0.001$. %FMD, percent flow-mediated dilation of brachial artery; IMT, intima-media thickness of common carotid artery; DHEA-S, dehydroepiandrosterone-sulfate.

Plasma Hormones

Blood sampling was performed in the morning of the vascular measurement after a 14-h overnight fast, to measure plasma hormones and other chemical parameters. Plasma total and free testosterone, dehydroepiandrosterone-sulfate (DHEA-S), estradiol and cortisol concentrations were determined using sensitive radioimmunoassays by a commercial laboratory (SRL Inc., Tokyo, Japan). The intra-assay coefficients of variation for these measurements were less than 5%.

Data Analysis

The values are expressed as the means \pm SD in the text. Pearson's simple correlation coefficients between age, vascular measurements and plasma hormones were determined. Standardized regression coefficients from multiple regression analysis of vascular measurements in relation to age, coronary risk factors and plasma hormones were determined. Differences between the groups were analyzed using one-factor ANOVA, followed by Newman-Keuls' test. A value of $p < 0.05$ was considered statistically significant.

Results

Changes in Plasma Hormones and Vascular Measurements According to Age and Coronary Risk Factors

Plasma levels of free testosterone and DHEA-S declined with age, while those of total testosterone and cortisol did not significantly change (Table 2). Conversely, estradiol showed a weak but significant positive correlation with age. %FMD decreased ($r = -0.365$, $p < 0.001$) and carotid IMT increased ($r = 0.546$, $p < 0.001$) with advancing age.

The subjects with hypertension, hyperlipidemia or diabetes mellitus showed impaired %FMD compared to those without these diseases (hypertension, 3.8 ± 2.4 vs. 6.3 ± 4.0 ; hyperlipi-

Table 4. Regression Coefficients between %FMD and Plasma Hormones Adjusted for Coronary Risk Factors

	Model 1	Model 2	Model 3	Model 4
Total testosterone	0.198 [†]	0.210 [†]	0.216 [†]	0.196 [†]
Free testosterone	0.247 [†]	0.266 [†]	0.255 [†]	0.227 [†]
DHEA-S	0.091	0.150	0.175	0.170
Estradiol	0.033	0.024	0.061	-0.001
Cortisol	0.012	-0.001	-0.004	-0.073

Standardized regression coefficients by multiple regression analyses with %FMD as a dependent variable and coronary risk factors (covariates used in each analysis are listed below) and each of the hormones as independent variables are shown. [†] $p < 0.01$. Model 1: age, body mass index, hypertension, hyperlipidemia, diabetes mellitus, and current smoking. Model 2: age, body mass index, systolic blood pressure, total cholesterol, HDL cholesterol, fasting plasma glucose and current smoking. Model 3: Model 2 plus carotid intima-media thickness. Model 4: Model 2 plus percent nitroglycerin-induced dilation of brachial artery. %FMD, percent flow-mediated dilation of brachial artery; DHEA-S, dehydroepiandrosterone-sulfate; HDL, high-density lipoprotein.

demia, 4.4 ± 3.4 vs. 6.1 ± 3.7 ; diabetes mellitus, 3.1 ± 2.4 vs. 5.9 ± 3.7 ; $p < 0.01$ for each). %FMD in the patients taking anti-hypertensive agents, statins or hypoglycemic agents was comparable to or smaller than that in the patients without medical agents (hypertension, 3.8 ± 2.5 vs. 4.6 ± 1.3 , n.s.; hyperlipidemia, 3.9 ± 2.7 vs. 6.3 ± 4.7 , $p < 0.05$; diabetes mellitus, 2.6 ± 2.0 vs. 4.9 ± 3.0 , $p < 0.05$), suggesting that the favorable effects of medical treatment on endothelial function, if present, might have been lost in patients with a long history of coronary risk factors. In contrast, no significant associations were found between any of the plasma hormones and either coronary risk factors or medications.

Relationship between Plasma Hormones and Vascular Measurements

First, simple correlation coefficients between plasma hormones and vascular measurements were determined. As shown in Table 2, %FMD was positively correlated with total testosterone, free testosterone and DHEA-S. Carotid IMT was negatively correlated with free testosterone and DHEA-S, and was positively correlated with estradiol. There was no significant correlation between cortisol and vascular measurements.

Next, age-adjusted regression coefficients were determined, because age was correlated with both hormones and vascular measurements, as mentioned above. The results showed that none of the hormones was significantly related to carotid IMT, and estradiol was not related to either of the vascular measurements (Table 3). In contrast, total testosterone, free testosterone and DHEA-S were significantly related to %FMD, independent of age.

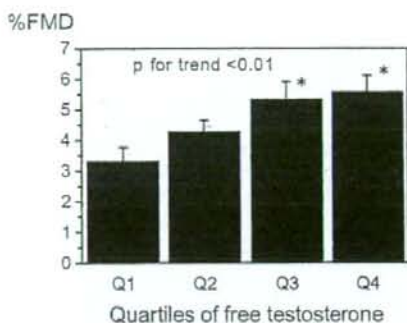


Fig. 1. Percent flow-mediated dilation of the brachial artery (%FMD) according to quartiles of plasma free testosterone. Values are expressed as the means \pm SEM. * $p < 0.05$ vs. Q1.

Finally, multiple regression analyses were performed to exclude the influence of coronary risk factors on the relationship between hormones and %FMD. As shown in Table 4, total and free testosterone were related to %FMD, independent of age, body mass index, hypertension, hyperlipidemia, diabetes mellitus and current smoking (Model 1), and were independent of age, body mass index, systolic blood pressure, total cholesterol, high-density lipoprotein cholesterol, fasting plasma glucose and current smoking (Model 2). Furthermore, the relationship between total and free testosterone and %FMD was significant after addition of carotid IMT to the model (Model 3), suggesting that the relationship was not attributable to the effect of testosterone on the development of subclinical atherosclerosis. Also, the statistical result was unchanged after %NTG of the brachial artery to the model (Model 4), indicating that testosterone is related to endothelial function independent of arterial compliance. DHEA-S, estradiol and cortisol were not significantly related to %FMD in similar multivariate analyses (Table 4). As shown in Fig. 1, %FMD showed a stepwise increment according to quartiles of free testosterone, and %FMD in the highest quartile of free testosterone was 1.7-fold higher than that in the lowest quartile.

Discussion

In this cross-sectional study, both total and free testosterone levels were positively correlated with %FMD, a surrogate marker of clinical atherosclerosis that reflects endothelial function (19, 20). Adjustment for potential confounders such as age, coronary risk factors and %NTG had little influence on the results. These results suggest that testosterone level is an independent determinant of endothelial vasomotor function in men.

A number of studies have shown an association between low testosterone level and cardiovascular disease (6, 21, 22)

or risk factors (4, 5, 7–10), but others have shown no association (23, 24) and have reported that a low level of DHEA-S (6, 25, 26) or estradiol (24) is associated with cardiovascular disease. Also, a positive association between the cortisol:testosterone ratio and the incidence of coronary heart disease has been reported (27). Therefore, we added DHEA-S, estradiol and cortisol to the present analysis. However, our results showed that estradiol and cortisol were not related to %FMD. The ratio of cortisol to total testosterone ($r = -0.162$, $p < 0.05$) and that of cortisol to free testosterone ($r = -0.194$, $p < 0.05$) were significantly related to %FMD in simple regression analyses, but statistical significance was not found in multiple regression analyses (data not shown). DHEA-S was positively correlated with %FMD, but the statistical significance disappeared after adjustment for coronary risk factors. Taking these results together, testosterone was the only steroid hormone that was significantly related to %FMD in the multivariate analyses.

