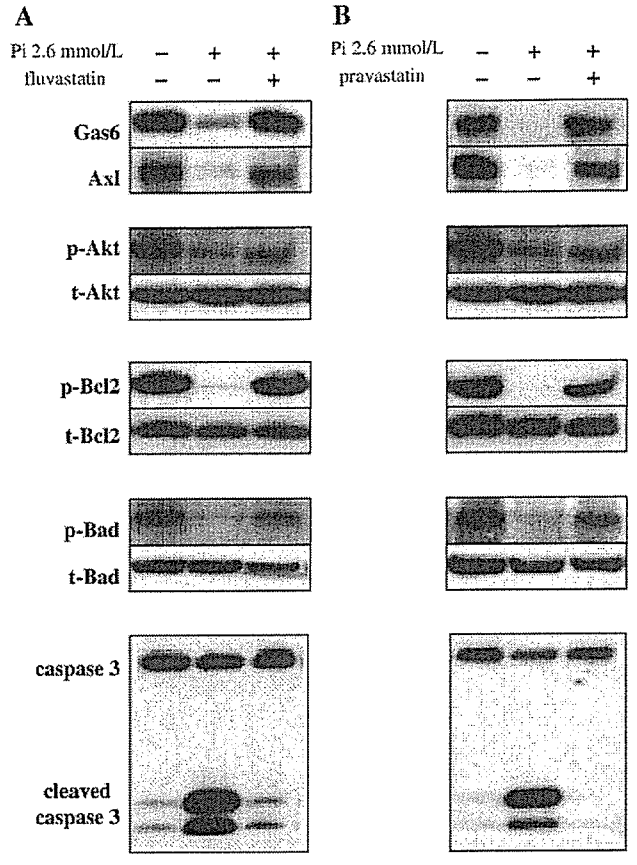

Fig. 5. Antiapoptotic effect of statins is associated with upregulation of Gas6-Axl survival pathway. After pretreatment with 0.1 μM fluvastatin (A) and 50 μM pravastatin (B) for 12 h, apoptosis was induced by 2.6 mM Pi. After 12 h, cell lysates were collected and subjected to SDS-PAGE followed by immunoblotting with antibodies that recognize Gas6 and Axl, with phospho-specific Akt (p-Akt) and total Akt (t-Akt) antibody, with phospho-specific Bcl2 (p-Bcl2) and total Bcl2 (t-Bcl2) antibody, or with phospho-specific Bad (p-Bad) and total Bad (t-Bad) antibody. Cell lysates were immunoblotted with an antibody that recognizes uncleaved caspase-3 (35 kDa) and the cleaved forms of caspase-3 (17 and 19 kDa).

Bad, with total expression unchanged. Pi-induced caspase 3 activation was also prevented by both statins. Taken together, these findings suggest that the inhibitory effect of statins on Pi-induced apoptosis is mediated by restoration of the Gas6-mediated survival pathway; PI3K-induced Akt phosphorylation, Bcl2 activation, Bad inactivation, and caspase 3 inactivation.

4. Discussion

In the present study, we found that both lipophilic fluvastatin and hydrophilic pravastatin protected against Pi-induced apoptosis and calcification in HASMC, as we found with atorvastatin previously. With regard to the different potency of statins, we found that the inhibitory effect of pravastatin was inferior to those of fluvastatin and atorvastatin, which exerted similar effects on calcification and apoptosis. This might relate to our previous finding that the inhibition of calcification by statins was not dependent on the mevalonate pathway (Son et al., 2006). Consequently, the inhibitory effect on calcification was not parallel to the cholesterol-lowering effect. We speculate that the difference between statins was derived from their affinity to *vascular smooth muscle cells* (VSMC), that is, lipophilic statins have stronger effects on VSMC calcification than hydrophilic statins.

The antiapoptotic effect of statins was induced by restoration of the Gas6-mediated survival pathway: PI3K-induced Akt phosphorylation, Bcl2 and Bad phosphorylation, and caspase 3 inactivation. Gas6 plays a crucial role in the effect of statins on Pi-induced apoptosis. Gas6, a secreted vitamin K-dependent protein, binds to the receptors of the mammalian Axl protein-tyrosine kinase family; Axl, Sky, and Mer, with different affinities (Nagata et al., 1996). Gas6 and Axl have been shown to localize in the neointima of the artery after balloon injury, in which they presumably modulate several cell functions such as differentiation, adhesion, migration, proliferation, and survival in a cell-specific manner (Melaragno et al., 1998). The Gas6-Axl interaction is also shown to upregulate scavenger receptor A expression in VSMC (Ming et al., 2001), and facilitates the clearance of apoptotic cells by macrophages (Ishimoto et al., 2000). Of the above functions, protection against apoptotic cell death has been most studied (Goruppi et al., 1996; Healy et al., 2001; Lee et al., 2002; Nakano et al., 1996). Consistently, the expression of Gas6 and Axl was downregulated by Pi, leading to apoptosis and subsequent calcification.

