cell death is activation of the caspase cascade. Activation of caspase 3 is required for internucleosomal DNA degradation (Woo et al., 1998), and caspase inhibition prevents the release of apoptotic bodies from cells (Zhang et al., 1999). In the present study, supplementation of the medium with rhGas6 prevented Pi-induced caspase 3 activation. These results clearly show that Pi downregulates Gas6-Axl, decreases PI3K-mediated Akt phosphorylation, inactivates Bcl2, activates Bad, and activates caspase 3, leading to apoptosis.

The present study demonstrated that statins restored the Gas6-mediated survival pathway. Consistent with these results, Akt phosphorylation has been reported to be an antiapoptotic mechanism of statins: pravastatin inhibited hypoxia-induced apoptosis through activation of Akt in cardiomyocytes (Bergmann et al., 2004), and simvastatin and pravastatin enhanced phosphorylation of Akt and promoted angiogenesis in endothelial cells (Kureishi et al., 2000). Recently, it was reported that statins inhibit caspase 3 activation driven by protein kinase C inhibitors in the process of apoptosis, suggesting that caspase 3 is also under the control of statins during apoptosis (Tanaka et al., 2004).

In this study, we performed experiments under both short-term (within 24 h) and long-term (up to 10 days) conditions. In general, short-term experiments are able to examine acute cell behavior, such as signaling and transcription. However, because obvious HASMC calcification takes at least 3 days, we also performed long-term experiments. Downregulation of Gas6, Axl expression and reduced phosphorylation of Akt, Bcl2, and Bad, and a beneficial effect of statins were consistently found in the long-term condition. This confirms that the Gas6-Axl survival signal is the key mechanism for Pi-induced calcification.

It is concluded that statins inhibit Pi-induced apoptosis via the Gas6/Axl-PI3K-Akt signal pathway, which has a crucial role in the prevention of HASMC calcification. This study adds further evidence of the pleiotropic effects of statins, suggesting a therapeutic strategy for the prevention of vascular calcification.

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ORIGINAL ARTICLE

# Multiple consultations and polypharmacy of patients attending geriatric outpatient units of university hospitals

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**Background:** Multiple consultations in older patients may increase the chance of overlapping prescriptions or inappropriate drug prescribing.

**Methods:** We carried out a survey investigating the status of multiple consultations and the polypharmacy of patients attending geriatric outpatient units of five university hospitals.

**Results:** The patients who received multiple consultations did not have a different number of diagnosed disorders and drugs prescribed by geriatricians compared with the patients who received a single consultation.

**Conclusions:** No significant difference in diagnostic and prescribing profiles between the patients with referrals and those without, together with the relatively smaller incidence of inappropriate prescriptions by referrals to non-geriatric specialists, suggest that multiple consultations per se may not necessarily increase the risk for adverse drug events in clinical settings.

**Keywords:** adverse drug reactions (ADR), multiple consultations, polypharmacy, university hospitals.

## Introduction

Because of comorbidity and the presence of various clinical manifestations, elderly patients are often characterized by their multiple consultations across specialties. Under the existing health care system in Japan, free access to specialists is granted to all patients even though consultations to specialists are encouraged only through primary care physicians' referrals, without which patients have to pay an extra fee for specialist consultations. Multiple consultations in older patients may not be desirable in terms of preventing inappropriate drug prescribing. They may increase the chance of overlapping prescriptions or unexpected drug interactions caused by polypharmacy, leading to an elevated risk of adverse drug reactions (ADR) or poor compliance to pharmacotherapeutics. Despite suggestions that ADR in older patients are commonly observed[1,2] and can become a cause of hospital admission,[3–9] inappropriate drug prescribing has been reported in various care settings for older adults.[10] In terms of optimal drug therapy for older patients, physicians must always take into consideration the unique aspects of age-related changes in pharmacokinetics/pharmacodynamics and the potential harm of prescribing inappropriate medication.[11] Since the Beers criteria for determining potentially inappropriate medication use by the elderly and its

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revised version,[12,13] much attention has been paid to the potential harm of prescribing drugs inappropriate for older adults, but awareness of this problem is mainly shared within geriatric specialists, and there has been insufficient outreach on this problem across specialties. Therefore, in terms of referring older patients to non-geriatric specialists, not all specialists may be aware of possible ADR in considering pharmacotherapy. University hospitals in particular have many clinical departments and sections, and the clinical environment thus encourages the referral of geriatric outpatients to other specialists, which may increase the risk of inappropriate drug prescribing. In this study, we carried out a survey investigating the status of multiple consultations and the polypharmacy of patients being treated at geriatric outpatient units of five university hospitals.

## Methods

### *Subjects*

We randomly sampled 660 patients who had been attending geriatric outpatient units of five university hospitals (Kanazawa Medical University Hospital, Kyoto University Hospital, Nagoya University Hospital, University of Tokyo Hospital, Kyorin University Hospital) from October 2003 to December 2003, and surveyed the patients' clinical background (age, identified diagnoses), prescribed drugs and consultations to other specialists within the university hospitals by chart reviews. Differences in continuous variables among the five institutions were determined by a one-way analysis of variance (ANOVA). Correlation coefficients between each of the variables were calculated by Pearson's method. The patients were divided into two groups, one group in which patients had received multiple consultations and one group in which they had not, and differences in the variables between the two groups were tested using the Student's *T*-test. Values of $P < 0.05$ were considered to indicate statistical significance. Inappropriate drug prescribing was identified based on the 2002 Beers criteria,[13] an updated version of the original Beers criteria,[12] which is an explicit criteria for determining potentially inappropriate medication use by the elderly. The original Beers criteria constructs guidelines on the inappropriate use of medications based on consensus from a panel of six nationally recognized experts in the US on the appropriate use of medication in the elderly. The updated Beers criteria review covered two types of statements: (i) 48 individual medications or medication classes that should generally be avoided in persons 65 years or older because they are either ineffective or they pose unnecessarily high risk for older persons and a safer alternative is available; and (ii) 20 diseases/conditions and medications that should not be used in older persons known to have specific medical conditions.

## Results

The mean age of the subjects sampled was 77 ± 9 (Male: 37%). The clinical profiles of all the study subjects are shown in Table 1. Table 2 compares the mean age, number of diagnosed disorders, and number of drugs prescribed in the patients attending the five geriatric outpatient units. There were no differences in all the parameters examined among the five institutions. Regarding the correlations between the parameters, although correlations of all the pairs showed statistical significance ($P < 0.001$), the correlation between the number of diagnosed disorders and that of prescribed drugs showed a much stronger correlation coefficient ($r = 0.768$) relative to the other pairs (age × number of diagnosed disorders: r = 0.246, age × number of

**Table 1** Clinical profile of the study subjects

| | |
|---|---|
| Cardiovascular disorders (including hypertension) | 406 (61.5%) |
| Cerebroneurologic disorders | 373 (56.5%) |
| Gastrointestinal disorders | 286 (43.3%) |
| Endocrine and metabolic disorders | 264 (40.0%) |
| Joint and muscle disorders (including osteoporosis) | 139 (21.1%) |
| Pulmonary disorders | 64 (9.7%) |
| Disorders of the genitourinary system | 54 (8.2%) |

**Table 2** Comparison of variables surveyed in five institutions

| | Number of cases | Age | Number of disorders | Number of drugs |
|---|---|---|---|---|
| Total | 660 | 77 ± 9 | 3.5 ± 1.9 | 4.4 ± 2.8 |
| Kanazawa | 217 | 77 ± 10 | 4.1 ± 1.9 | 4.5 ± 2.5 |
| Kyoto | 120 | 76 ± 6 | 2.7 ± 1.5 | 4.1 ± 2.5 |
| Nagoya | 120 | 78 ± 7 | 3.3 ± 1.8 | 5.0 ± 3.4 |
| Kyorin | 88 | 74 ± 11 | 3.0 ± 1.6 | 3.2 ± 2.3 |
| Tokyo | 115 | 76 ± 8 | 3.5 ± 2.0 | 5.0 ± 2.9 |

All data except number of patients are expressed as mean ± SD.

prescribed drugs: r = 0.191). Regarding multiple consultations, 148 patients (22%) were referred from geriatricians to specialists within the same institution. The distribution of specialist referrals is shown in Figure 1. Patients who received multiple consultations did not have a different number of diagnosed disorders and number of drugs prescribed by geriatricians than the patients who received a single consultation (geriatrician only) (Table 3). Because patients who had multiple consultations were prescribed with a mean of 1.8 ± 2.1 drugs by other specialists, their total number of drugs prescribed was greater than that of the patients who had received a single consultation. As for overlapping prescriptions across specialties, only one case, in which vitamin B12 was prescribed by both the geriatrician and otorhinolaryngologist at the same time, was found in this survey.