Several studies (4, 8) have assayed bioavailable testosterone, non-globulin-bound or free plus albumin-bound testosterone (28), whereas others have measured total (5, 7, 21–24) or free (6, 9, 10, 21, 22) testosterone in the plasma. These differences in assays might influence the results. In this study, we did not analyze bioavailable testosterone, because a direct assay is not available in Japan, and we did not measure the levels of sex hormone binding globulin and albumin, which are needed to estimate the value of bioavailable testosterone. However, both total and free testosterone levels were positively associated with %FMD, although free testosterone showed a stronger impact throughout the statistical analyses. Accordingly, we believe that the assays do not affect our conclusion that testosterone level is an independent determinant of endothelial vasomotor function in men.

The mechanisms by which testosterone regulates vasomotor function should be discussed. Short-term intracoronary administration of testosterone has been reported to elicit vasodilation and increased blood flow in men (29) and in animals (30). A supra-physiologic dose of testosterone induced relaxation of isolated blood vessels *in vitro* (31). These direct vasodilator actions of testosterone observed at higher concentrations seem to be endothelium- and androgen receptor-independent, and to be mediated *via* membrane ion channels of smooth muscle cells (31). On the other hand, both acute (13) and chronic (14) supplementation of testosterone in men enhanced %FMD without affecting the basal diameter of the brachial artery, suggesting an endothelium-dependent vasodilator action of testosterone. We also showed that the relation between %FMD and testosterone was not altered after adjustment for %NTG, further supporting the action of testosterone on endothelial function. Although the existence of androgen receptors in endothelial cells is recognized (32), the cellular and molecular mechanism linking testosterone to endothelial release of vasoactive agents such as nitric oxide is uncertain. We recently found that ginsenoside Rb1 stimulated nitric oxide production and endothelial nitric oxide synthase activ-

ity via androgen receptors in human aortic endothelial cells (33). To date, however, there has been no experimental evidence showing a direct effect of testosterone on endothelial nitric oxide synthesis. Another less likely hypothesis is that estradiol converted from testosterone by aromatase might exhibit vasoreactivity. Although the plasma level of estradiol was not correlated with %FMD in the present study, tissue conversion of testosterone into estradiol might play a role. Further *in vitro* and animal studies will be needed to clarify these issues.

The results of this study do not imply that testosterone has favorable effects in women. In fact, in a preliminary study, we observed that the plasma testosterone level was not related to FMD in postmenopausal women (unpublished observation). It has been reported that testosterone may impair endothelial function in women, and especially in young women with polycystic ovary syndrome (34) and women taking high-dose androgens (35). Aortic rings obtained from female rats treated with testosterone showed a significant decrease in prostacyclin synthesis (36), supporting the idea that testosterone influences vasoconstriction in women. Taken together, these results indicate that the vascular responses to testosterone are clearly different between men and women. Gender differences in the steroid hormone receptor expression in arteries (37, 38) might play a mechanistic role.

This study has some limitations. First, since this was a cross-sectional study, the causal relationship between testosterone and vasomotor function could not be determined. Endothelial dysfunction might be associated with a reduction in blood flow of endocrine organs, leading to decreased hormone production. Longitudinal studies following the subjects might add some information. Secondly, a population bias was possible. The study subjects ranged from young to elderly men with or without coronary risk factors. Consequently, the results might have been different if homogeneous subjects in terms of age and health status had been studied. In our subgroup analyses according to age and coronary risk factors and in multiple regression analyses including drug classes, comparable regression coefficients were obtained between testosterone and %FMD, although the statistical power was weakened (data not shown).

In summary, a low plasma testosterone level was associated with endothelial dysfunction in men independent of other risk factors, suggesting a protective effect of testosterone on the endothelium. This finding provides mechanistic insight into the role of endogenous testosterone in the development of cardiovascular disease in men.

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Potent free radical scavenger, edaravone, suppresses oxidative stress-induced endothelial damage and early atherosclerosis

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Abstract

Objective: Effects of potent free radical scavenger, edaravone, on oxidative stress-induced endothelial damage and early atherosclerosis were investigated using animal models and cultured cells.

Methods and results: Endothelial apoptosis was induced by 5-min intra-arterial exposure of a rat carotid artery with 0.01 mmol/L H₂O₂. Edaravone treatment (10 mg/kg i.p.) for 3 days suppressed endothelial apoptosis, as evaluated by chromatin staining of *en face* specimens at 24 h, by approximately 40%. Similarly, edaravone dose-dependently inhibited H₂O₂-induced apoptosis of cultured endothelial cells in parallel with the inhibition of 8-isoprostane formation, 4-hydroxy-2-nonenal (4-HNE) accumulation and VCAM-1 expression. Next, apolipoprotein-E knockout mice were fed a high-cholesterol diet for 4 weeks with edaravone (10 mg/kg i.p.) or vehicle treatment. Edaravone treatment decreased atherosclerotic lesions in the aortic sinus (0.18 ± 0.01 to 0.09 ± 0.01 mm², *P* < 0.001) and descending aorta (5.09 ± 0.86 to 1.75 ± 0.41 mm², *P* < 0.05), as evaluated by oil red O staining without influence on plasma lipid concentrations or blood pressure. Dihydroethidium labeling and cytochrome c reduction assay showed that superoxide anions in the aorta were suppressed by edaravone. Also, plasma 8-isoprostane concentrations and aortic nitrotyrosine, 4-HNE and VCAM-1 contents were decreased by edaravone treatment.

Conclusions: These results suggest that edaravone may be a useful therapeutic tool for early atherosclerosis, pending the clinical efficacy. © 2006 Elsevier Ireland Ltd. All rights reserved.

Keywords: Atherosclerosis; Reactive oxygen species; Free radical scavenger; Edaravone; 4-HNE; Apolipoprotein E knockout mouse

1. Introduction

Accumulating evidence has shown that stress-induced injury of vascular endothelial cells (ECs) is an initial event in the development of atherosclerosis [1]. In particular, oxidative stress has been implicated in endothelial injury caused by oxidized LDL and smoking as well as hypertension, diabetes and ischemia-reperfusion [1–3]. This notion is supported by the findings that the production of reactive oxygen species (ROS) is upregulated in vascular lesions [4,5], and that lesion formations such as endothelial dysfunction [6]

and atherosclerosis [7] are accelerated by superoxide anion (O₂^{•-}).

Experimental studies have shown the protective effects of antioxidants on atherosclerosis and endothelial injury. Dietary antioxidants were reported to preserve endothelial function [8,9] and inhibit atherosclerosis [10] in cholesterol-fed rabbits. In a well employed animal model of atherosclerosis, apolipoprotein E knockout (ApoE-KO) mouse fed a high fat diet, it has been shown that there was a significant increase in basal superoxide products [11,12], and that both O₂^{•-} levels and aortic lesion areas were attenuated by treatment with Vitamin E [11] or superoxide dismutase [13]. By contrast, it has been reported that elimination of NAD(P)H oxidase [14] or disruption of its subunit p47phox [15] had no effect on lesion size in ApoE-KO mice. Clinical experiments have

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also shown that antioxidants such as Vitamins C and E can ameliorate endothelial dysfunction in patients with hypercholesterolemia or atherosclerosis [16,17], although recent clinical trials have failed to prove the protective effects of Vitamin E on cardiovascular events in patients with risk factors [18] and in healthy subjects [19].

Edaravone is a potent free radical scavenger that has been clinically used to reduce the neuronal damage following ischemic stroke [20]. Edaravone has promising property to quench hydroxyl radical ($\cdot\text{OH}$) and show inhibitory effects on peroxynitrite (ONOO^-) and both water-soluble and lipid-soluble peroxy radical ($\text{LOO}\cdot$) [21,22]. Accordingly, this compound exerts a wide range of antioxidant activity on ROS beyond the effects of water-soluble or lipid-soluble antioxidant vitamins. Based on this idea, we hypothesized that edaravone would inhibit the process of atherosclerosis.