Several intracellular signaling pathways mediated by Gas6-Axl interaction have been shown previously (Goruppi et al., 1999; Lee et al., 2002; Ming et al., 2001). Akt, which is necessary for Gas6-dependent survival, is a critical downstream effector of the PI3K-dependent antiapoptotic pathway. In VSMC, it has been reported that the PI3K-Akt pathway mediates Gas6 induction of scavenger receptor A (Ming et al., 2001). Consistent with these reports, our study provides evidence that the PI3K-Akt pathway is a target of Gas6-Axl interaction, and downregulation of Akt phosphorylation is associated with Pi-induced apoptosis and calcification. Moreover, it is known that PI3K-Akt affects the cell death program through the Bcl2 family of proteins. This protein family is a critical regulator of apoptosis in a variety of cell types, and the balance of antiapoptotic members, such as Bcl2, versus proapoptotic mediators, such as Bad, determines cell fate (Reed, 1997). Bcl2, whose phosphorylation is required for its anti-apoptotic activity (Ruvolo et al., 2001), inhibits programmed cell death by several mechanisms: It binds to caspase CED-4 (Apaf-1) and prevents the cell execution cascade; Bcl2 alters mitochondrial membrane potential and inhibits the release of cytochrome c. On the other hand, Bad plays a proapoptotic role in its dephosphorylated form by binding to Bcl2 and reversing its anti-apoptotic effect; phosphorylation of Bad results in its cytosolic sequestration by 14-3-3 and hampers its binding to Bcl2 (Zha et al., 1996). It was also reported that Bad is directly phosphorylated by PI3K-Akt (del Peso et al., 1997). In the present study, Bcl2 was inactivated and Bad was activated (both proteins were dephosphorylated) by Pi, directing the cells to apoptosis, and rhGas6 restored phosphorylation of Bcl2 and Bad. During apoptosis, one of the final biochemical events leading to programmed

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.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (No. 15390239), Mitsui Sumitomo Insurance Welfare Foundation, Ono Medical Research Foundation, Kanzawa Medical Research Foundation, Novartis Foundation for Gerontological Research, and Takeda Research Foundation. We thank Yuki Ito for technical assistance.

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Geriatr Gerontol Int 2006; 6: 244–247

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

Accepted for publication 9 July 2006.

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 doi: 10.1111/j.1447-0594.2006.00355.x

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.*

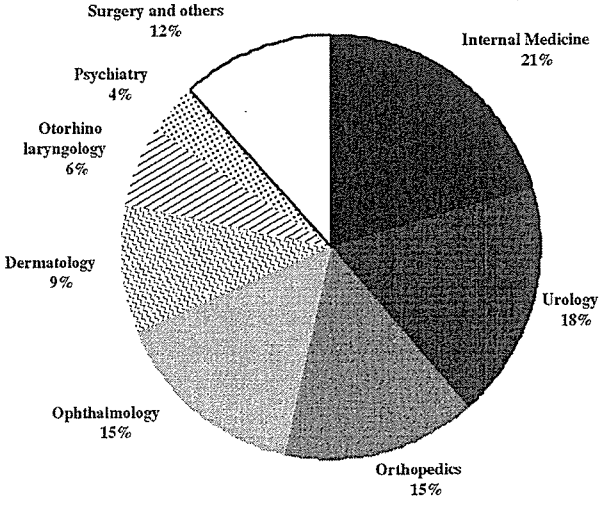

Figure 1 Distribution of specialist referrals.

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 191 (2007) 281–289

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

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Received 28 November 2005; received in revised form 9 May 2006; accepted 19 May 2006
Available online 27 June 2006

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 ± 0.01 to 0.09 ± 0.01 mm^2, $P < 0.001$) and descending aorta (5.09 ± 0.86 to 1.75 ± 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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doi:10.1016/j.atherosclerosis.2006.05.040

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 (•OH) and show inhibitory effects on peroxynitrite ($ONOO^-$) and both water-soluble and lipid-soluble peroxyl radical ($LOO^•$) [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.

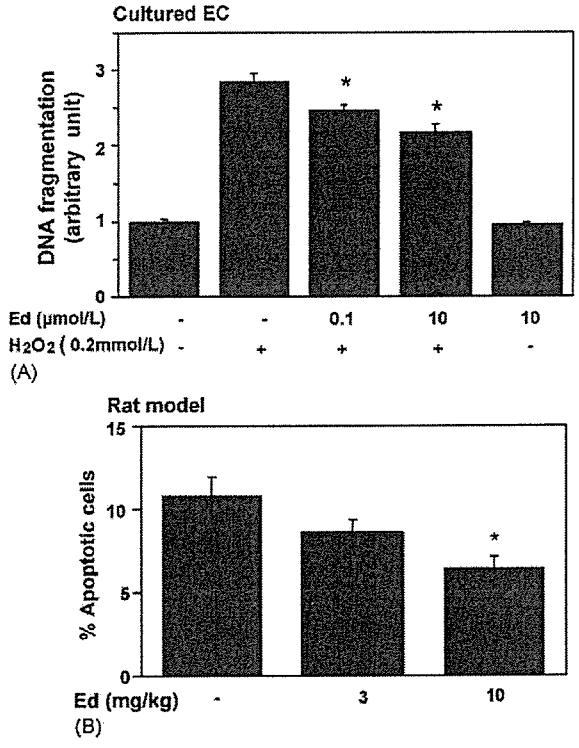


Fig. 1. Effects of edaravone (Ed) on H_2O_2-induced EC apoptosis in culture (A) and in a rat model (B). (A) Ed or its vehicle was added to the culture medium 24 h before H_2O_2 treatment until assay. EC apoptosis was evaluated 24 h after H_2O_2 treatment (0.2 mmol/L) by means of DNA fragmentation. Values 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

