p21 or p16 in cancer cells (Zhu *et al.*, 1997; Chang *et al.*, 1999a, b; Collado *et al.*, 2000; Wainwright *et al.*, 2001; Wright and Shay, 2001; te Poele *et al.*, 2002; Beausejour *et al.*, 2003; Mallette *et al.*, 2004; Munro *et al.*, 2004). p27 was shown to be required for premature senescence mediated by Rb (Alexander and Hinds, 2001) or PI3-K inhibitor (Collado *et al.*, 2000). In accordance with previous findings (Chang *et al.*, 1999a; Collado *et al.*, 2000; Wainwright *et al.*, 2001; Mallette *et al.*, 2004), Sirtinol induced senescence-like growth arrest in p16-deficient MCF-7 and p53-deficient H1299 cells. These results indicate that p53 and p16 are not required for Sirtinol-induced senescence-like growth arrest in H1299 and MCF-7 cells, respectively.

Recently, senescence-like growth arrest has been proposed as a new target of cancer therapy. Since p53 and p16 are not expressed or mutated in many types of malignancies, the effectiveness of Sirtinol to induce senescence-like growth arrest in p53- and p16-deficient cancer cells may be of clinical significance for the treatment of patients with malignancy. Taken together, the present study highlights Sirt1 inhibitor as an antitumor drug candidate.

Materials and methods

Materials
Sirtinol, Splitomicin, IGF-I, EGF (Calbiochem, La Jolla, CA, USA), trichostatin A, cisplatin, propidium iodide, RNase, tamoxifen (Sigma, St Louis, MO, USA), anti-phospho-ERK (Thr202/Tyr204), ERK, phospho-JNK/SAPK (Thr183/Tyr185), phospho-p38 (Thr180/Tyr182), p38, phospho-Raf (Ser259), Raf-1, phospho-MEK1/2 (Ser217/221), MEK1/2, phospho-SEK1/MKK4 (Ser80), SEK1/MKK4, MKK7, phospho-Elk1 (Ser383), Elk1, phospho-ATF2 (Thr71), ATF2, phospho-Rb (Ser795), Rb, phospho-Akt (Ser473), Akt, p53 (Cell Signaling, Beverly, MA, USA), acetylated p53, EGFR, phospho-MKK7 (Thr275/Ser277) (Upstate, Lake Placid, NY, USA), phospho-EGFR (Tyr1173), p16, p21, phospho-c-Jun (Ser63), c-Jun, JNK1 (Santa Cruz, Santa Cruz, CA, USA), p27 (BD Transduction Laboratories, Lexington, KY, USA), PAI-1 (Molecular Innovations, Southfield, MI, USA), acetylated histone H3, histone H3 (Upstate, Charlottesville, VA, USA), phospho-IGF-I receptor (Tyr1162/1163), IGF-I receptor (Biosource, Camarillo, CA, USA) and pan-Ras antibodies (Oncogene, San Diego, CA, USA) were purchased commercially.

Cell culture
Human breast cancer MCF-7 cells and non-small lung cancer H1299 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 supplemented with 10% fetal bovine serum (FBS, Sigma), respectively. Logarithmically growing cells were treated with the indicated concentrations of Sirtinol or Splitomicin for 24 h. After exposure to Sirtinol or Splitomicin for 24 h, the cells were washed three times with inhibitor-free medium and cultured for an additional 9 days with the complete media in the absence of inhibitor. Cell viability was determined by the Trypan blue exclusion test, and viable cells were counted. At 9 days after the addition of Sirtinol to the culture media, the cells were serum-deprived for overnight, and then treated with EGF (50 ng/ml) or IGF-I (100 ng/ml) for 2, 10 or 20 min.

SA-β-gal staining
SA-β-gal staining was performed as previously described (Dimri *et al.*, 1995) (see Supplementary section).

BrdU incorporation assay
At 10 days after the addition of Sirtinol, BrdU incorporation was assayed as previously described (Takahashi *et al.*, 1992). In cells treated with siRNA, at 10 days after the transfection of siRNA, BrdU incorporation was evaluated using a commercial kit (Roche, Indianapolis, IN, USA).

Gene knockdown with siRNA
Cells were plated in six-well plates at 20–30% confluency, and 24 h later transfected with 200 pmol of siRNA for Sirt1 ('5-GAT GAA GTT GAC CTC CTC A-3' (Picard *et al.*, 2004) and 5'-TGA AGT GCC TCA GAT ATT A-3') or control siRNA (Dharmacom, Chicago, IL, USA), using siIMPORTER (Upstate).