To test this hypothesis, we investigated the effects of edaravone in two experimental models. First, we examined whether edaravone could inhibit hydrogen peroxide (H_2O_2)-induced EC apoptosis in a rat model [23] and cultured ECs. Second, we examined whether edaravone could suppress the atherosclerotic lesion formation in ApoE-KO mice.

2. Methods

2.1. Animals

Male Wistar rats aged 10–12 weeks (Japan Clea), and male C57BL/6 mice and ApoE-KO mice on C57BL/6 background aged 4–6 weeks (Jackson Laboratory) were used in this study. All of the experimental protocols were approved by the Animal Research Committee of the Kyorin University School of Medicine.

2.2. H_2O_2 -induced EC apoptosis in rats and in culture

EC apoptosis was induced by 5-min intra-arterial treatment of a rat carotid artery with 0.01 mmol/L H_2O_2 as previously described [23]. Briefly, edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one; 3 or 10 mg/kg; donated by Mitsubishi Pharma Corporation, Japan) or its vehicle was intraperitoneally injected daily for 3 days before H_2O_2 treatment. A catheter was placed in the common carotid artery via the external carotid artery. The lumen was flushed with saline, replaced with 0.01 mmol/L H_2O_2 diluted with saline for 5 min and recovered. At 24 h after H_2O_2 treatment, EC apoptosis was evaluated by chromatin staining of *en face* specimens of the carotid artery using Hoechst 33342 dye. Apoptotic cells were identified by their typical morphological appearance; chromatin condensation, nuclear fragmentation, or apoptotic bodies. The numbers of apoptotic cells and intact cells were counted in 10 high-power fields for each specimen by an observer blinded to the treatment group.

Apoptosis of ECs isolated from a bovine carotid artery was induced as previously described [24]. Briefly, subconfluent ECs were pretreated for 24 h with culture medium containing edaravone or vehicle. After washing twice with Hank's balanced salt solution, the cells were exposed to H_2O_2 (0.2 mmol/L) diluted in Hank's balanced salt solution for 1.5 h at 37 °C to induce apoptosis. Then ECs were cultured in culture medium containing edaravone or vehicle until assay. Apoptosis was evaluated at 24 h after H_2O_2 treatment as histone-associated DNA fragments using a photometric enzyme immunoassay (Cell Death Detection ELISA, Roche), according to the manufacturer's instructions.

2.3. Atherosclerosis in ApoE-KO mice

ApoE-KO mice received a high-cholesterol diet (1% cholesterol, 10% fat in CE-2 standard diet; Japan Clea) for 4 weeks. Simultaneously, edaravone (10 mg/kg) or its vehicle was intra-peritoneally injected daily throughout the experiments. Body weight and systolic blood pressure were recorded every week in a conscious state by the tail cuff method (BP-98A; Softron, Tokyo).

At 4 weeks of treatment, mice were sacrificed with an overdose of diethyl ether and perfusion-fixed. Atherosclerotic lesions in the aortic sinus were quantified according to the method described previously [25]. We also measured the surface area of atherosclerotic lesions in the whole descending aorta including the abdominal aorta just proximal to the iliac bifurcation. *En face* specimens of the descending aorta were stained with oil red O, photographed and analyzed using the NIH image software. Total cholesterol, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol in mice plasma were determined by a commercial laboratory (SRL, Japan).

2.4. Measurement of ROS

Aortic samples for ROS measurements were prepared separately from those for atherosclerosis evaluation. At 4 weeks of treatment, ApoE-KO mice were sacrificed with CO_2 inhalation. Descending aortas were rapidly removed and placed into chilled modified Krebs/HEPES buffer. C57BL/6 mice fed a standard diet were also used as the control. To determine superoxide production *in situ*, frozen cross-sections of the aorta were stained with 10 $\mu\text{mol/L}$ dihydroethidium (DHE; Molecular Probes), followed by fluorescent microscopy [26]. Also, superoxide production in aortic rings was quantified using the superoxide dismutase-inhibitable cytochrome *c* reduction assay as previously described [27]. Immunohistochemical detection of 3-nitrotyrosine in the aorta was visualized by diaminobenzidine as reported previously [28].

Intracellular production of superoxide anions was measured using DHE as described previously [29], and the intensity values were calculated using the Metamorph software [24]. Concentrations of 8-isoprostane (8-iso prostaglandin

F_{2a}) in the culture supernatants and mouse plasma were measured using a commercially available EIA kit (Cayman Chemical). Culture supernatants were directly applied to EIA, while plasma was applied to EIA after solid phase extraction purification according to the manufacturer's instructions.

2.5. Western blotting

Western blotting was performed as previously described [30], to detect the expression of VCAM-1 and 4-HNE in cultured ECs and mouse aortas. Descending aortas were prepared as described in ROS measurements. The antibodies used in this study were anti-4-HNE monoclonal antibody (JaICA, Shizuoka, Japan), anti-VCAM-1 polyclonal antibody (Santa Cruz Biotechnology) and anti-3-nitrotyrosine monoclonal antibody (Upstate). Densitometric analysis was performed using an image scanner and the NIH software.

2.6. Data analysis

All values are expressed as mean \pm S.E.M. Data were analyzed using one-factor ANOVA. If a statistically significant effect was found, Newman-Keuls' test was performed to isolate the difference between the groups. Differences with a value of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of edaravone on H₂O₂-induced EC apoptosis and ROS

As shown in Fig. 1A, edaravone dose-dependently inhibited EC apoptosis in culture, which was induced 24 h after H₂O₂ treatment. Edaravone was then employed in a rat model of H₂O₂-induced EC apoptosis. Consistent with the *in vitro* experiment, edaravone of 10 mg/kg/day decreased EC apoptosis of the rat carotid artery by approximately 40% (Fig. 1B).

We next examined whether edaravone decreased ROS production in the process of H₂O₂-induced EC apoptosis. For this purpose, DHE fluorescent, a marker of intracellular production of superoxide anions, release of 8-isoprostane into the culture supernatants and accumulation of 4-HNE, a pivotal end-product of lipid peroxidation [31], were measured using cultured ECs. We also examined the expression of VCAM-1 as a marker of endothelial injury or activation [32]. Edaravone decreased DHE fluorescent, 8-isoprostane formation and VCAM-1 expression at 3 h after H₂O₂ treatment in a dose-dependent manner (Fig. 2A–C). As shown in Fig. 2D, multiple bands showing 4-HNE-Michael protein adducts [33,34] were accumulated after H₂O₂ treatment in a time-dependent manner. Consequently, the effect of edaravone on 4-HNE expression was examined at 3 h after H₂O₂ treatment (4.5 h after H₂O₂ was initially added). Edaravone decreased 4-HNE expression in a dose dependent manner.