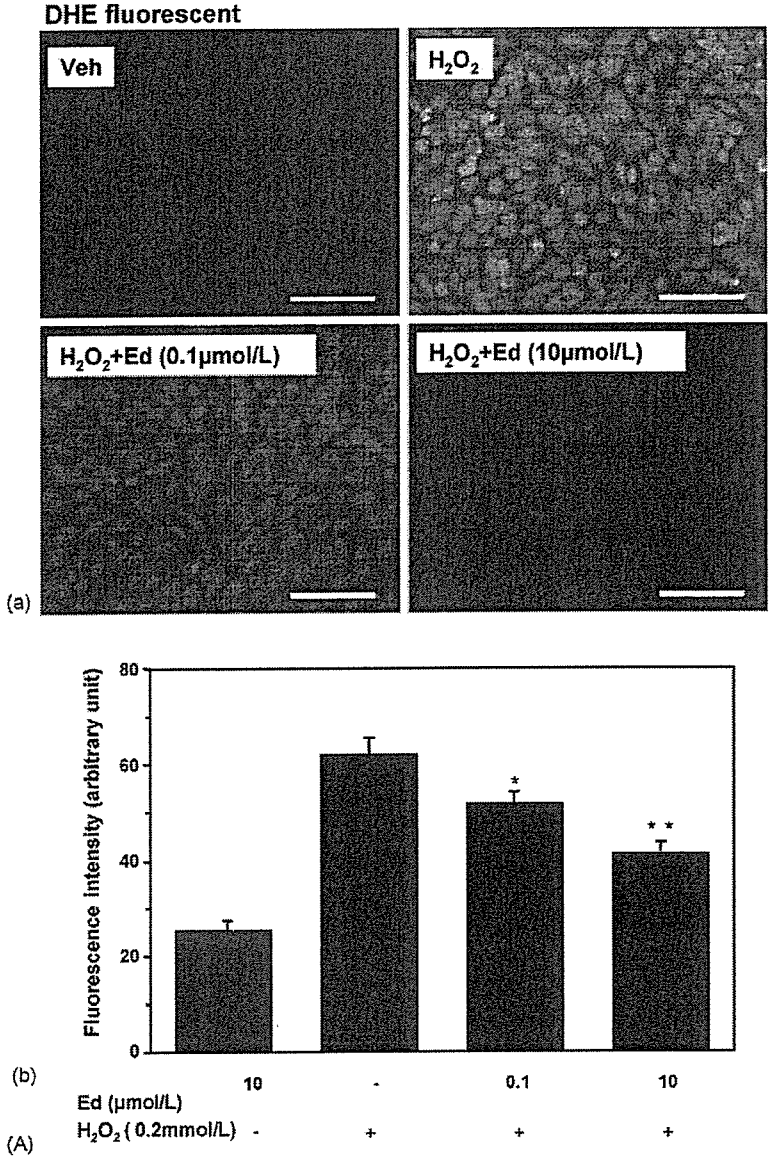
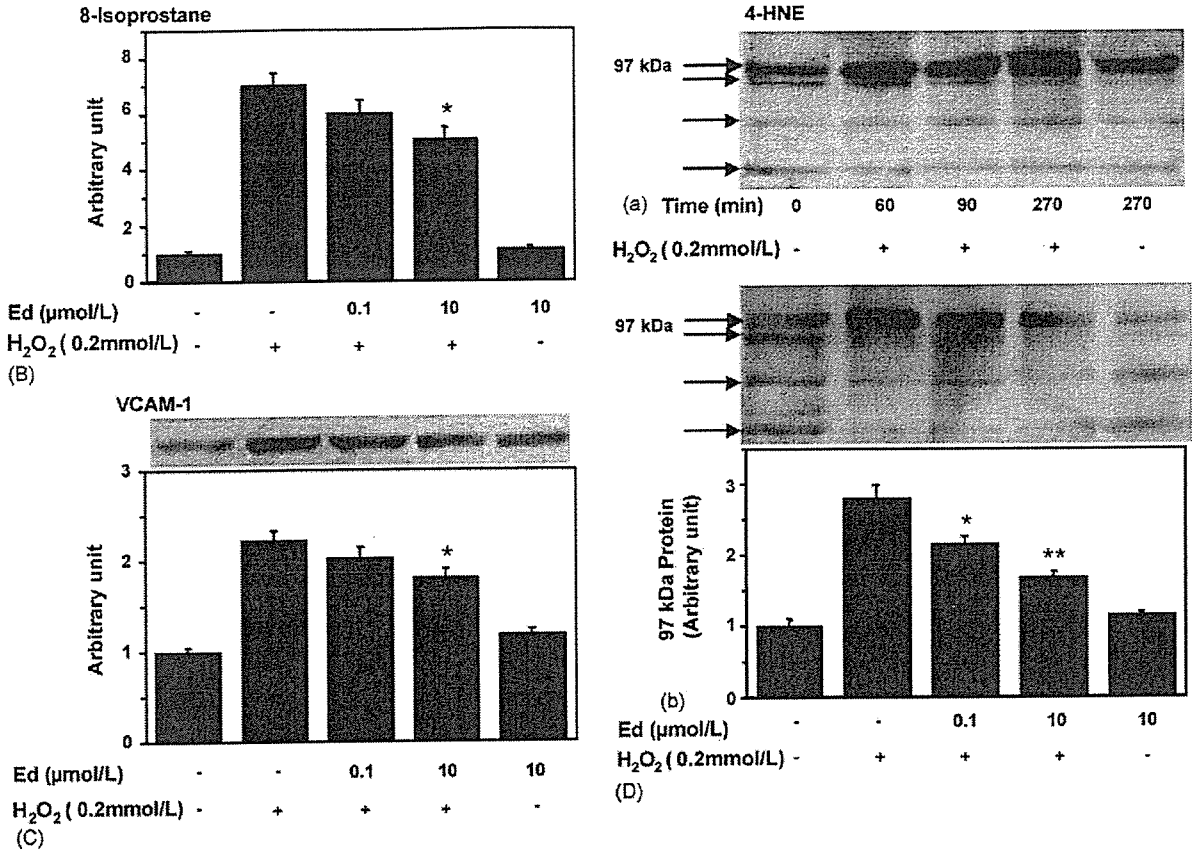


Fig. 2. Effects of edaravone (Ed) on DHE fluorescent (A) and 8-isoprostane formation (B), VCAM-1 expression (C) and 4-HNE expression (D) in cultured EC. Ed or its vehicle was added to the culture medium 24 h before H_2O_2 treatment until assay. DHE fluorescent ($n = 6$), 8-isoprostane concentration ($n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