Regarding inappropriate drug prescribing based on the Beers criteria,[13] 98 inappropriate cases (14.8% of all the patients) were prescribed by geriatricians, while 14 cases (9.4% of all the patients with referrals) were prescribed by other specialists. The number of identified inappropriate prescribing of drugs included in the Beers criteria is shown in Table 4.

## Discussion

Although the recent dissemination of electronic chart review systems in general hospitals would seem to enable physicians to find out what medications are being prescribed to their patients, the inaccuracy of computerized medication histories can be suggested given the substantial numbers of omissions for over-the-counter products or a variety of supplements available elsewhere.[14] Under the current health care system in Japan, older patients enjoy free access to medical practitioners at their own discretion. Unless older patients are placed in certain types of care facilities such as nursing homes, where prescribing is sometimes restricted, presumably because of financial reasons, they can easily be at risk for polypharmacy, which has recently been identified as a medication safety issue. It has been reported that the risk for ADR increases as the number of medications a patient takes increases.[15] Although multiple definitions are use in the literature to define polypharmacy, if the most stringent criteria is applied,[16] the medication profiles of the patients being treated at all five institutions in the present survey fall into the category of polypharmacy, in which more than three drugs are prescribed regularly. However, as reported by Arai *et al.* [17] who investigated the incidence of ADR in geriatric inpatients of six university hospitals, the average incidence rate (9.2% of all cases) was lower than would be expected from the average number of medications, which exceeded five. Underestimations or neglect by attending physicians of symptoms related to adverse drug events might account for the discrepancy in the results from a previous report by Prybys *et al.*

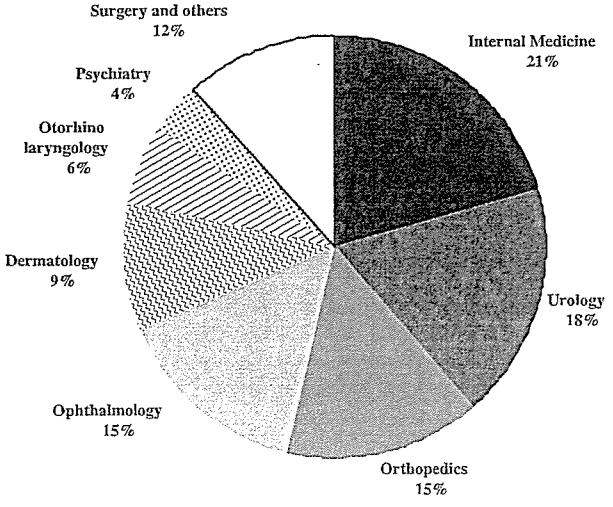


**Figure 1** Distribution of specialist referrals.

**Table 4** Number of inappropriate prescribing for the drugs listed in the Beers criteria

| Prescriber | Geriatricians | Other specialists |
|---|---|---|
| Ticlopidine | 47 | |
| Mid-long acting benzodiazepines | 20 | 5 |
| Oxybutynine | 13 | 3 |
| Dipyridamole | 6 | 1 |
| Alfacalcidol | 5 | 1 |
| Digoxin | 5 | |
| Tricyclic antidepressant | 4 | 1 |
| Disopyramide | 3 | 1 |
| Diclofenac | | 1 |
| Indometacin | | 1 |
| Phenobarbital | | 1 |

**Table 3** Comparison of variables with or without referral to non-geriatric specialists

| | Number of cases | Age | Number of disorders | Number of drugs |
|---|---|---|---|---|
| Referral (–) | 511 | 77 ± 9 | 3.5 ± 1.9 | 4.5 ± 2.7 |
| Referral (+) | 148 (22%) | 76 ± 8 | 3.3 ± 1.8 | 4.1 ± 2.9 |

All data except number of patients are expressed as mean ± SD.

showing that the risk for ADR increases to 58% for five medications.

Apart from cases of referrals within the same institution, physicians do not always monitor prescriptions made by other doctors, and because prescriptions can be changed over time, there is a possibility of medications overlapping or the inappropriate use of drugs. Contrary to our expectations, there was only one overlapping prescription in the present survey. Even though this study surveyed subjects who were attending geriatric outpatient units of university hospitals, where most of the referrals usually take place within the same institutions and medication records are shared across specialties by computerized prescription systems, a survey for tracking the record of consultations outside of each institution (e.g. consultation with local general practitioners) has not been implemented, and thus some overlapping or inappropriate use of drugs might have been overlooked.

As shown in Figure 1, referrals of the patients from the geriatric outpatient unit vary across specialties depending on the needs of each patient. Other than Internal Medicine, the majority of referrals are to specialists, whose expert knowledge and skills are helpful for the management of the common symptoms older patients exhibit (e.g. urinary incontinence, osteoporosis and related fractures, cataract, decubitus ulcers). Considering the circumscribed cases of referrals confirmed in the present survey, older patients attending geriatric outpatient units seem to regard geriatricians as their primary physicians responsible for the overall management of various clinical symptoms. To gain a view of this from the opposite perspective, it would be interesting to survey the status of referrals from other specialists to geriatricians. Regarding the adequacy of medications, geriatricians seem to prescribe more inappropriate drugs for older patients than other specialists in this survey. However such conclusions cannot readily be drawn, given the limited number of referral cases and drugs prescribed by other specialists. Comparing prescribing status to older patients by geriatricians with those by non-geriatric attending physicians under the matched clinical settings would address the question of whether geriatricians are more aware of potential ADR in older patients relative to other general/specialist physicians.

In conclusion, our results showing no significant difference in diagnostic and prescribing profiles between the patients who were referred and those who weren't, together with the relatively smaller incidence of inappropriate prescriptions by referrals to non-geriatric specialists, suggests that multiple consultations per se may not necessarily increase the risk for ADR in clinical settings.

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ATHEROSCLEROSIS

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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 $H_2O_2$. 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 $H_2O_2$-induce 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 ($0.18 \pm 0.01$ to $0.09 \pm 0.01$ mm$^2$, $P < 0.001$) and descending aorta ($5.09 \pm 0.86$ to $1.75 \pm 0.41$ mm$^2$, $P < 0.05$), 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.


*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 ($O_2^{\bullet -}$).

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 $O_2^{\bullet -}$ 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 ($^{\bullet}OH$) and show inhibitory effects on peroxynitrite ($ONOO^-$) and both water-soluble and lipid-soluble peroxyl radical ($LOO^{\bullet}$) [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 μmol/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

$F_{2\alpha}$) 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 express as mean ± 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 $H_2O_2$-induced EC apoptosis and ROS

As shown in Fig. 1A, edaravone dose-dependently inhibited EC apoptosis in culture, which was induced 24 h after $H_2O_2$ treatment. Edaravone was then employed in a rat model of $H_2O_2$-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 $H_2O_2$-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 $H_2O_2$ 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 $H_2O_2$ treatment in a time-dependent manner. Consequently, the effect of edaravone on 4-HNE expression was examined at 3 h after $H_2O_2$ treatment (4.5 h after $H_2O_2$ was initially added). Edaravone decreased 4-HNE expression in a dose dependent manner.