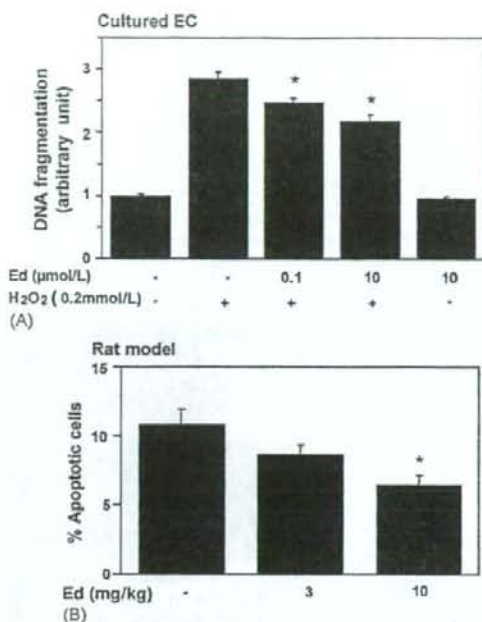


Fig. 1. Effects of edaravone (Ed) on H₂O₂-induced EC apoptosis in culture (A) and in a rat model (B). (A) Ed or its vehicle was added to the culture medium 24 h before H₂O₂ treatment until assay. EC apoptosis was evaluated 24 h after H₂O₂ treatment (0.2 mmol/L) by means of DNA fragmentation. Values are expressed as mean \pm S.E.M. ($n = 3$). * $P < 0.05$ vs. H₂O₂ (+) + Ed (-). (B) Ed or its vehicle was intraperitoneally injected once a day for 3 days before H₂O₂ treatment. At 24 h after H₂O₂ treatment, apoptotic ECs were counted per high power field and the ratio of the apoptotic cell number to the intact cells was calculated using *en face* specimens of the carotid artery stained with Hoechst 33342. Values are expressed as mean \pm S.E.M. ($n = 7$). * $P < 0.05$ vs. vehicle.

3.2. Effects of edaravone on atherosclerotic lesions and ROS in ApoE-KO mice

In the next set of experiments, we examined whether edaravone could suppress the atherosclerotic lesions in ApoE-KO mice fed a high cholesterol diet for 4 weeks. As shown in Fig. 3A and B, atheromatous lesions both in the aortic sinus and the descending aorta were smaller in mice treated with 10 mg/kg/day edaravone than in those with vehicle. This dose of edaravone did not influence body weight, blood pressure or plasma LDL and HDL cholesterol levels (Table 1).

Then, we examined whether the anti-atherogenic effects of edaravone were associated with the decrease in ROS production. Peroxynitrite formation was assessed as 3-nitrotyrosine accumulation in the aorta [28]. Both immunohistochemistry and Western blotting showed that edaravone inhibited nitrotyrosine accumulation in the aorta of ApoE-KO mice (Fig. 4A(a) and A(b)). Superoxide production *in situ* was examined using DHE staining of the descend-

Table 1
Body weight, blood pressure and plasma lipid levels in ApoE-KO mice treated with edaravone or vehicle

	Vehicle	Edaravone
Body weight (g)	21.4 ± 0.5	21.0 ± 0.5
Systolic blood pressure (mmHg)	106 ± 2	103 ± 3
Total cholesterol (mg/dL)	1967 ± 38	1872 ± 66
HDL cholesterol (mg/dL)	66 ± 6	82 ± 9
LDL cholesterol (mg/dL)	602 ± 24	602 ± 12

The values are shown as mean ± S.E. (n = 14). There were no significant differences in the values between the two groups.

ing aorta. As shown in Fig. 4B, ethidium fluorescence, which was amplified in ApoE-KO mice, was decreased by edaravone treatment. A quantitative analysis by the superoxide dismutase-inhibitable cytochrome *c* reduction assay revealed that $O_2^{\bullet-}$ levels in aortic rings of ApoE-KO mice were decreased by 43% in edaravone-treated ApoE-KO mice compared to those in vehicle-treated mice (Fig. 4C). Consistent with these results, plasma 8-isoprostane levels and 4-HNE expression in the descending aorta, both of which were elevated in ApoE-KO mice compared to

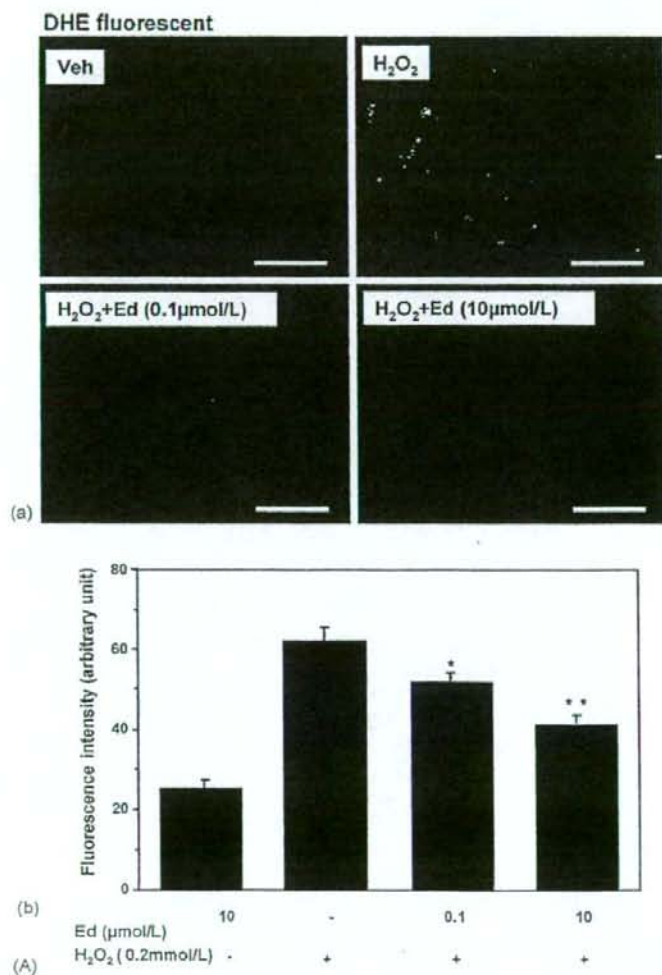


Fig. 2. Effects of edaravone (Ed) on DHE fluorescent (A) and 8-isoprostane formation (B), VCAM-1 expression (C) and 4-HNE expression (D) in cultured EC. Ed or its vehicle was added to the culture medium 24 h before H_2O_2 treatment until assay. DHE fluorescent (n = 6), 8-isoprostane concentration (n = 3) and VCAM-1 expression (n = 3) in the cell lysate were measured 3 h after H_2O_2 treatment. Values are expressed as mean ± S.E.M. Time dependent changes of 4-HNE expression after H_2O_2 treatment was detected by Western blotting. Representative image showed that 4-HNE-Michael protein adducts were accumulated after treatment (D(a)). The major 97 kDa band was measured 4.5 h after H_2O_2 treatment in the presence or absence of edaravone (D(b)). Values are expressed as mean ± S.E.M. (n = 3). * $P < 0.05$, ** $P < 0.01$ vs. H_2O_2 (+) + Ed (-).

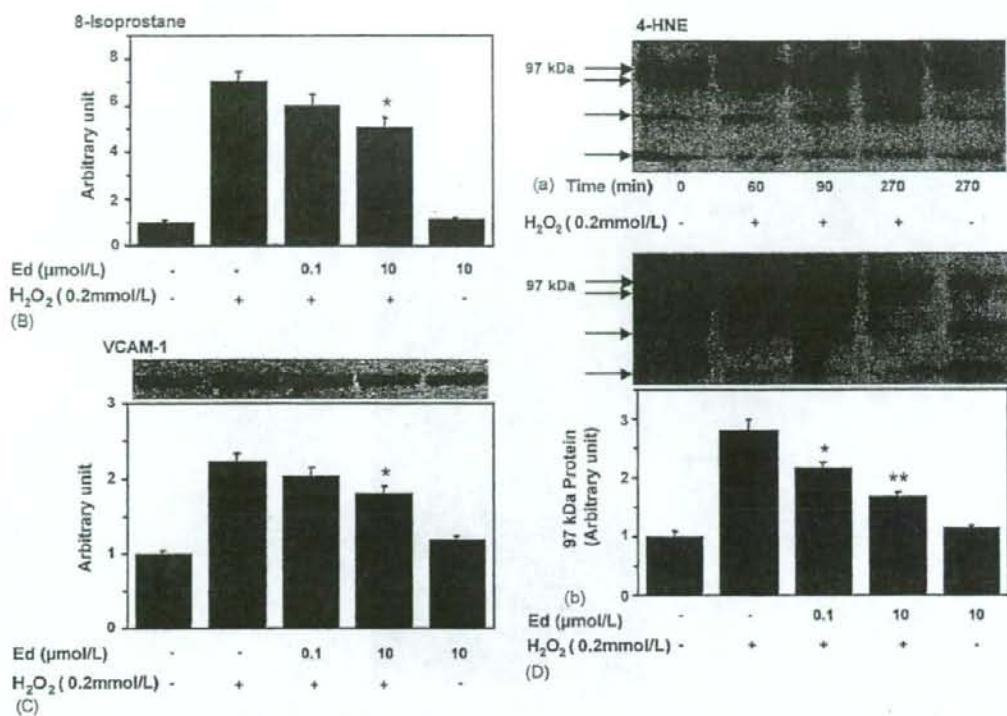


Fig. 2. (Continued).