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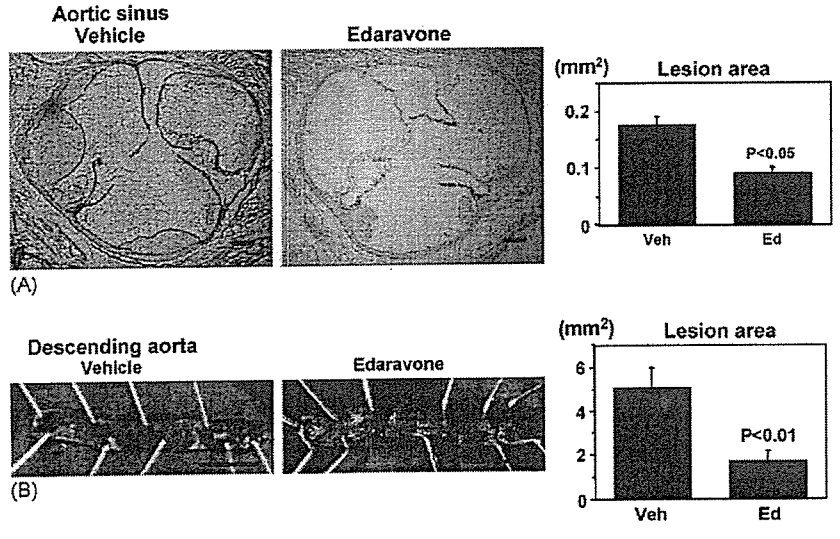

Fig. 3. Effects of edaravone on atherosclerotic lesion in ApoE-KO mice. ApoE-KO mice were fed a high-cholesterol diet for 4 weeks with the administration of edaravone (10 mg/kg daily) or its vehicle by i.p. injection. (A) Oil red O-stained cross-sections of the aortic sinus (bar = 100 μm) and morphometric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

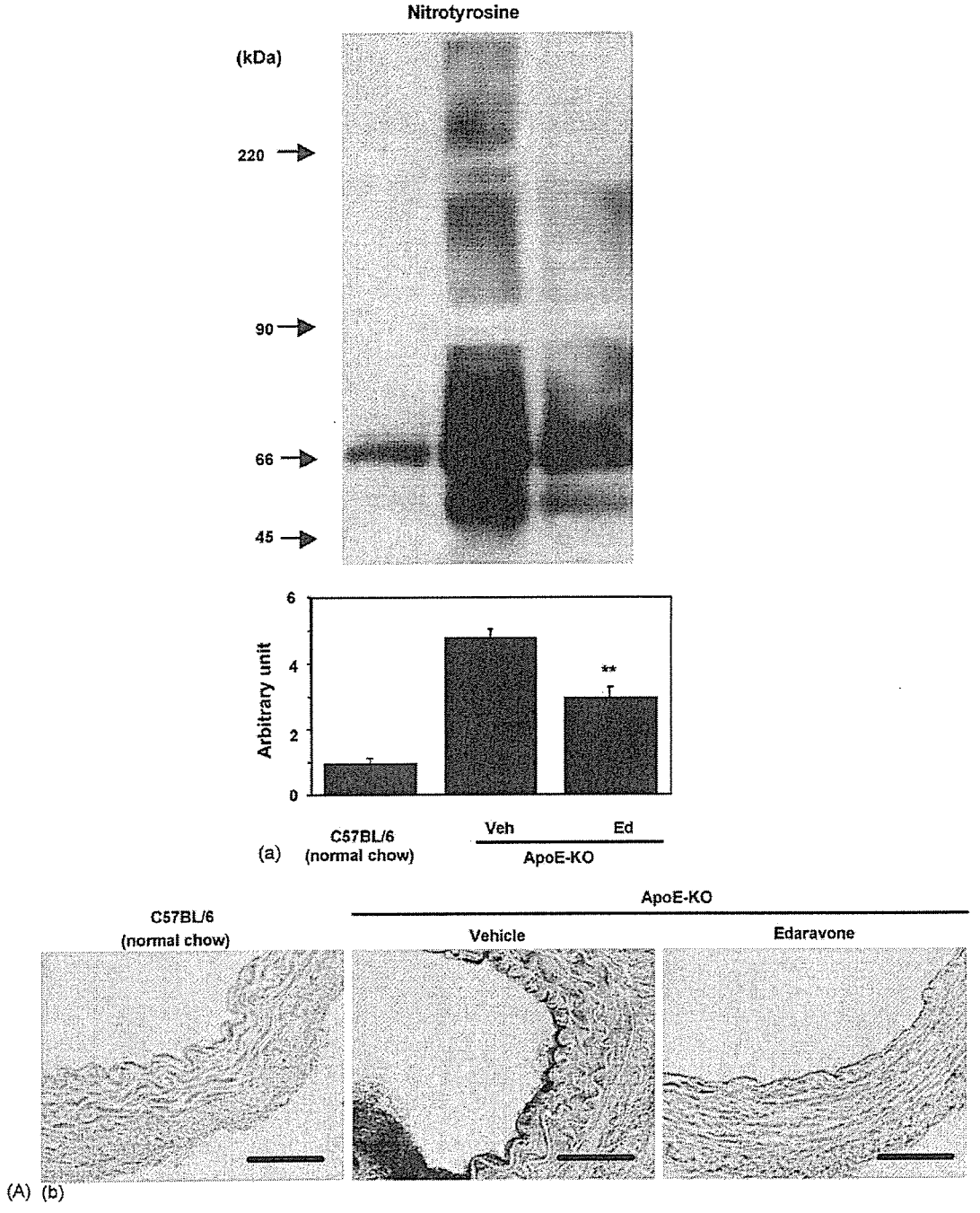

Fig. 4. Effects of edaravone (Ed) on ROS production (A–E) and VCAM-1 expression (F) in ApoE-KO mice. (A) Nitrotyrosine contents in the aorta was examined by Western blot analysis (A(a), $n=6$) and immunohistochemistry (A(b)). Bar = 50 μm. (B) Fresh-frozen cross-sections of the aorta were stained with 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.