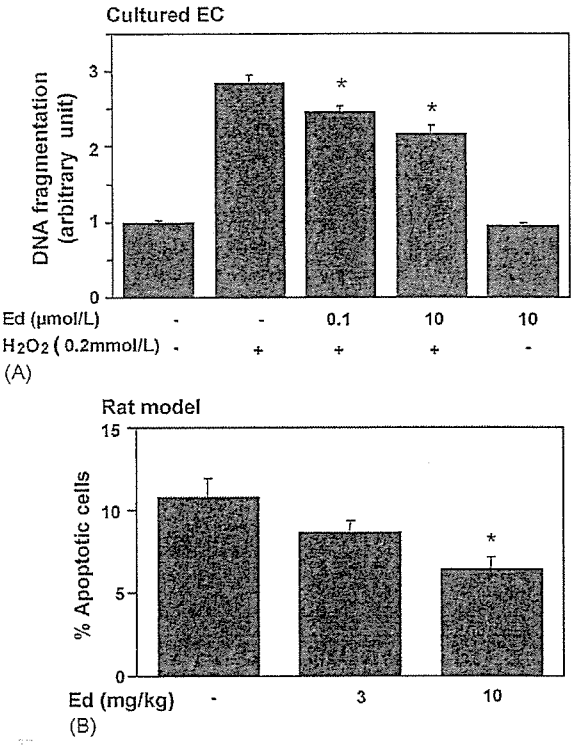


Fig. 1. Effects of edaravone (Ed) on $H_2O_2$-induced EC apoptosis in culture (A) and in a rat model (B). (A) Ed or its vehicle was added to the culture medium 24 h before $H_2O_2$ treatment until assay. EC apoptosis was evaluated 24 h after $H_2O_2$ treatment (0.2 mmol/L) by means of DNA fragmentation. Values are expressed as mean ± S.E.M. ($n = 3$). $^*P < 0.05$ vs. $H_2O_2$ (+) + Ed (−). (B) Ed or its vehicle was intraperitoneally injected once a day for 3 days before $H_2O_2$ treatment. At 24 h after $H_2O_2$ 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 ± 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

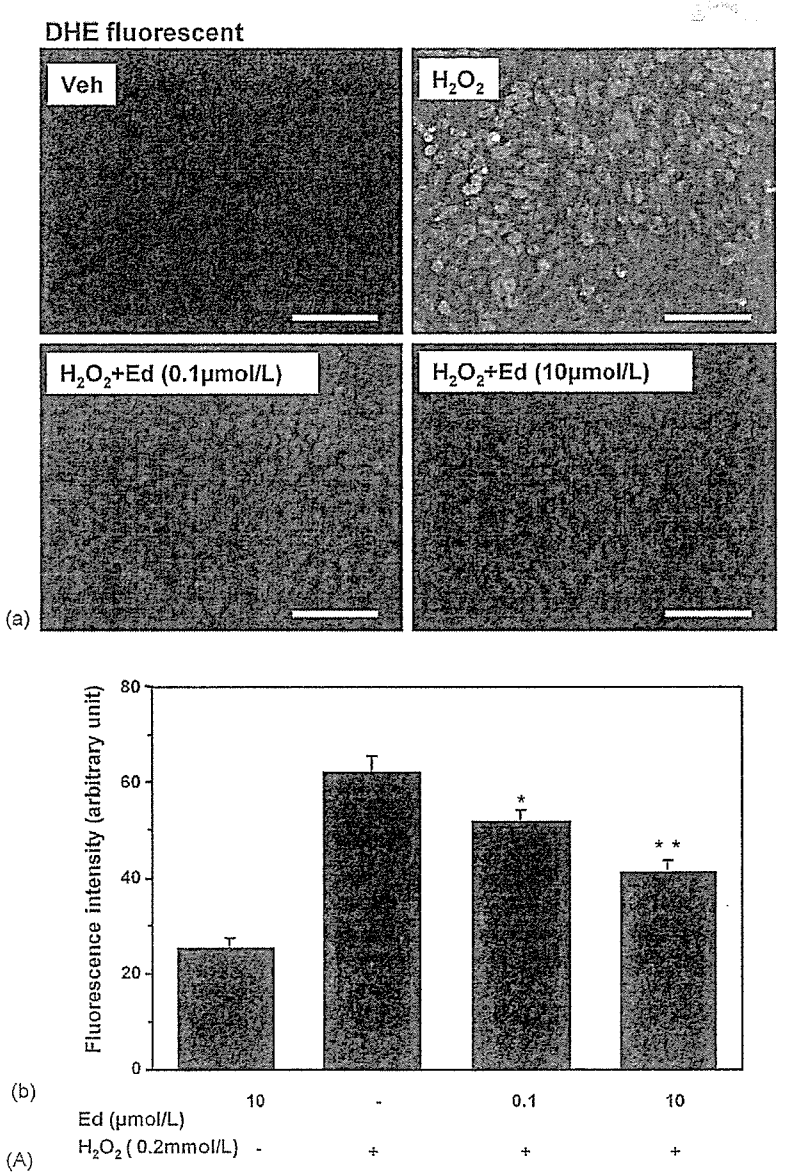


Fig. 2. Effects of edaravone (Ed) on DHE fluorescent (A) and 8-isoprostane formation (B), VCAM-1 expression (C) and 4-HNE expression (D) in cultured EC. Ed or its vehicle was added to the culture medium 24 h before $H_2O_2$ treatment until assay. DHE fluorescent ($n = 6$), 8-isoprostane concentration ($n = 3$) 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 (−).

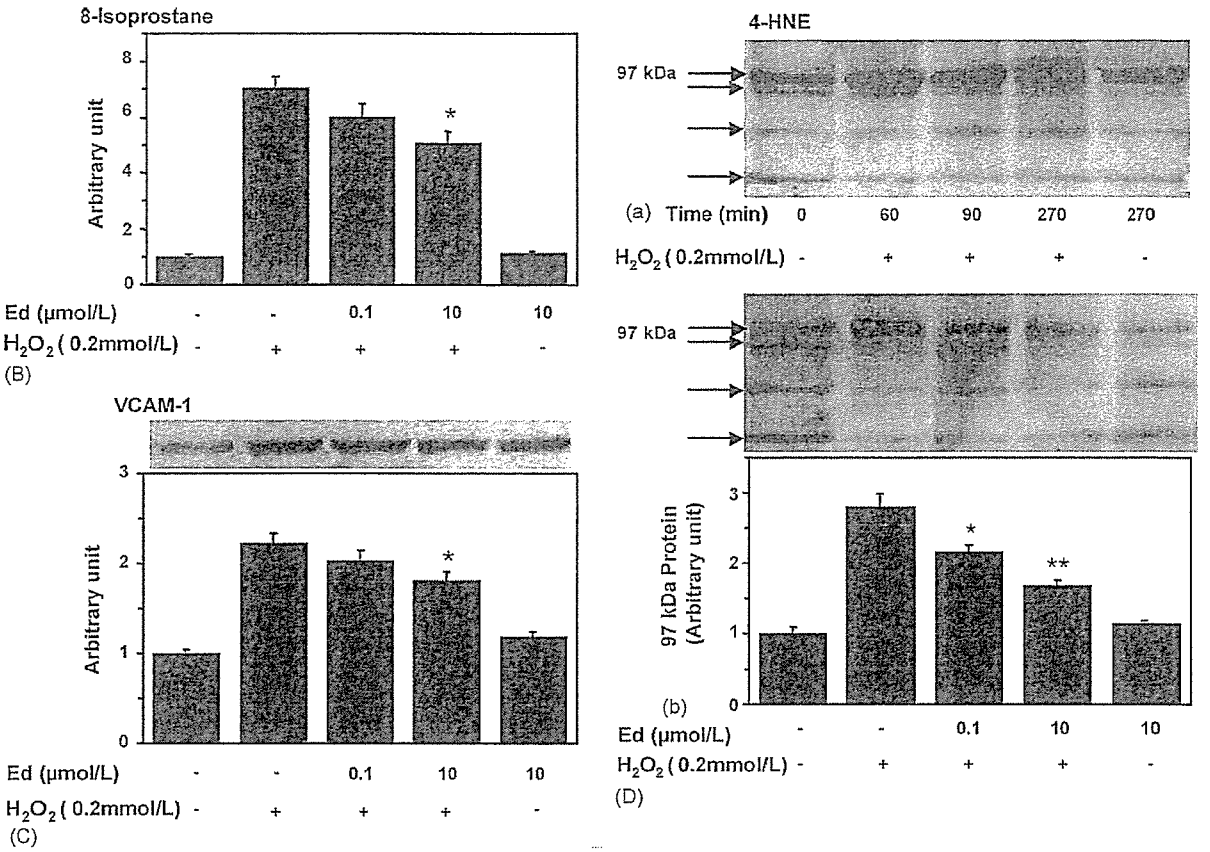


Fig. 2. (*Continued*).