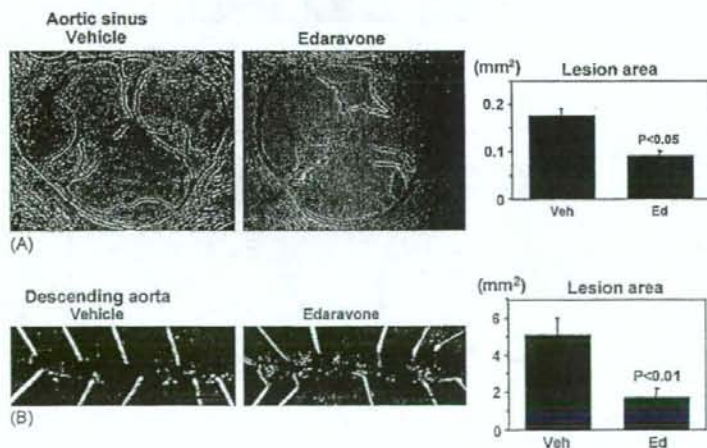


Fig. 3. Effects of edaravone on atherosclerotic lesion in ApoE-KO mice. ApoE-KO mice were fed a high-cholesterol diet for 4 weeks with the administration of edaravone (10 mg/kg daily) or its vehicle by i.p. injection. (A) Oil red O-stained cross-sections of the aortic sinus (bar = 100 μm) and morphometric analysis of the lesions are shown. (B) Oil red O-stained *en face* specimens of the descending aorta (bar = 5 mm) and morphometric analysis of the lesions are shown. Values are expressed as mean \pm S.E.M. ($n = 14$).

those in wild-type C57BL/6 mice fed a normal chow, were decreased by edaravone treatment (Fig. 4D and E). Finally, the increase in VCAM-1 expression in the aorta of ApoE-KO mice was attenuated by edaravone as well (Fig. 4F).

4. Discussion

A number of studies have shown that ROS contribute to the pathogenesis of endothelial dysfunction and atherosclerosis formation. In addition to $O_2^{\bullet-}$ that is predominantly pro-

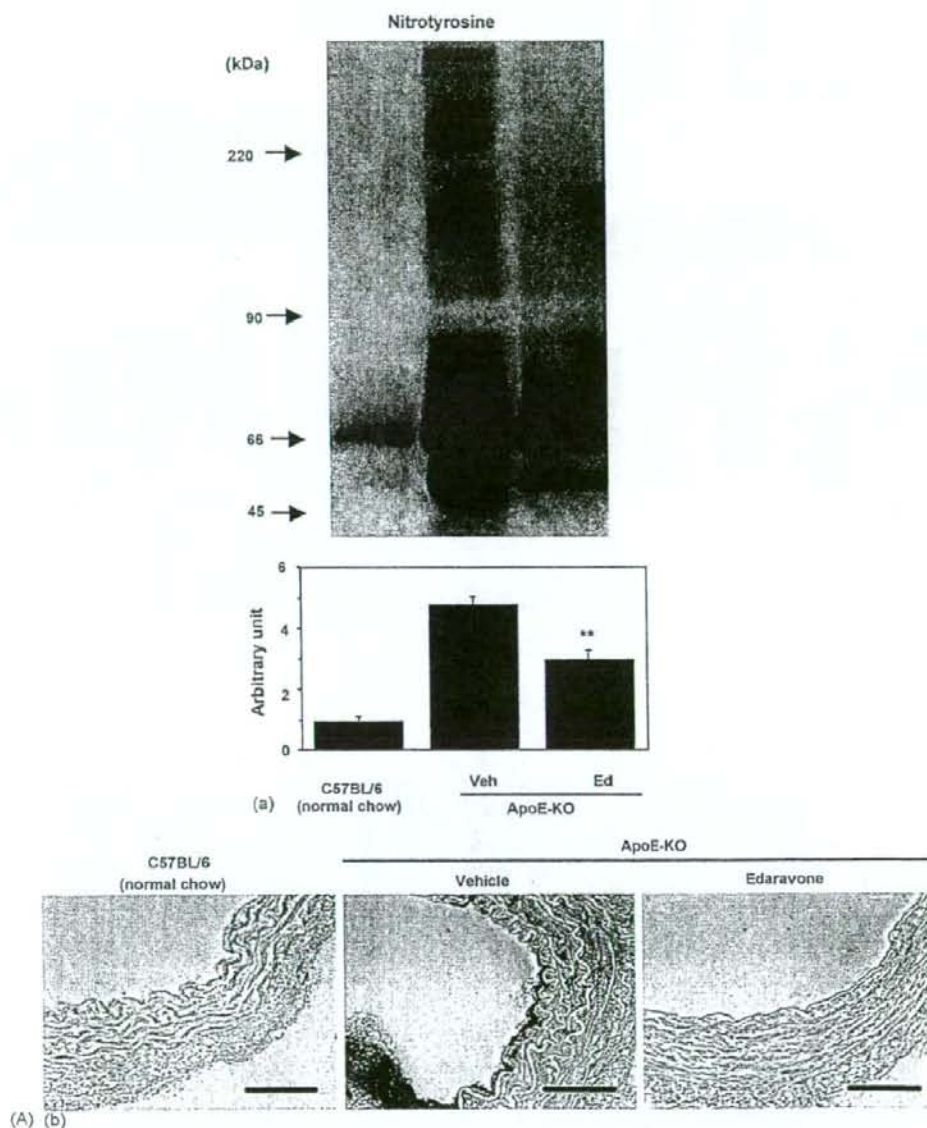


Fig. 4. Effects of edaravone (Ed) on ROS production (A–E) and VCAM-1 expression (F) in ApoE-KO mice. (A) Nitrotyrosine contents in the aorta was examined by Western blot analysis (A(a), $n=6$) and immunohistochemistry (A(b)). Bar = 50 μ m. (B) Fresh-frozen cross-sections of the aorta were stained with DHE, and representative fluorescent micrographs are shown (bar = 100 μ m). (C) Superoxide anion in aortic rings was determined using SOD-inhibitable-cytochrome *c* reduction assay ($n=6$). (D) 8-Isoprostane level in mouse plasma was measured with EIA ($n=6$). (E and F) Representative Western blotting for 4-HNE (97 kDa band) and VCAM-1 expression in the aorta and densitometric analysis are shown ($n=3$). Values are expressed as mean \pm S.E.M. * $P < 0.05$, ** $P < 0.01$ vs. vehicle (Veh). C57BL/6 mice fed a normal chow serve as the control.