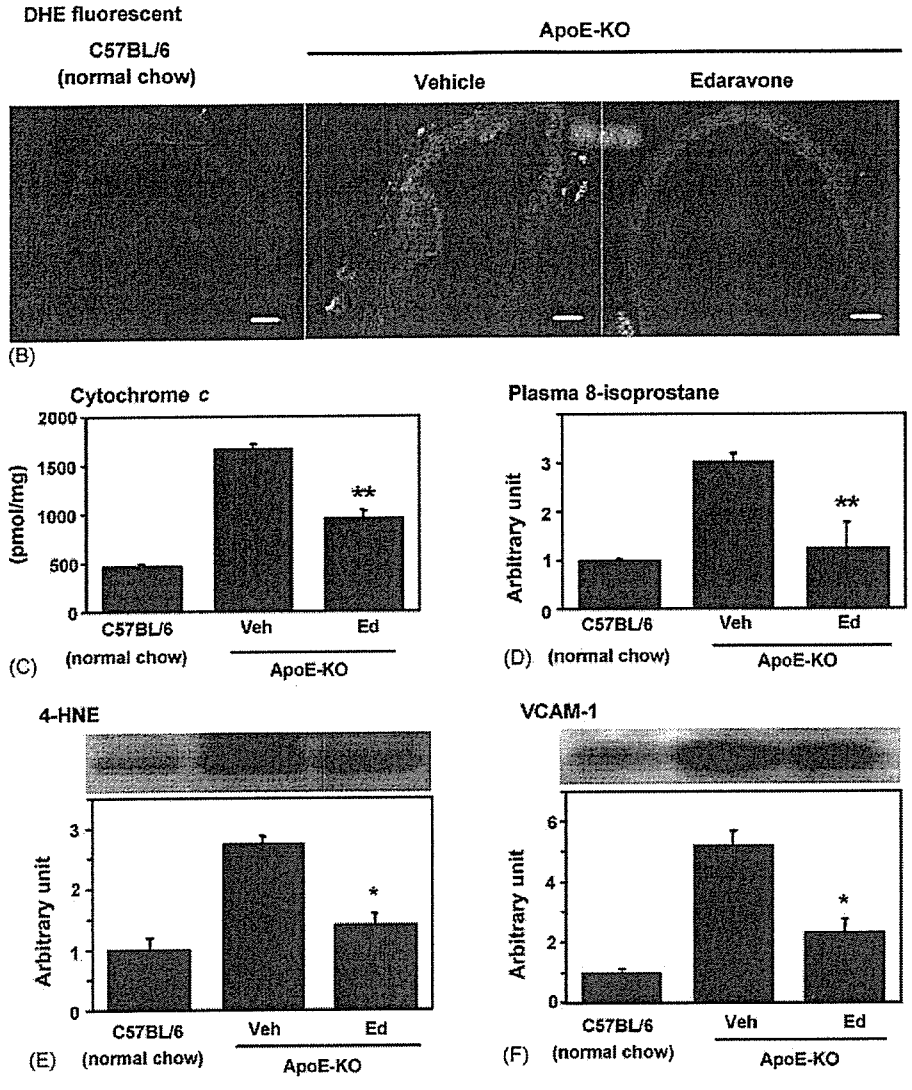

Fig. 4. (*Continued*).

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.

Acknowledgements

We thank Ms. Mariko Sawano for her excellent technical assistance. This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sports of Japan (13670741), and by Health and Labour Sciences Research Grants (H15-Choju-013, H15-Choju-015 and H17-Choju-046) from the Ministry of Health, Labour and Welfare of Japan.

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AT2 receptor mediates the cardioprotective effects of AT1 receptor antagonist in post-myocardial infarction remodeling

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Received 11 May 2006; accepted 23 August 2006

Abstract

There are two subtypes of angiotensin (Ang) II receptors, AT1R and AT2R. It is established that clinical use of specific AT1R blocker (ARB) improves the long-term prognosis of heart failure. However, scientific basis for such effects of ARB is incompletely understood. The present study was designed to determine whether ARB inhibits the left ventricular (LV) remodeling that occurs early after myocardial infarction (MI) and whether the benefit of ARB is mediated by blockade of AT1R itself or by stimulation of AT2R resulting from AT1R blockade. MI was induced in AT2R-knockout mice and wild-type mice. Administration of valsartan, an ARB, or vehicle was started soon after the surgery and continued for two weeks. Infarction caused significant increase in end diastolic and end systolic LV dimensions, LV/body weight ratio, and myocyte cross-sectional area (MCSA) in both strains to a similar extent. Lung/body weight ratio, an index of pulmonary congestion, was also significantly increased in both strains, but the magnitude of increase was significantly larger in knockout mice. Valsartan significantly reduced LV dimensions, LV/body weight ratio, MCSA, and lung/body weight ratio in wild-type mice. In knockout mice, however, valsartan failed to inhibit the increases in LV dimensions and LV/body weight ratio. After the treatment, lung/body weight ratio in the mutant strain was significantly larger than that in the wild-type mice. Valsartan attenuates acute phase post-infarction remodeling and ameliorates heart failure, and a large part of its cardioprotective effect was mediated by AT2R.