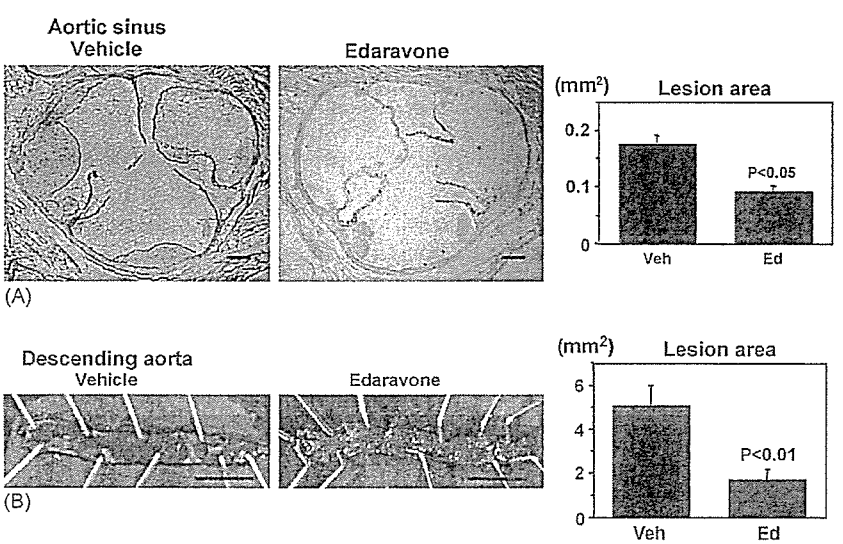


Fig. 3. Effects of edaravone on atherosclerotic lesion in ApoE-KO mice. ApoE-KO mice were fed a high-cholesterol diet for 4 weeks with the administration of edaravone (10 mg/kg daily) or its vehicle by i.p. injection. (A) Oil red O-stained cross-sections of the aortic sinus (bar = 100 μm) and morphometric 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 ± 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-

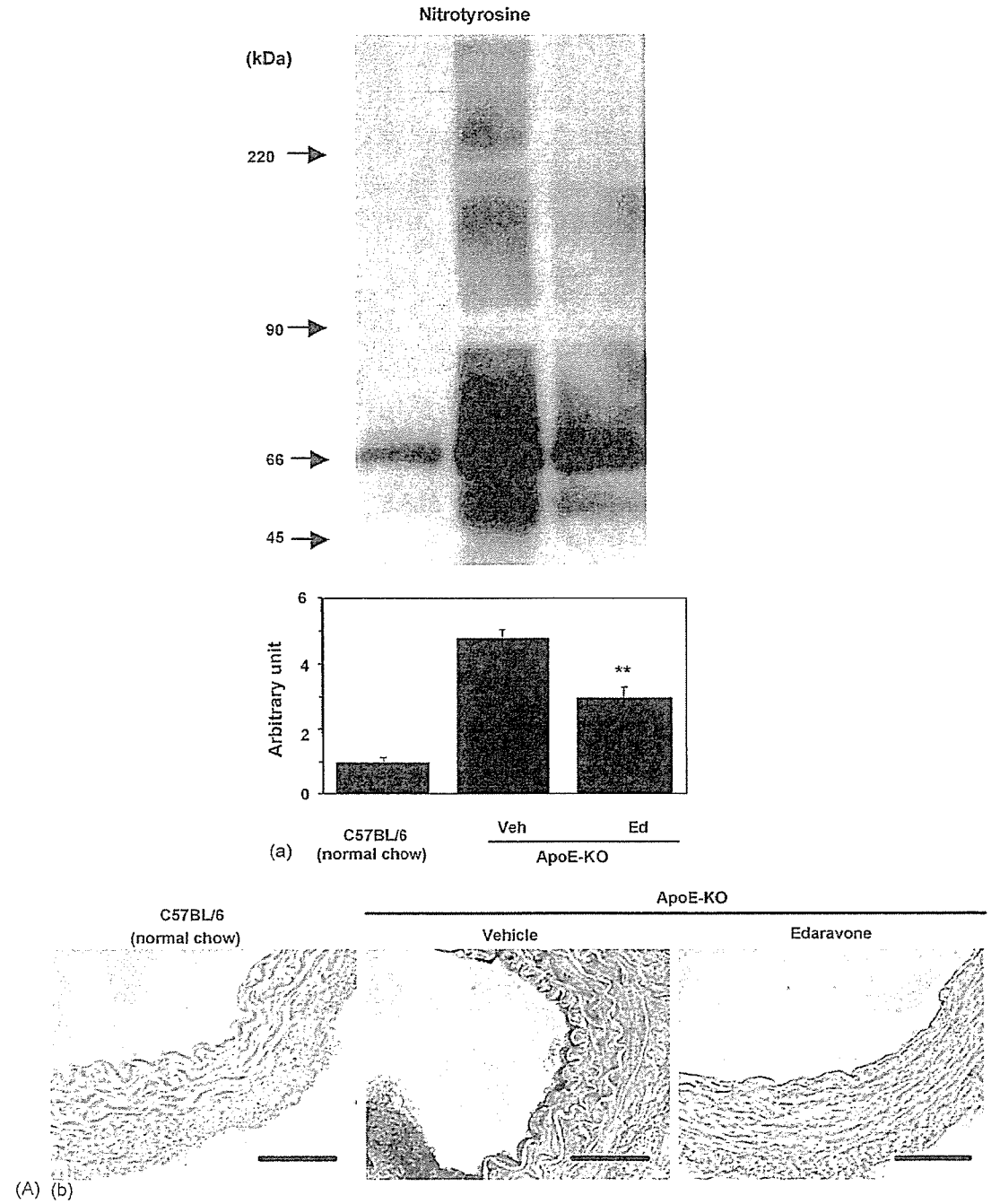


Fig. 4. Effects of edaravone (Ed) on ROS production (A–E) and VCAM-1 expression (F) in ApoE-KO mice. (A) Nitrotyrosine contents in the aorta was examined by Western blot analysis (A(a), $n=6$) and immunohistochemistry (A(b)). Bar = 50 μm. (B) Fresh-frozen cross-sections of the aorta were stained with DHE, and representative fluorescent micrographs are shown (bar = 100 μm). (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 ± S.E.M. $^{*}P<0.05$, $^{**}P<0.01$ vs. vehicle (Veh). C57/BL6 mice fed a normal chow serve as the control.

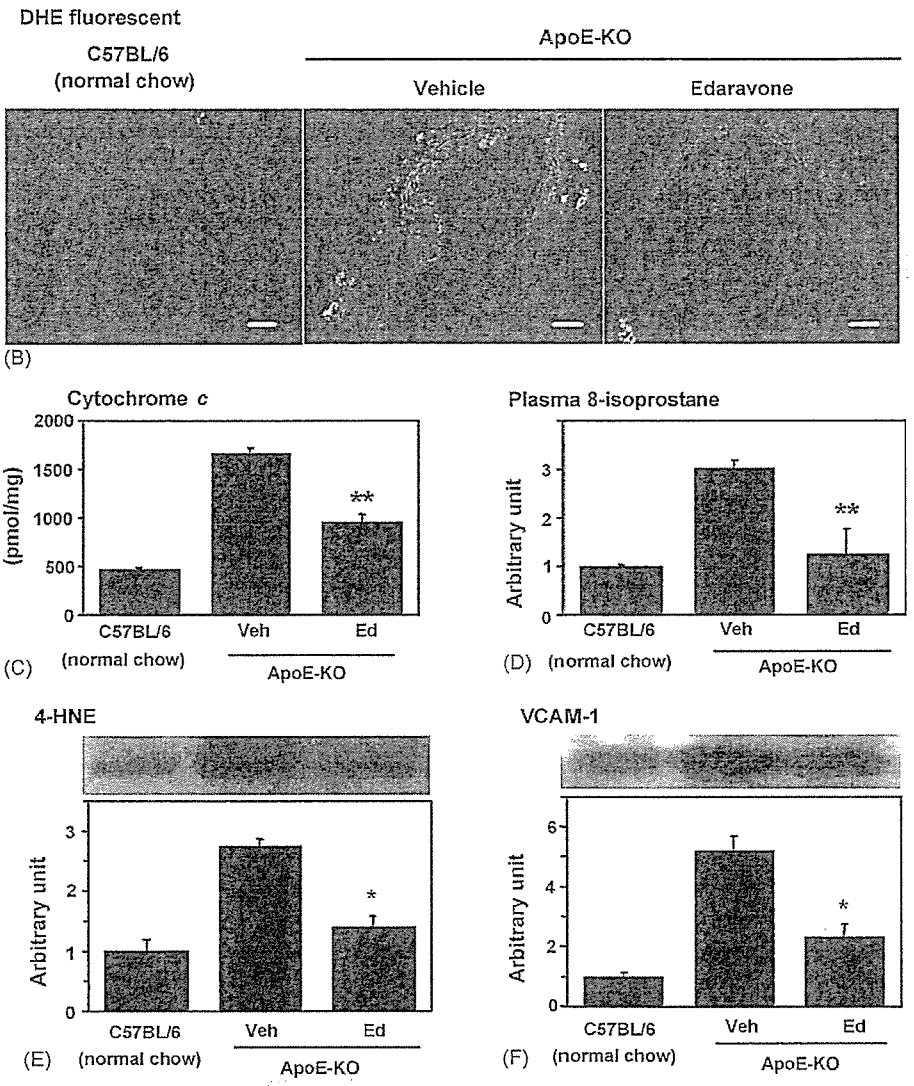


Fig. 4. (*Continued*).