Edaravone has been clinically used as a neuroprotectant in the treatment of ischemic stroke in Japan from 2001. The dose of edaravone used in this study (intraperitoneal injection of 10 mg/kg) has been reported to be comparable to that of intravenous injection in clinical use in terms of plasma concentration [42]. This compound has been reported to preserve endothelial function in ischemic brain [43] and ameliorate ischemia-reperfusion injury in various organs such as kidney [44] and heart [45]. Also, edaravone has been shown to inhibit pressure overload-induced cardiac hypertrophy [42]. To our knowledge, however, the effect of edaravone on atherosclerosis has never been reported till now.

The effects of edaravone on endothelial injury and atherosclerosis were associated with the decrease in ROS production including peroxynitrite, superoxide anion and 8-isoprostane, suggesting the mechanistic role of antioxidation in vascular protection. Edaravone also inhibited the expression of 4-HNE in vascular tissues, further indicating the antioxidant activity and suggesting the signaling cascade leading to endothelial injury, because 4-HNE triggers cellular damages through the MAP kinase pathway as an end-product of ROS [34]. Antioxidant effects of edaravone on lipoproteins were not determined in the present study because of the methodological limitation in mice. It has been reported, however, that edaravone can inhibit oxidative modification of low-density lipoprotein *in vitro* and in rats [46]. Consequently, it is likely that reduced lipoprotein oxidation would have played a role in the anti-atherosclerotic effects of edaravone in ApoE-KO mice. Furthermore, edaravone has been reported to stimulate the expression of endothelial nitric oxide synthase in cultured ECs [46] and the artery [47], leading to the increased production of nitric oxide. Taken together with the effects on peroxynitrite formation, edaravone might synergistically increase the availability of nitric oxide, which exerts vasoprotective and anti-atherosclerotic action.

The effects of edaravone on advanced and complicated lesions of atherosclerosis were not investigated in this study. Neither, the effects on plaque ruptures nor consequent cardiovascular events are known. This study demonstrated that edaravone might be a potential new therapeutic agent for the prevention and treatment of early atherosclerosis. For the purpose of chronic use, however, the innovation of drug preparation for oral administration is necessary. Another application of edaravone might be the prevention of restenosis after percutaneous coronary interventions, since ROS plays an important role in neointimal formation after angioplasty [48]. Intravenous injection of edaravone for several days might inhibit neointimal formation in addition to ischemia reperfusion injury of cardiomyocytes [45]. Taken together, edaravone is expected to show protective effect on ROS-related vascular diseases beyond cerebral infarction.

In summary, edaravone, a free radical scavenger with unique properties, attenuated oxidative stress-induced endothelial damage in rats and early atherosclerosis in ApoE-KO mice in association with the inhibition of ROS formation.

These findings provide new information on the role of ROS in atherogenesis and the therapeutic strategy for atherosclerosis.

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Signaling pathway of nitric oxide production induced by ginsenoside Rb1 in human aortic endothelial cells: A possible involvement of androgen receptor

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Abstract

Ginsenosides have been shown to stimulate nitric oxide (NO) production in aortic endothelial cells. However, the signaling pathways involved have not been well studied in human aortic endothelial cells. The present study was designed to examine whether purified ginsenoside Rb1, a major active component of ginseng could actually induce NO production and to clarify the signaling pathway in human aortic endothelial cells. NO production was rapidly increased by Rb1. The rapid increase in NO production was abrogated by treatment with nitric oxide synthetase inhibitor, L-NAME. Rb1 stimulated rapid phosphorylation of Akt (Ser473), ERK1/2 (Thr202/Thr204) and eNOS (Ser1177). Rapid phosphorylation of eNOS (Ser1177) was prevented by SH-5, an Akt inhibitor or wortmannin, PI3-kinase inhibitor and partially attenuated by PD98059, an upstream inhibitor for ERK1/2. Interestingly, NO production and eNOS phosphorylation at Ser1177 by Rb1 were abolished by androgen receptor antagonist, nilutamide. The results suggest that PI3kinase/Akt and MEK/ERK pathways and androgen receptor are involved in the regulation of acute eNOS activation by Rb1 in human aortic endothelial cells. © 2006 Elsevier Inc. All rights reserved.

Keywords: Ginsenoside Rb1; Endothelial cells; Nitric oxide; eNOS; Androgen receptor; PI3-kinase; Akt; ERK; MEK; Phosphorylation

Ginseng, the root of *Panax ginseng* C.A. Meyer (Araliaceae), is a well-known and popular herbal medicine used worldwide. Among more than 30 ginsenosides, the active ingredient of ginseng, ginsenoside Rb1 is regarded as the main compound responsible for many pharmaceutical actions of ginseng. The oral administration of ginseng caused a decrease in blood pressure in essential hypertension [1]. Intravenous administration of ginsenosides (a mixture of saponin from *Panax ginseng* C.A. Meyer) lowered blood pressure in a dose-dependent manner in anesthetized rats [2]. Although these reports suggest that ginsenosides could stimulate the production of nitric oxide (NO) by aortic vascular endothelial cells, the precise mechanisms of the

ginsenoside actions have not been fully elucidated [3]. NO released from endothelial cells via the endothelial nitric oxide synthetase (eNOS) is a pivotal vasoprotective molecule. In addition to its vasodilating feature, endothelial NO has anti-atherosclerotic properties, such as inhibition of platelet aggregation, leukocyte adhesion, smooth muscle cell proliferation, and expression of genes involved in atherosclerosis [4].

The present study aims at investigating the signaling pathways involved in NO production by purified ginsenoside Rb1 in human aortic endothelial cells *in vitro*.

Materials and methods

Materials. Rb1, nilutamide, L-NAME (hydrochloride), Hanks' balanced salt solution (HBSS) were purchased from Sigma (St. Louis, MO,

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USA). ICI182780 was from Zeneca Pharmaceuticals (Macclesfield, UK). 4,5-diaminofluorescein diacetate (DAF-2 DA) was purchased from Daiichi (Daiichi Pure Chemicals Co., Ltd, Tokyo, Japan). PD98059, SH-5, wortmannin and Nitric Oxide Synthase Assay Kit were from Calbiochem (EDM Biosciences, Inc., La Jolla, CA, USA and Germany). t -[3 H]Arginine was purchased from Amersham (Amersham Biosciences, Uppsala, Sweden). Antibody of phospho-eNOS (Ser1177) was from upstate (Upstate Inc., Lake Placid, NY). Antibody for eNOS/NOS type III was purchased from BD Transduction Laboratories (BD Biosciences, Franklin Lakes, NJ, USA). All other antibodies were from Cell Signaling Technology (Beverly, MA, USA). LumiGLO Reserve Chemiluminescent Substrate Kit was from KPL (Gaithersburg, MD, USA). EBM-2 (endothelial cell base medium) was from Clonetics (Walkersville, MD, USA). Human aortic endothelial cells (HAECs) were from Cambrex (Cambrex BioScience Walkersville, Inc. Walkersville, MD, USA). Fetal bovine serum (FBS) was from CCT (Sanko Junyaku Co., Ltd, Tokyo, Japan). Fetal bovine serum charcoal stripped was from MultiSer (ThermoTrace Ltd., Melbourne, Australia).

Cell culture. HAECs were cultured in a 37 °C humidified atmosphere of 95% air/5% CO₂ in EGM-2 (endothelial cell growth medium 2) medium supplemented with 10% FBS. The EGM-2 medium consisted of 0.1% EGF, 0.04% hydrocortisone, 0.4% hFGF-B, 0.1% VEGF, 0.1% R³-IGF-1, 0.1% ascorbic acid, 0.1% GA-1000, and 0.1% heparin. Experiments were performed with cells from passages 5 to 7. For all experiments, HAECs were plated at a concentration of 1×10^4 /mL and grown to confluence. Then cells were serum-starved for 6 h in phenol red free EBM-2 containing 1% DCC-FBS, that was removed the steroid by processing it with dextran-coated charcoal (DCC-FBS). In some inhibitory experiments, the inhibitors were added to cells 60 min before the stimuli. DMSO was used as a solvent for Rb1, PD98059, wortmannin, SH-5, L-NAME, nitroglycerin, and DAF-2 DA present at equal concentrations (0.01%) in all groups, including the vehicle.