Keywords: AT2; Receptor; Valsartan; Myocardial infarction; Ventricular remodeling

Introduction

MI induces global changes in ventricular architecture, called post-infarction LV remodeling. The process of LV remodeling involves acute phase LV dilatation due to lengthening of infarct and non-infarcted myocardium and subsequent development of myocyte hypertrophy and interstitial fibrosis in the non-infarct region. Consequently, LV function deteriorates with increasing rate of mortality (Pfeffer and Braunwald, 1990; White et al., 1987). A substantial body of evidence (Dickstein and Kjekshus, 2002; Flather et al., 2000; Pfeffer et al., 2003) has suggested that Ang II plays a critical role in post-infarction LV remodeling. Ang II has two major receptor subtypes, AT1R and AT2R, both of which are expressed in the heart (Ozono et al., 2000). It has been suggested that AT1R signaling mediates vasoconstriction, aldosterone secretion, cardiomyocyte hypertrophy, proliferation of fibroblasts, interstitial collagen deposition, and catecholamine release, all of which are implicated in the progression of LV remodeling (Harada et al., 1999). AT2R is thought to have the opposite effect to that of AT1R, and has been shown to suppress myocardial hypertrophy (Booz and Baker, 1996), fibroblast proliferation (Tsutsumi et al., 1998), and vascular cell hyperplasia (Stoll et al., 1995). We have recently demonstrated

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0024-3205/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2006.08.033

in mice that deletion of AT2R gene caused deterioration of heart failure and increased mortality during 14 days after MI (Oishi et al., 2003), suggesting that AT2R is protective against the acute phase post-infarction LV remodeling.

Recent clinical trials (Dickstein and Kjekshus, 2002; Pfeffer et al., 2003) have demonstrated that long-term administration of ARBs reduces cardiovascular mortality and morbidity effectively in patients with heart failure after MI. However, it is unclear whether ARBs may benefit the survival of patients with heart failure that frequently occurs within the first 3–4 days after the onset of infarction. Specific blockade of AT1R with ARBs results in the elevation of circulating Ang II and thus an overstimulation of AT2R (Levy, 2004; Spinale et al., 1997), indicating that the effect of ARBs could be partly mediated by its effect on AT2R. Regarding the role of AT2R, activation of AT2R does not always lead to cardioprotection, but could exert deleterious effects in certain contexts (Levy, 2004). It is therefore important to address whether ARB is beneficial in the acute phase after MI, and whether the effects of ARBs are brought about by blockade of AT1R or by stimulation of AT2R. Of note, a relative abundance of AT2R to AT1R in the heart is highest in humans compared to rodents and other animal models (Tsutsumi et al., 1998; Wharton et al., 1998).

In the present study using mice lacking AT2R, we investigated the effects of valsartan on LV remodeling, focusing on the LV dilatation and development of heart failure in the acute phase after MI. By comparing the effect of valsartan between wild-type and the knockout mice, we can determine the contribution of AT2R.

Methods

Animals

Adult male AT2R-knockout mice (*Agtr2*−)(Akishita et al., 2000; Hein et al., 1995; Oishi et al., 2003) and wild-type mice (*Agtr2*+) littermates were used in this study. These mice were back-crossed for at least 6 generations onto a FVB/N background (Akishita et al., 2000; Hein et al., 1995). All experimental procedures were approved and carried out in accordance with the Guidelines of Hiroshima University Graduate School of Biomedical Sciences.

Table 1
Hemodynamic parameters

			Vehicle	
			Day 0	Day 14
Systolic BP, mmHg	*Agtr2*+	(*n*=5)	114±2	117±7
	Agtr2−	(*n*=5)	113±5	117±4
Diastolic BP, mmHg	*Agtr2*+	(*n*=5)	74±4	77±3
	Agtr2−	(*n*=5)	79±4	83±4
Heart rate, bpm	*Agtr2*+	(*n*=5)	489±32	459±34
	Agtr2−	(*n*=5)	500±61	492±13
			Valsartan	
			Day 0	Day 14
Systolic BP, mmHg	*Agtr2*+	(*n*=5)	116±7	118±4
	Agtr2−	(*n*=6)	116±2	115±2
Diastolic BP, mmHg	*Agtr2*+	(*n*=5)	83±6	81±5
	Agtr2−	(*n*=6)	75±3	82±5
Heart rate, bpm	*Agtr2*+	(*n*=5)	494±28	495±46
	Agtr2−	(*n*=6)	489±27	488±43

Values are mean±SEM.

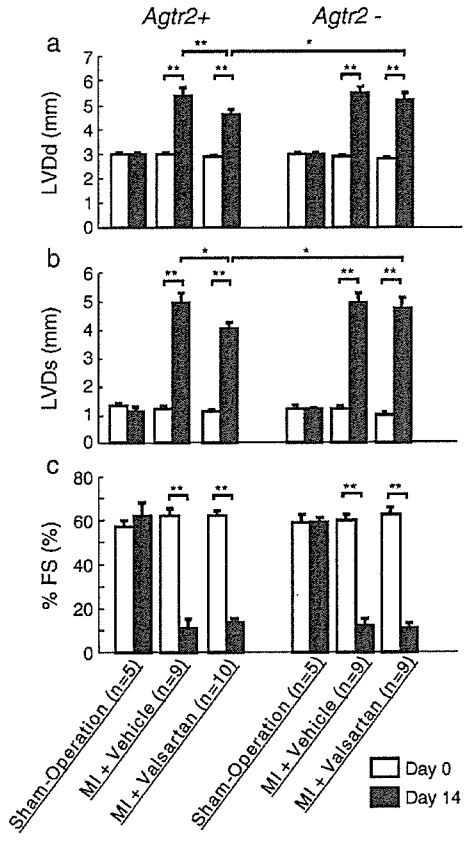

Fig. 1. Changes in (a) end-diastolic LV dimension (LVDd), (b) end-systolic LV dimension (LVDs), and (c) percentage of fractional shortening (%FS) of LV dimensions on day 0 (open column)and day 14 (closed column). Each data point is the mean of pooled data from 5–10 mice. MI; myocardial infarction.**$P<0.001$, *$p<0.05$.