duced via NAD(P)H oxidase [35], •OH as well as LOO• [36] and $ONOO^-$ [37] play a role in atherogenesis. In particular, •OH is extremely strong in terms of oxidative activity and cellular damage [38]. Therefore, it might be essential to scavenge the wide range of ROS for the prevention of atherosclerosis. As a matter of fact, recent clinical trials have denied the protective effects of Vitamin E, which predominantly reacts with LOO• [39], on cardiovascular events [18,19].

Edaravone, a potent free radical scavenger with unique properties, works by donating an electron from edaravone anion to free radicals [22]. Edaravone quenches •OH and inhibits both •OH-dependent and •OH-independent lipid peroxidation [22]. Edaravone shows inhibitory effects on both water-soluble and lipid-soluble LOO-induced peroxidation systems [22]. Edaravone also inhibits $ONOO^-$-induced tyrosine nitration [22]. These properties are different from those of water-soluble Vitamin C and lipid-soluble Vitamin E.

In the present study, we demonstrated that edaravone suppressed endothelial apoptosis and fatty streak formation. Reduced expression of VCAM-1, a marker of vascular injury and activation [32], were corroborated with these results. In cultured ECs, protein expression of VCAM-1 was induced as early as 3 h after $H_2O_2$ treatment (actually 4.5 h after addition of $H_2O_2$, Fig. 2C). This is reasonable based on our time course experiments (data not shown), and is consistent with the previous reports that VCAM-1 protein has been induced 4–6 h after cytokine stimulation through an antioxidant-sensitive mechanism [40,41]. Although the experimental conditions were different between the cell culture and animal studies, edaravone inhibited both the rapid induction of VCAM-1 in cultured ECs and the chronic upregulation of VCAM-1 in the aorta of ApoE-KO mice, further supporting the vasoprotective effects of edaravone.

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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# Statins Protect Human Aortic Smooth Muscle Cells From Inorganic Phosphate-Induced Calcification by Restoring Gas6-Axl Survival Pathway

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*Abstract*—Vascular calcification is clinically important in the development of cardiovascular disease. It is reported that hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors (statins) inhibited vascular calcification in several clinical trials. However, the mechanism is poorly understood. Recently, it has been suggested that apoptosis is one of the important processes regulating vascular smooth muscle cell (VSMC) calcification. In this study, we investigated the effect of statins on VSMC calcification by testing their effect on apoptosis, focusing in particular on regulation of the survival pathway mediated by growth arrest-specific gene 6 (Gas6), a member of the vitamin K–dependent protein family, and its receptor, Axl. In human aortic smooth muscle cells (HASMC), statins significantly inhibited inorganic phosphate (Pi)-induced calcification in a concentration-dependent manner (reduced by 49% at 0.1 μmol/L atorvastatin). The inhibitory effect of statins was mediated by preventing apoptosis, which was increased by Pi in a concentration-dependent manner, and not by inhibiting sodium-dependent phosphate cotransporter (NPC) activity, another mechanism regulating HASMC calcification. Furthermore, the antiapoptotic effect of statins was dependent on restoration of Gas6, whose expression was downregulated by Pi. Restoration of Gas6 mRNA by statins was mediated by mRNA stabilization, and not by an increase in transcriptional activity. Suppression of Gas6 using small interfering RNA and the Axl-extracellular domain abolished the preventive effect of statins on Pi-induced apoptosis and calcification. These data demonstrate that statins protected HASMC from Pi-induced calcification by inhibiting apoptosis via restoration of the Gas6-Axl pathway. (***Circ Res.* 2006;98:1024-1031.**)

**Key Words:** calcification ■ statins ■ apoptosis ■ Gas6 ■ Axl

Vascular calcification, such as coronary and aortic calcification, is a significant feature of vascular pathology, because this lesion is associated with cardiovascular disease.[1,2] It has been recognized that statins exhibit various protective effects against atherosclerosis, including modification of endothelial function,[3] decreased inflammation,[4] and inhibition of vascular smooth muscle cell (VSMC) proliferation and migration,[5] all of which cannot be accounted for by lipid reduction. One of the interesting pleiotropic effects of statins is the inhibition of vascular calcification. Results from clinical trials suggest an association of statin use with slowed progression of calcific aortic stenosis[6–8] and coronary artery calcification.[9] Statins also inhibited calcification of atherosclerotic plaques in experimental hyperlipidemic animals.[10,11] On the other hand, some recent clinical trials were not able to find such an inhibitory effect.[12,13] To clarify these discrepancies, it is important to identify the detailed regulatory mechanism of vascular calcification and the target of effect of statins.

Based on clinical findings,[14] inorganic phosphate (Pi) has been shown to be an important inducer of VSMC calcification, which is morphologically similar to that observed in calcified human heart valves and the aortic media. Transport of Pi into VSMC has been suggested to play an important role in the initiation of extracellular matrix calcification.[15] Recently, it has been shown that similar structures to matrix vesicles, derived from apoptotic VSMC, have been identified in human calcified arteries.[16] These vesicles have the capacity to concentrate and crystallize Ca, initiating calcification. Pi has been shown to induce apoptosis of hypertrophic chondrocytes, which is associated with cell maturation and extracellular matrix mineralization.[17] However, it is not clear whether or not apoptosis plays a regulatory role in the occurrence of VSMC calcification induced by Pi.

Recently, it was shown that growth arrest-specific gene 6 (Gas6), a member of the vitamin K–dependent protein family, and its receptor, Axl, a membrane receptor tyrosine kinase, are decreased on calcification of vascular pericytes.[18]

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Gas6 is a secreted protein that harbors a γ-carboxylglutamic acid–rich domain and 4 epidermal growth factor–like repeats.[19] Gas6-Axl interaction has been shown to be implicated in the regulation of multiple cellular functions, including growth, survival, adhesion, and chemotaxis.[20–23] In particular, they are known to protect a range of cell types from apoptotic death. However, there is no evidence that Gas6-Axl interaction is involved in Pi-induced apoptosis and calcification of VSMC.

In the present study, we found that statins inhibited Pi-induced calcification by preventing apoptosis in human aortic smooth muscle cells (HASMC). The effect of statins was dependent on restoration of the Gas6-Axl pathway. Furthermore, this beneficial effect was mediated by Gas6 mRNA stabilization, and not by increasing the transcription rate. Our results reveal a novel pathway by which statins regulate Pi-induced calcification in HASMC.

## Materials and Methods

### Materials

Pravastatin, atorvastatin, and fluvastatin were supplied by Sankyo Co Ltd, Pfizer Inc (New York), and Tanabe Seiyaku Co Ltd, respectively. Recombinant human Gas6 (rhGas6) and Axl-ECD were prepared as described previously.[22,24] All other reagents were of analytical grade.

### Cell Culture

HASMC were obtained from Clonetics. They were cultured in DMEM supplemented with 20% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in a humidified atmosphere with 5% $CO_2$. HASMC were used up to passage 8 for the experiments.