Western blot analysis. After treatment with reagents, confluent monolayers of cells were washed two times in ice-cold phosphate-buffered saline and lysed with buffer containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton-X, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β -glycerophosphate, 1 mmol/L Na₂VO₄, 1 μ g/mL Leupeptin, 1 mM PMSF. For western blot analysis, total cell lysate was subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and proteins were transferred to polyvinylidene difluoride (PVDF) membrane. The antibodies used in this study were anti-phospho-ERK1/2 (Thr202/Thr204), anti-ERK1/2, anti phospho-Akt (Ser473), anti-Akt, anti-phospho-eNOS (ser1177) and anti-NOS. Antibodies were detected by means of a horseradish peroxidase-linked secondary antibody and immunoreactive bands were visualized using LumiGLO Reserve Chemiluminescent Substrate Kit.

Endothelial NO synthase activity assay. Endothelial cell NO synthase (eNOS) activity was quantified by measuring the conversion of t -[3 H]-arginine to t -[3 H]-citrulline by the use of a NO synthase assay kit.

Measurement of intracellular production of NO. Production of NO was assessed using the NO-sensitive fluorescent dye DAF-2 DA [5]. Briefly, confluent cells were serum-starved for 6 h. Because NOS generates O₂⁻ instead of NO in the absence of t -arginine, so t -arginine (100 μ M/L) was added 1 h prior to all solutions, except for the experiment with *N*-nitro- t -arginine methyl ester (L-NAME; a NOS inhibitor)-treated cells. Cells were loaded with DAF-2 DA (final concentration 5 μ M/L, 30 min 37 °C) and then rinsed three times with HBSS, kept in the dark, and maintained at 37 °C in 1% EBM-2 medium with a warming stage. After 30 min, cells were then treated with Rb1 or other stimuli. In some inhibitory experiments, the inhibitors were administered 30 min before loading with DAF-2 DA. Green fluorescence intensity was measured with a laser scanning confocal microscopy system (LSCM) (Bio-Rad Laser Sharp2000). The fluorescence image was obtained as a 512 \times 512 pixel frame. Ex = 488 nm, EM = 510 nm. All other settings, including scanning speed, pinhole diameter, and voltage gain, remained the same for all experiments.

Statistics. Data are means \pm SEM. Statistical comparisons were performed by Student's *T* test between two groups. A value of *P* < 0.05 was considered significant.

Results

Rb1 stimulates rapid production of NO in human aortic endothelial cells

We used the NO-specific fluorescent dye DAF-2 DA to evaluate the effect of Rb1 on NO production in HAECs. 5, 10, 15, 30, 60, 120 and 180 min after Rb1 treatment, cells were fixed and then viewed using a fluorescence microscope. Emission of green light (510 nm) from cells excited by light at 488 nm is indicative of NO production. A significant increase in green fluorescence was observed >15 min after the addition of Rb1 and lasted for 60 min in HAECs (Fig. 1A). Maximal stimulation of NO production was obtained at 30 min.

To verify that the rapid increase in green fluorescence in response to Rb1 treatment specifically reflected NO production, we compared results from HAECs treated with acetylcholine (1 μ M/L) or Rb1 (1 μ M/L) for 5 min. Reassuringly, treatment with either acetylcholine and calcium ionophore or Rb1 resulted in an increase in green fluorescence (Fig. 1B). We next examined the effects of the NOS inhibitor L-NAME to determine whether the NO increase was attributable to NOS derived de novo synthesis. As shown in Fig. 1C, the Rb1-induced DAF-2 DA fluorescence was completely suppressed by pretreatment with L-NAME (0.5 mmol/L). The results suggested that the rapid increase in NO production after Rb1 treatment was mediated by an increase in NOS activity.

Rb1 stimulates phosphorylation of eNOS (Ser1177) and increases NOS activity

To examine involvement of eNOS in the NO increase, the effect of Rb1 on eNOS phosphorylation at Ser-1177 was tested by Western blotting. As shown in Fig. 2, Rb1 induced rapid eNOS phosphorylation after 10 min of incubation, maximal eNOS phosphorylation by Rb1 was observed from 30 to 60 min of incubation. The relative magnitude of eNOS phosphorylation falls subsequently but is still significantly greater than control after 120 min of Rb1 incubation (Fig. 2A, upper blots). The acute effect by Rb1 on eNOS phosphorylation was concentration dependent (Fig. 2B, upper blots). Rb1 did not affect eNOS protein expression (Fig. 2A and B, lower blots).

To see whether Rb1 actually activates NOS in HAECs, we measured NOS activity after 30 min of treatment with Rb1. As shown in Fig. 2C, Rb1 significantly increased NOS activity in HAECs.

PI3-kinase/Akt and MEK/ERK pathways are involved in eNOS phosphorylation and NO production

Previous studies have demonstrated that PI3-kinase/Akt and MEK/ERK pathways are two important signaling cascades mediating eNOS activation by many stimuli in vascular endothelial cells [6,7]. Therefore, we examined

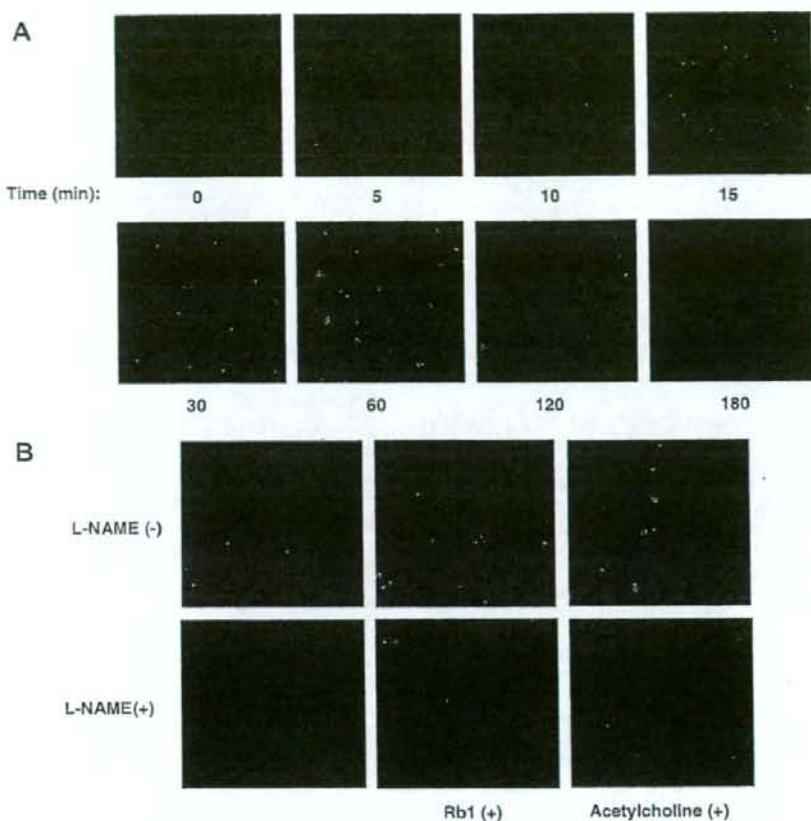


Fig. 1. Effect of Rb1 on production of NO. HAECs were starved and loaded with DAF-2 DA (5 $\mu\text{mol/L}$) as described in Materials and methods prior to treatment with either Rb1 (1 $\mu\text{mol/L}$) for 0, 5, 10, 15, 30, 60, 120, and 180 min (A) or acetylcholine (1 $\mu\text{mol/L}$) for 5 min (B). After Rb1 treatment, cells were fixed in 2% paraformaldehyde for 10 min at 4 $^{\circ}\text{C}$ and then viewed using a fluorescent microscope. Emission of green light (510 nm) from cells excited by light at 488 nm is indicative of NO production. In some groups of cells, L-NAME (0.5 mmol/L) was added 30 min before loading cells with DAF-2 DA (B). A representative time course experiment is shown for experiments that were repeated independently for three times. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