Experimental protocols

Myocardial infarction was induced as previously described (Oishi et al., 2003). Soon after the surgery, sub-pressor dose of valsartan (1 mg/kg/day) or a vehicle was given via an osmotic pump for two weeks. This dose of valsartan effectively inhibited vascular injury in the same strains of mice without affecting blood pressure (Wu et al., 2001). Valsartan was kindly provided by Novartis Pharma Corp. The natural prognosis after surgery was examined for 14 days. In total, 23 *Agtr2*+ and 19 *Agtr2*− mice were allocated to a valsartan treatment group, 31 *Agtr2*+ mice and 27 *Agtr2*− mice were allocated to a vehicle treatment group, and 5 *Agtr2*+ and 5 *Agtr2*− mice were allocated to a sham-operation group. Since the focus of the present protocol was assessment of post-infarction cardiac remodeling, the number of animals might not be sufficient for analysis of survival rate. Physiological profiles were examined using

transthoratic echocardiography before (day 0) and 14 days after surgery. Mice that survived for14 days after surgery were sacrificed and subjected to analysis of heart and lung weight and histology. Therefore, the numbers of animals used for physiological profile analysis and necropsy varied and are individually indicated in tables and figure legends.

Hemodynamic and physiological assessments

Blood pressure (BP) and heart rate (HR) of sham-operated animals were measured before and 14 days after surgery by the tail-cuff method as previously described (Oishi et al., 2003; Ozono et al., 2000). Cardiac geometry and function were evaluated using an echocardiographic system (Toshiba SSA 550A) equipped with a 14-MHz linear transducer as previously described (Oishi et al., 2003; Ozono et al., 2000). LVDd and LVDs were measured at the distal level of the papillary muscle using short-axis M-mode images. Three beats were averaged for each measurement. Percent fractional shortening (%FS) was calculated as [(LVDd−LVDs)/LVDd]×100.

Morphological and histopathological assessments

Mice were sacrificed by KCl injection via the jugular vein. (Oishi et al., 2003) Hearts were fixed with 10% buffered formalin and embedded in paraffin. One to two-μm-thick sections were cut and stained with Masson's trichrome and sirius red, for measurements of MCSA (Oishi et al., 2003; Ozono et al., 2000) and interstitial fibrosis, respectively. For measurement of MCSA, 100 myocytes with circular profiles were chosen from non-infarcted myocardium and the areas were traced. Averaged MCSA was calculated. Interstitial fibrosis index (IFI) was calculated as the ratio of interstitial fibrosis area to the connective-tissue area plus myocyte area in non-infarcted myocardium. All the parameters were quantitatively analyzed using Scion Image 1.62 software (NIH Service Branch).

Table 2
Infarct size (%) and infarct length (%)

	Agtr2+		*Agtr2−*	
	Vehicle	Valsartan	Vehicle	Valsartan
n	11	10	9	8
Infarct size (%)	24.2±3.4	25.1±2.3	25.5±2.3	24.5±5.5
Infarct length (%)	64.5±5.4	63.9±4.1	65.9±6.0	65.3±3.0

Values are mean±SEM. Both infarct size and infarct length were analyzed in paraffin sections of LV, in which the LVs were cut into 4 transverse slices. Infarct size was expressed as a ratio (%) of the sum of infarct area to total left ventricular area and infarct length as % of the sum of circumferential infarct length to total cardiac circumferential length in the 4 slices. Circumferential lengths were analyzed along both of the endocardial and epicardial surfaces.

Measurement of infarct size

Infarct size was measured in the same paraffin sections as those used for histological assessment. The excised hearts were cut into four transverse slices and embedded in paraffin so that the four slice levels appear on the slides. Typically, the apex slice and the 2nd (and often 3rd) slice of the apical side contained

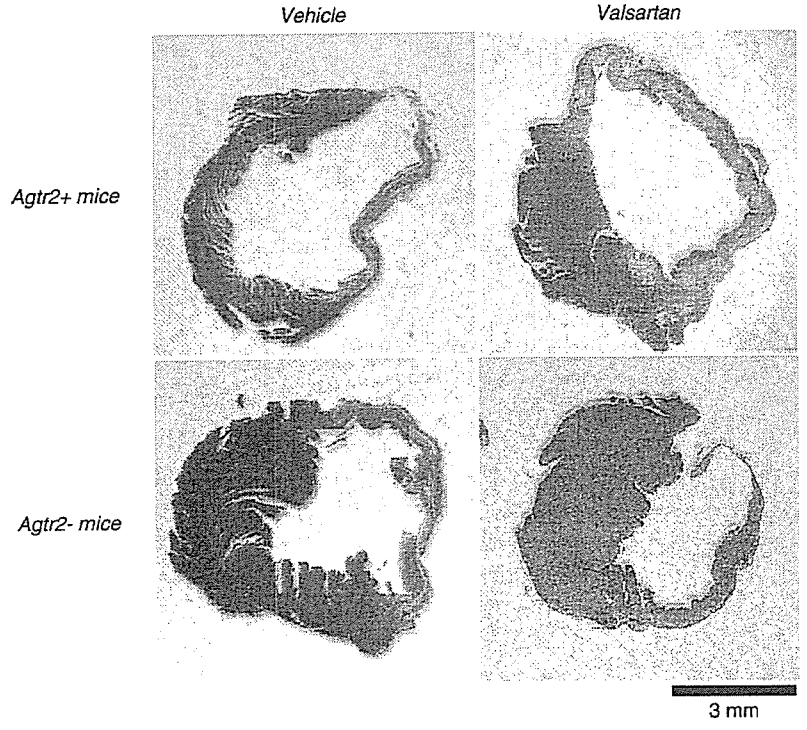

Fig. 2. Transverse sections of LV (post-MI 14 days) from Agtr2+ and Agtr2− mice treated with a vehicle or valsartan. Masson's trichrome staining. Excised LVs were cut into 4 transverse slices, embedded in paraffin, and the sections were cut. The second slices from the apical side are shown. The infarct sizes were not different among the groups.