### Induction and Quantification of Calcification

For Pi-induced calcification, Pi (a mixed solution of $Na_2HPO_4$ and $NaH_2PO_4$ whose pH was adjusted to 7.4) was added to serum-supplemented DMEM to final concentrations of 2.0, 2.6, and 3.2 mmol/L ("calcification medium"). After the indicated incubation period, cells were decalcified with 0.6 mol/L HCl, and Ca content in the supernatant was determined by the *o*-cresolphthalein complexone method (C-Test, WAKO). The remaining cells were solubilized in 0.1 mol/L NaOH/0.1% SDS, and cell protein content was measured by Bio-Rad protein assay. Calcification was visualized by von Kossa's method. Briefly, the cells were fixed with 4% formaldehyde and exposed to 5% aqueous $AgNO_3$.

### Induction of Apoptosis

Two different time courses were tested to investigate Pi-induced apoptosis and examine the effect of statins. (1) Short-term condition: Pi was added at final concentrations of 2.0, 2.6, and 3.2 mmol/L for 24 hours at confluence, after the cells were incubated with serum-free DMEM for 48 hours. To test the effect of statins on apoptosis, they were added 24 hours after incubating the cells with serum-free DMEM (12 hours before adding Pi). (2) Long-term condition: at confluence, the medium was switched to calcification medium and cells were cultured for up to 10 days. The medium was changed every 2 days. To test the effect of statins, each was added simultaneously when the medium was switched to the calcification medium.

### RNA Extraction, Northern Blot, and mRNA Stability Analysis

The 304-bp product of the Gas6 cDNA probe (forward, 5′-GCGTGGCCAAGAGTGTGAAGT-3′; reverse, 5′-CGCCACTCC-TCAACAGAGAT-3′) was amplified by RT-PCR. For Northern blot analysis, harvested RNA (≈5 to 10 μg) was fractionated on 1.4% formaldehyde-agarose gel and transferred to a nylon filter. The filter was hybridized at 68°C for 2 hours with $^{32}$P-labeled Gas6 cDNA and 18S probe in QuickHyb solution (Stratagene) and autoradiographed. To examine Gas6 mRNA stability, serum-starved HASMC were incubated with actinomycin D (Act D, 5 μg/mL) in the presence of 2.6 mmol/L Pi after 12 hours of atorvastatin (0.1 μmol/L) treatment. Total RNA was harvested at 0, 1, 3, and 6 hours for Northern blot analysis. Signal density of the Gas6 mRNA was normalized to that

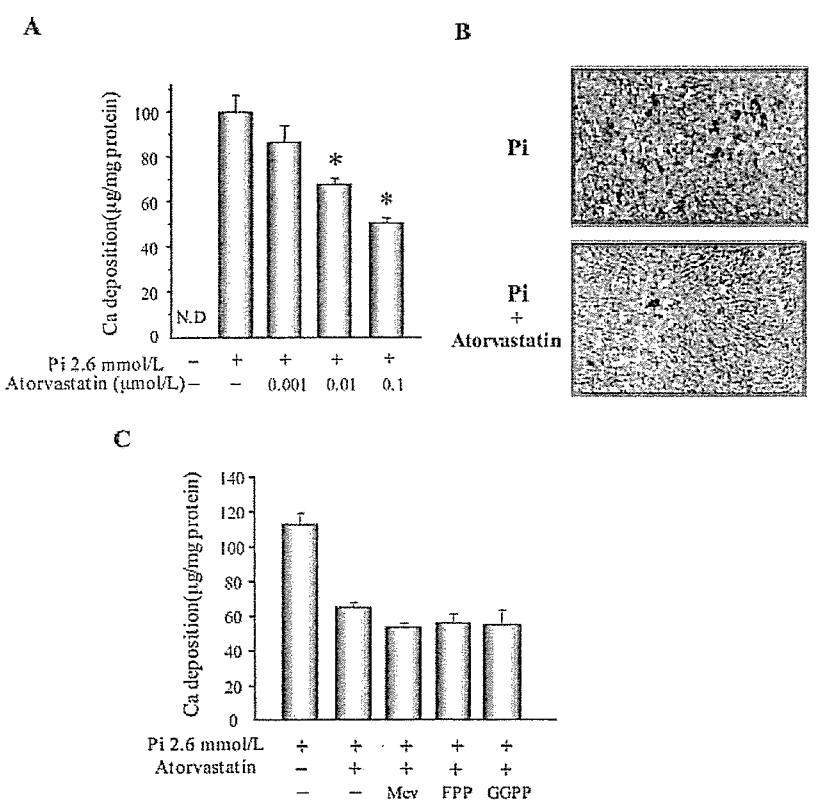


**Figure 1.** Statins prevent HASMC calcification. A, HASMC were cultured with the indicated concentrations of atorvastatin in the presence of 2.6 mmol/L Pi for 6 days. Ca deposition was measured by *o*-cresolphthalein complexone method and normalized by cell protein content. All values are presented as mean±SEM (n=6). *$P<0.05$ vs statin (-) by Fisher's test. N.D. indicates not detected. B, On day 6, the inhibitory effect of atorvastatin (0.1 μmol/L) on 2.6 mmol/L Pi-induced Ca deposition was evaluated at the light microscopic level with von Kossa's staining. The arrow points to an area of Ca deposition. C, HASMC were cultured with mevalonate (100 μmol/L), farnesylpyrophosphate (1 μmol/L), or geranylgeranylpyrophosphate (1 μmol/L) in the presence of atorvastatin (0.1 μmol/L) and 2.6 mmol/L Pi for 6 days. All values are presented as mean±SEM (n=6).

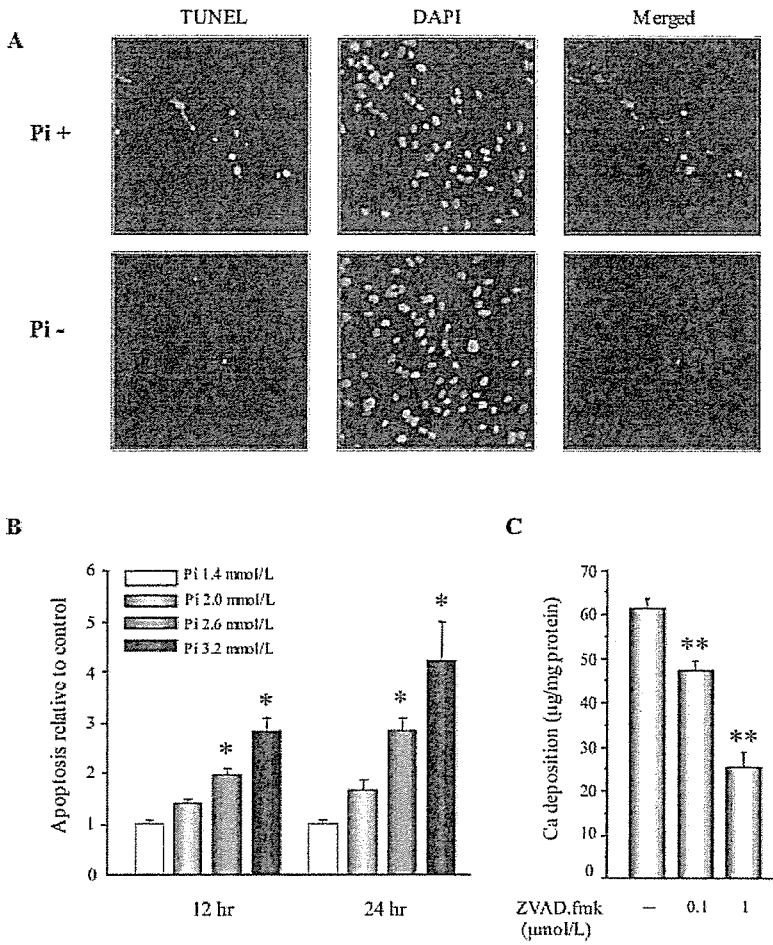


**Figure 2.** Pi induces apoptosis, and ZVAD.fmk inhibits Pi-induced calcification. A, After incubation with 1.4 (Pi-) and 3.2 mmol/L (Pi+) Pi for 10 days, apoptotic cells were identified by TUNEL staining (green). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). B, Serum-starved HASMC were cultured with the indicated concentration of Pi for 24 hours. A quantitative index of apoptosis, determined by ELISA, is presented as the relative value to that with 1.4 mmol/L Pi. All values are presented as mean±SEM (n=3). *$P<0.05$ vs 1.4 mmol/L Pi by Fisher's test. C, HASMC were incubated with the indicated concentration of ZVAD.fmk in the presence of 2.6 mmol/L Pi for 6 days. Ca content was measured and normalized by cell protein content. All values are presented as mean±SEM (n=6). **$P<0.01$ vs 2.6 mmol/L Pi, ZVAD.fmk(-) by Fisher's test. Experiments were performed with at least 3 different cell populations.

of the 18S RNA at each time point, and the half-life was calculated by linear extrapolation.