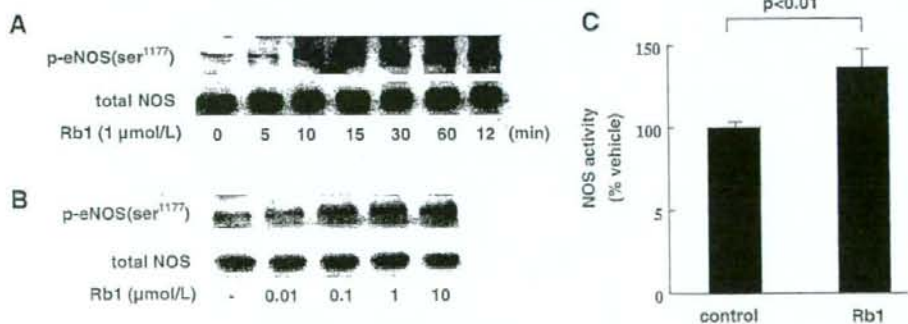


Fig. 2. Effects of Rb1 on eNOS phosphorylation and NOS activity. Phosphorylation of eNOS in HAECs. Starved HAECs were treated with the vehicle (0.01% DMSO) or Rb1 (1 $\mu\text{mol/L}$) for indicated times (A) or with various concentrations of Rb1 for 30 min (B). Western analysis performed to detect phospho-eNOS (Ser1177) and total eNOS. The experiments were repeated three times in triplicates, with equal result. NOS activity in HAECs homogenates. Rb1 (1 $\mu\text{mol/L}$) were added to the starved medium for 30 min, then activity of NOS was measured by the conversion of L-arginine to L-citrulline at 37 $^{\circ}\text{C}$ for 60 min (C). Histograms and error bars represent means \pm SEM of four independent experiments performed in duplicate. * $P < 0.01$ vs control.

whether Rb1 activated Akt and ERK1/2. We used phospho-specific antibodies to evaluate the ability of Rb1 to stimulate phosphorylation of Akt and ERK1/2 in HAECs. Rb1 rapidly increased phosphorylation of Akt (Ser473) and ERK1/2 (Fig. 3A, upper blots) in HAECs > 5 min after the addition of Rb1. Maximal phosphorylation was attained at 30 min in Akt and at 15 min in ERK1/2. The relative magnitude of the Rb1 response falls subsequently but is still significantly greater than control after 120 min of Rb1 treatment. Rb1 did not affect total Akt and ERK protein expression (Fig. 3A, lower blots).

We next examined the rapid phosphorylation of eNOS at Ser1179 by Rb1 either in the absence or presence of PI3 kinase inhibitor wortmannin, and Akt inhibitor SH-5 or MEK (ERK kinase) inhibitor PD98059. As shown in Fig. 3B, the rapid eNOS phosphorylation was abolished by pretreatment of cells with wortmannin (5 μmol/L) or SH-5, and partially attenuated by MEK inhibitor PD98059 (10 μmol/L). NO production viewed by fluorescent microscopy showed the similar inhibition by these inhibitors (Fig. 3C). These results suggest that acute activation of eNOS and NO production by Rb1 were mediated through activation of PI3-kinase/Akt and ERK1/2.

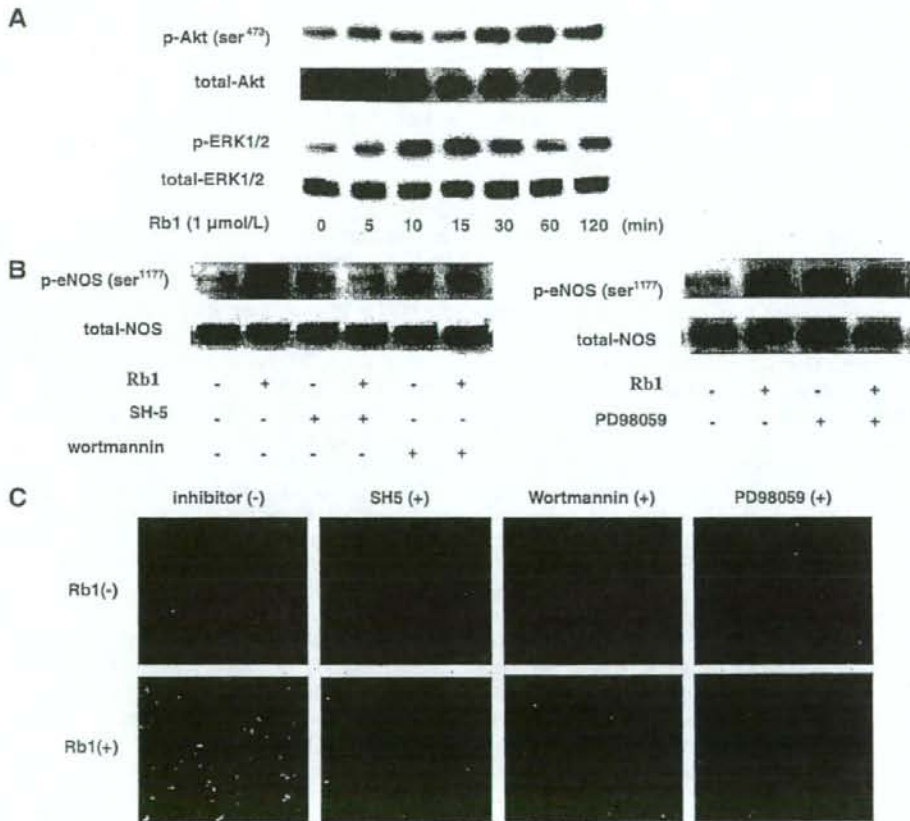


Fig. 3. Effects of inhibitors for PI3kinase/Akt or MEK (ERK kinase) on eNOS phosphorylation and NO production. Starved HAECs were treated with the vehicle (0.01% DMSO) or Rb1 (1 μmol/L) for indicated times (A). In some groups, cells were pretreated with SH-5 (10 μmol/L), wortmannin (5 μmol/L) or PD98059 (10 μmol/L) for 1 h, then cells were treated without or with Rb1 (1 μmol/L) for 30 min (B). Cell lysates were analyzed by Western blot as described in Materials and methods. Anti-phospho-Akt (Ser473) antibody and anti-Akt antibody; anti-phospho-ERK1/2 antibody and anti-ERK1/2 antibody (A), anti-phospho-eNOS (Ser1177) antibody and anti-eNOS antibody (B) were used for western blot analysis. Experiments were repeated three times, with equivalent result. Starved cells were loaded with DAF-2 DA as described in Materials and methods before treatment with Rb1 (1 μmol/L). In some groups of cells, SH-5 (10 μmol/L), wortmannin (5 μmol/L) or PD98059 (10 μmol/L) were added 60 min before cells were loaded with DAF-2 DA. After Rb1 treatment, cells were fixed in 2% paraformaldehyde for 10 min at 4 °C and then viewed using a fluorescent microscope (C). Emission of green light (510 nm) from cells excited by light at 488 nm is indicative of NO production. A representative set of experiments is shown for experiments that were repeated independently three times. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)