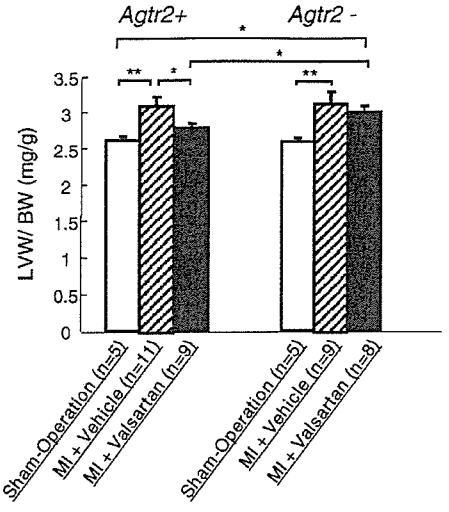

Fig. 3. Left ventricle/body weight ratio(LVW/BW) on day 14 was significantly increased both in *Agtr2*+ and *Agtr2*− mice treated with a vehicle. Valsartan significantly decreased LVW/BW in *Agtr2*+ mice but not in *Agtr2*− mice. Each data point is the mean of pooled data from 5–11 mice. MI; myocardial infarction, $^{**}P<0.001$, $^{*}p<0.05$.

transverse sections of the infarct. The apex slice always had full circumferential infarction. The images of the section were captured and digitalized, and then the area and the length of infarct were measured in all of the 4 slices using computer-assisted planimeter (Scion Image software program). The infarct size was expressed as a ratio of the sum of infarct area relative to the entire LV area. The infracted myocardium was identified by histology and staining of scar tissue.

Infarct size was also estimated by infarct length as previously described (Fuchs et al., 2003; Matsushima et al., 2006). Infarct length was measured along the endocardial and epicardial surface in each of the transverse cardiac sections, and values from all specimens were summed. Infarct length (as a percentage) was calculated as infarct circumference divided by total cardiac circumference.

The surgery to induce MI was performed by a single investigator. The average infarct size and infarct length 24 h after surgery, evaluated in a preliminary experiment, were 28.9±4% and 71.1±28% ($n=10$, mean±SD), respectively.

Statistical analysis

All results are expressed as means±SEM. Analyses of survival after MI were carried out by the Kaplan–Meier method. Multiple comparisons among 3 or more groups were carried out by one-way ANOVA and Fisher's exact test for post-hoc analyses. Statistical significance was accepted at a value of $P<0.05$.

Results

Survival rate after myocardial infarction

Consistent with our previous observation (Oishi et al., 2003), the survival rate of *Agtr2*− mice treated with a vehicle (33%) was lower than that of *Agtr2*+ mice treated with a vehicle (41%), although the difference did not reach statistical significance. Valsartan treatment improved the survival rates of both strains of mice (61% for *Agtr2*+ mice and 53% for *Agtr2*− mice).

Hemodynamic and physiological assessments

There was no difference between BPs and HRs in the two strains of mice on day 0 (Table 1). Treatment with valsartan or a vehicle did not affect BP or HR of sham-operated *Agtr2*+ and *Agtr2*− mice.

There were no differences in basal values of LVDd, LVDs and %FS between the groups of *Agtr2*+ mice and *Agtr2*− mice (Fig. 1). Fourteen days after surgery, LVDd and LVDs in both groups of *Agtr2*+ mice ($n=9$) and *Agtr2*− mice ($n=9$) treated with a vehicle were markedly enlarged by approximately 1.8 fold compared with those in the sham-operated animals ($n=5$, each group). The extent of LV dilatation was not significantly different between the strains. Treatment of *Agtr2*+ mice with valsartan ($n=10$) significantly attenuated the dilatations of LVDd and LVDs by 15% and 18%, respectively, compared with those in the vehicle-treated group. On the other hand, valsartan had no effect on the LV dimensions in *Agtr2*− mice ($n=9$), suggesting that the protective effect of valsartan against LV dilatation is brought about by stimulation of AT2R rather than by inhibition of AT1R. There was no difference in %FS between the strains with or without valsartan treatment.

Morphological and histopathological assessment

Fig. 2 shows the transverse sections of excised LVs 14 days after induction of MI. The average infarct sizes in the 4 groups were 24.2–25.5% of total LV area, and the average infarct lengths were 63.9–65.9% (Table 2), showing no difference

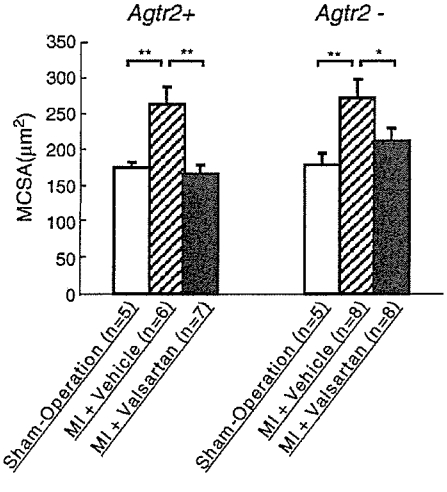

Fig. 4. Myocyte cross-sectional area (MCSA) on day 14 was significantly increased both in *Agtr2*+ and *Agtr2*− mice treated with a vehicle. Valsartan significantly decreased MCSA in both strains of mice. Each data point is the mean of pooled data from 5–11 mice. MI; myocardial infarction, $^{**}P<0.001$, $^{*}p<0.05$.