### Preparation of Small Interfering RNA Targeting Gas6 and Transfection

Two small interfering RNAs (siRNAs) were designed to target human Gas6 (accession no. NM_000820) using siRNA design software (Dharmacon). The sequences for Gas6 were 5′-GGACCTGCCAAGACATAGA-3′ and 5′-ACCTCGTGCAGCCT-ATAAA-3′. Nonspecific control siRNA was synthesized using standard templates (Dharmacon). Twenty-four hours after HASMC seeding onto 12-well plates, cells were cultured in serum-free medium for an additional 24 hours, then transfected with Gas6 (100 nmol/L) and control siRNA using transfection reagent (Upstate). To evaluate the effect of Gas6 siRNA on Ca deposition, siRNA was transfected when HASMC had reached 80% to 90% confluence and then transfected every time the medium was changed (every 2 days) up to 6 days. The loss of Gas6 by transfection of siRNA was validated by immunoblotting for Gas6 protein in the cell lysates 48 hours and 6 days after siRNA transfection.

### Statistical Analysis

All results are presented as mean±SEM. Statistical comparisons were made by ANOVA, unless otherwise stated. A value of $P<0.05$ was considered to be significant.

An expanded Materials and Methods section can be found in the online data supplement available at http://circres.ahajournals.org.

## Results

### Statins Inhibit Pi-Induced HASMC Calcification

To induce HASMC calcification, cells were incubated with calcification medium for 10 days. We confirmed that high phosphate (≥2.6 mmol/L) induced Ca deposition in a concentration- and time-dependent manner, whereas 1.4 mmol/L Pi, equivalent to the human physiological serum phosphate level, was not able to induce Ca deposition up to 10 days. To investigate the effect of statins on Pi-induced calcification, HASMC were incubated with atorvastatin in the presence of 2.6 mmol/L Pi. On day 6, Ca deposition was significantly suppressed by atorvastatin in a concentration-dependent manner (51.1±1.9% of control at 0.1 μmol/L) (Figure 1A). An inhibitory effect of the statins on Ca deposition was also found by von Kossa's staining (Figure 1B). Atorvastatin was able to be added at as high a concentration as 0.1 μmol/L without cell damage. The inhibitory effect was also observed with fluvastatin (0.001 to 0.1 μmol/L) and pravastatin (0.01 to 50 μmol/L) (data not shown). The inhibitory effect of statins was not blocked by mevalonate (100 μmol/L), farnesylpyrophosphate (1 μmol/L), or geranylgeranylpyrophosphate (1 μmol/L), suggesting that the effect is not dependent on the mevalonate pathway (Figure 1C).

### Inhibitory Effect of Statins on Calcification Is Caused by Preventing Apoptosis, Not by Inhibiting Sodium-Dependent Phosphate Cotransporter Activity

Two different time courses were tested to examine the effect of Pi on HASMC apoptosis: short-term (up to 24 hours) and long-term (up to 10 days; practical time course of calcifica-

tion process). During calcification, Pi increased the rate of apoptotic cell death detected by terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) assay (Figure 2A). Furthermore, cytoplasmic histone-associated DNA fragments determined by ELISA, as a quantitative index of apoptosis, were also increased by Pi in a concentration- and time-dependent manner in both short-term (Figure 2B) and long-term conditions (supplemental Figure I). In addition, caspase 3 activation, detected by immunoblotting, by 2.6 mmol/L Pi was observed in short-term and long-term conditions (data not shown). To investigate the relationship between apoptosis and calcification, we used ZVAD.fmk, a general caspase inhibitor. We found that ZVAD.fmk significantly inhibited Pi-induced apoptosis as well as calcification in a concentration-dependent manner (Figure 2C).

It has been reported that sodium-dependent phosphate cotransporter (NPC) activity is an important pathway regulating Pi-induced HASMC calcification.[25] We confirmed that type III NPC (Pit-1) was expressed in the HASMC that we used, and its activity was enhanced by Pi treatment. Furthermore, a specific inhibitor of NPC, phosphonoformic acid (PFA), inhibited Ca deposition (reduced by 90.4% at 0.1 μmol/L), indicating that NPC-mediated Pi uptake is also essential for HASMC calcification.

To investigate the mechanisms of these statins, we examined the effect of atorvastatin on apoptosis and NPC activity. Atorvastatin, at concentrations exerting inhibition of calcification, reduced apoptosis in a concentration-dependent manner (Figure 3A). A beneficial effect of statins was also observed in the long-term condition (supplemental Figure II). On the other hand, statins did not inhibit NPC activity induced by Pi treatment (Figure 3B).

## Downregulation of Gas6-Axl Interaction Is Associated With Pi-Induced Apoptosis

Immunoblot analysis showed that the expression of Gas6 and Axl was markedly downregulated by 2.6 mmol/L Pi in both short-term (Figure 4A) and long-term (supplemental Figure III) conditions. To further examine whether Pi affects the secretion of Gas6 by HASMC, conditioned medium was collected after Pi treatment. Gas6 production in the medium was reduced by 2.6 mmol/L Pi, along with a reduction in its intracellular expression (Figure 4B). Gas6 production was also reduced in an immunoprecipitation-immunoblotting study on day 10 (Figure 4C). Next, to investigate the role of Gas6-Axl interaction in the process of apoptosis and calcification, rhGas6 and Axl-ECD were supplemented in Pi-treated HASMC. The addition of rhGas6 significantly inhibited both Pi-induced apoptosis and calcification. Addition of Axl-ECD to block the binding of Gas6 to Axl clearly abrogated the inhibitory effect of rhGas6 (Figure 4D and 4E). These results indicate that Pi-induced apoptosis and calcification are associated with downregulation of the Gas6-Axl interaction.

## Statin-Mediated Induction of Gas6 Expression Is Dependent on mRNA Stabilization, Not on Transcription

To investigate whether the antiapoptotic effect of statins is dependent on restoration of the Gas6-Axl interaction, we first assessed the effect of statins on Gas6 expression. As shown in Figure 5A, atorvastatin increased Gas6 expression, which was downregulated by Pi at both the mRNA and protein levels. Upregulation of Gas6 expression was also observed in the long-term condition (supplemental Figure IV). Furthermore, to elucidate the mechanism of statins on restoration of Gas6 mRNA, a promoter study was undertaken. Reporter assay using the −1.9 kb Gas6-luciferase DNA construct revealed that atorvastatin did not have a significant effect on Gas6 promoter activity (supplemental Figure V), as well as mRNA expression under the condition in which it was significantly inhibited by PDGF-BB (data not shown). Next, we investigated the effect of atorvastatin on mRNA stabilization using an RNA polymerase inhibitor, actinomycin D (ActD). As shown in Figure 5B, Gas6 mRNA expression was more stable in the presence of atorvastatin than in its absence under Pi and ActD treatment. The half-life was 15.9 hours with atorvastatin and 5 hours without atorvastatin, suggesting the capacity of statins to improve Gas6 mRNA stabilization (Figure 5C). Taken together, these findings suggest that the restoration of Gas6 mRNA by statins appears to be mediated by decreasing the mRNA degradation rate, and not by stimulating transcriptional activity.

Furthermore, to determine whether Gas6 is required for statin-mediated effects, we tried to knock down the action of

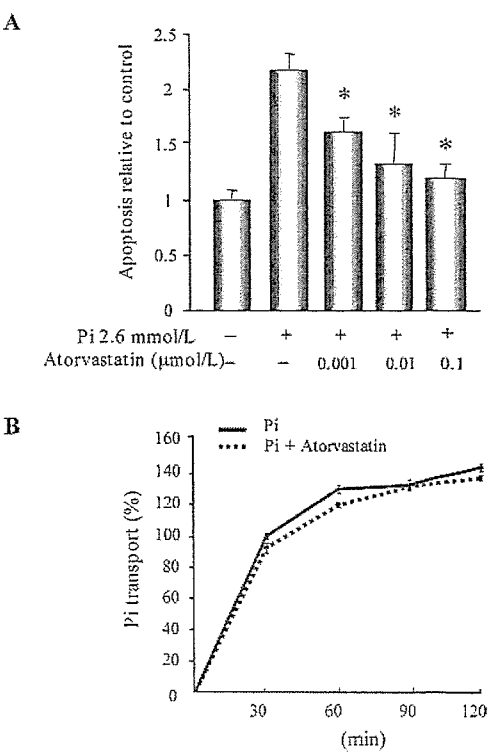


Figure 3. Effect of atorvastatin on Pi-induced apoptosis and NPC activity. A, HASMC were cultured with the indicated concentration of atorvastatin for 12 hours and then incubated with 2.6 mmol/L Pi for an additional 24 hours. All values are presented as mean±SEM (n=3). *$P<0.05$ vs 2.6 mmol/L Pi, statin (-) by Fisher's test. B, HASMC were treated with (dotted line) or without (solid line) 0.1 μmol/L atorvastatin in the presence of 2.6 mmol/L Pi. On day 6, NPC activity was determined in Earl's balanced salt solution containing 0.1 mmol/L $H_3{}^{32}PO_4$ (1 μCi/mL) with 143 mmol/L sodium chloride for the indicated period. All values are presented as mean±SEM (n=6).

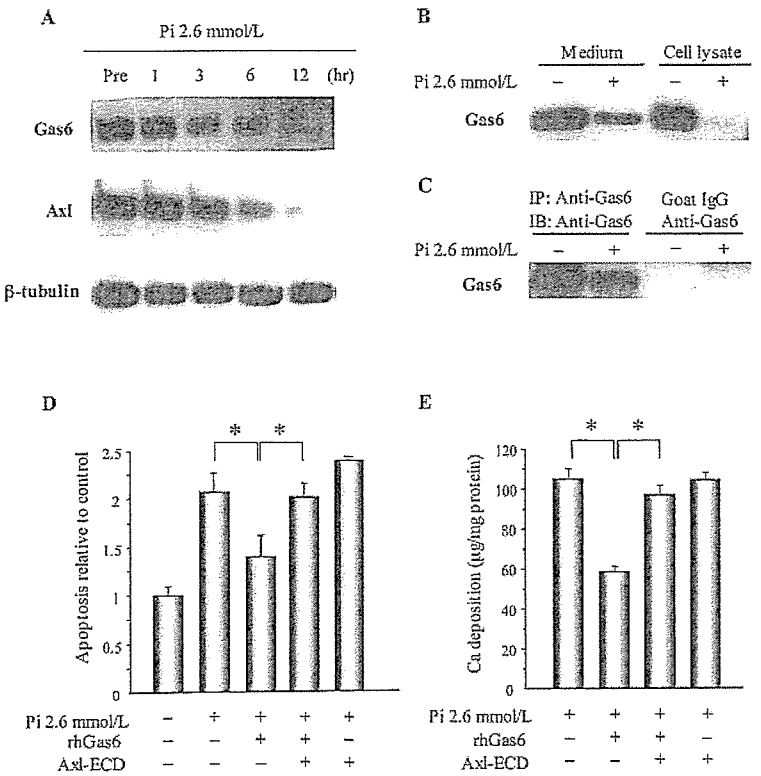


**Figure 4.** Pi reduces production of Gas6 and Axl, and rhGas6 inhibits Pi-induced apoptosis and calcification via Axl. A, HASMC were cultured in the presence of 2.6 mmol/L Pi for 12 hours. Cell lysates were subjected to SDS-PAGE followed by immunoblotting with antibodies to Gas6, Axl, or β-tubulin. B, Conditioned medium of HASMC in the absence (lane 1) or presence (lane 2) of 2.6 mmol/L Pi at 12 hours was concentrated and separated by SDS-PAGE along with cell lysates. C, Conditioned medium of HASMC on day 10 in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 2.6 mmol/L Pi was subjected to immunoprecipitation with anti-Gas6 antibody (lanes 1 and 2) or control goat IgG (lanes 3 and 4). Precipitates were immunoblotted with anti-Gas6 antibody. D, After pretreatment with rhGas6 (400 ng/mL) with or without Axl-ECD (1 μg/mL), apoptosis was induced by 2.6 mmol/L Pi. All values are presented as mean±SEM (n=3). *$P<0.05$ by Fisher's test. E, For measurement of Ca deposition, HASMC were cultured with rhGas6 (400 ng/mL) with or without Axl-ECD (1 μg/mL) in the presence of 2.6 mmol/L Pi for 6 days. All values are presented as mean±SEM (n=6). *$P<0.05$ by Fisher's test. Experiments were performed with at least 3 different cell populations.

Gas6 and examined the effect of atorvastatin on Pi-induced apoptosis and calcification. Transfection of Gas6 siRNA markedly decreased Gas6 expression in the short-term and long-term conditions (Figure 6A). The inhibitory effect of atorvastatin on Pi-induced apoptosis and calcification was reversed by Gas6 siRNA (Figure 6B and 6C). Similarly, the beneficial effect of atorvastatin was also abolished by blocking the binding of Gas6 to Axl using Axl-ECD (Figure 6D and 6E). These data support a critical role of Gas6 in the preventive effect of statins on apoptosis and calcification.

## Discussion

The present study demonstrated that statins protected HASMC from Pi-induced calcification. The clinical effect of statins on vascular calcification is controversial. Many retrospective clinical studies[6,7,9] and a prospective study[8] have shown beneficial effects, whereas recent prospective studies were unable to show such effects.[12,13] The reason is not yet clear, and the time window of statin use has been raised as an important matter. The discrepancy may also derive from the complex in vivo effects of statins. In this regard, it is important to analyze the detailed regulatory mechanism of statins in a simple model.

In Pi-induced calcification, HASMC undergo apoptosis. A causal link between apoptosis and calcification was evident from the finding that both apoptosis and calcification were inhibited by the general caspase inhibitor, ZVAD.fmk. As reported previously,[25] we confirmed that NPC-mediated Pi uptake is another essential mechanism for HASMC calcification. Given that apoptosis does not always lead to calcification, Pi-induced HASMC calcification is presumably dependent on both an NPC-mediated phenotypic transition from SMC to an osteoblastic phenotype and apoptotic cell death. With respect to the mechanism of action of statins, they clearly inhibited Pi-induced apoptosis, although they did not have an effect on Pi-induced NPC activity or osteoblastic differentiation; Pi-induced upregulation of matrix Gla protein (MGP) mRNA was not inhibited by atorvastatin (supplemental Figure VI). These results suggest that apoptosis is the target of statins in inhibiting HASMC calcification.

Another important signal in Pi-induced calcification is an increase in intracellular Ca ($[Ca^{2+}]_i$). Statins have been shown to inhibit VSMC proliferation[5] and reduce the acute increase of $[Ca^{2+}]_i$ in a mevalonate and isoprenoid pathway–independent manner.[26] On the other hand, $[Ca^{2+}]_i$ is reported to modulate Pi-induced apoptosis of terminally differentiated chondrocytes.[27] Therefore, modulation of $[Ca^{2+}]_i$ is another possible mechanism of the inhibition of apoptosis by statins. In this study, we investigated the association of proliferation with Pi-induced apoptosis and calcification. We found that Pi did not affect proliferation, measured by the incorporation of 5-bromo-2′-deoxyuridine (BrdU) during calcification (data not shown). We also found that the inhibitory effect of statins on calcification was not affected by an inhibitor of Rho kinase (Y-27632), an important modulator of the mevalonate and isoprenoid pathway affecting proliferation and apoptosis (supplemental Figure VII). These results suggest that proliferation is not associated with Pi-induced calcification. The inhibitory effect of statins on calcification was not blocked by mevalonate, farnesylpyrophosphate, geranylgeranylpyrophosphate, or Rho kinase inhibitor, suggesting that the effect of statins is not dependent on the mevalonate and isoprenoid pathways. Indeed, a mevalonate pathway–independent effect of statins has been reported previously,[26,28–30] although the precise mechanism has not been shown. The pleiotropism of statins is of continuing interest.