Flow cytometric analysis
At 10 days after the addition of Sirtinol, the cells were fixed with 70% ethanol and treated with 5 μg/ml (RNase) for 30 min. After staining with 50 μM propidium iodide, the cells were subjected to flow cytometric analysis with FACS Calibur and Cell Quest software (Becton-Dickinson, Franklin Lakes, NJ, USA).

Immunoblot analysis
Immunoblot analysis was performed as previously described (Yasukawa *et al.*, 2005) (see Supplementary section).

Determination of activation status of Ras
Active, GTP-bound Ras was assayed using the Ras activation assay kit (Upstate) according to the manufacturer's instructions (see Supplementary section).

Colony formation assay
Colony formation assay was performed as previously described (Elegbede *et al.*, 2002) (see Supplementary section).

Northern blotting
The mRNA level of p27 was evaluated by Northern blotting as previously described (Sugita *et al.*, 2005), using cDNA probe for p27 that was kindly provided by Dr N Fujita (Fujita *et al.*, 2002).

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Angiotensin converting enzyme inhibitor attenuates oxidative stress-induced endothelial cell apoptosis via p38 MAP kinase inhibition

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Abstract

Background: The effects of angiotensin converting enzyme (ACE) inhibitors on oxidative stress-induced apoptosis of endothelial cells and the intracellular signaling were investigated.
Methods: Cultured endothelial cells derived from a bovine carotid artery were treated with H_2O_2 or TNF-α to induce apoptosis. Apoptosis was evaluated by DNA fragmentation and cell viability, p38 MAP kinase activity by Western blotting, and oxidative stress by formation of 8-isoprostane. The effects of ACE inhibitors were examined by adding them into the medium throughout the experiments.
Results: Apoptosis was attenuated by ACE inhibitors, temocapril and captopril, in a dose-dependent manner (1–100 μmol/l). H_2O_2 (0.2 mmol/l for 1.5 h) or TNF-α (10 ng/ml for 72 h) treatment stimulated the activities of p38 MAP kinase. Temocapril and captopril decreased the activity of p38 MAP kinase as well as 8-isoprostane formation induced by H_2O_2. A p38 MAP kinase inhibitor, SB203580, partially inhibited the effect of temocapril on apoptosis.
Conclusions: These results suggest that ACE inhibitors protect endothelial cells from oxidative stress-induced apoptosis, and that p38 MAP kinase plays a critical role in the process.

Keywords: Apoptosis; ACE inhibitor; Endothelial cell; p38 MAP kinase

1. Introduction

Stress-induced injury of vascular endothelial cells (ECs) is considered to be 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 is upregulated in vascular lesions [4,5], and that lesion formation such as endothelial dysfunction is accelerated by superoxide anion [6] and, in contrast, is attenuated by free radical scavengers including vitamin E [7] and superoxide dismutase [8].

Angiotensin converting enzyme (ACE) inhibitors effectively interfere with the renin angiotensin system and exert various beneficial actions on vascular structure and function beyond their blood pressure-lowering effects [9,10]. ACE inhibitors attenuate neointimal formation after vascular injury in animals [11] and endothelial dysfunction in humans [12]. It has been demonstrated that ACE activation induces oxidative stress [13]. However, it has not been elucidated whether ACE inhibitors could attenuate oxidative stress-induced EC apoptosis, an initial and important process in atherosclerosis [14,15].

In this study, we examined the effects of ACE inhibitors, temocapril and captopril, on H_2O_2- and TNF-α-induced EC apoptosis and the pro-apoptotic intracellular signaling, p38 mitogen-activated protein (MAP) kinase, to clarify the underlying mechanism.

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2. Materials and methods

2.1. Induction of EC apoptosis

ECs derived from a bovine carotid artery [16] was cultured in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum. Cells were maintained at 37 °C in a 95% air/5% CO_2 atmosphere. ECs of the 5th to 7th passage were used in the experiments. When the cells had grown to 70–80% confluence, ECs were pretreated for 24 h with culture medium containing the reagents that were tested in the experiments. Subsequently, after washing twice with Hank's balanced salt solution (Gibco), the cells were exposed to H_2O_2 (0.1–0.4 mmol/l) diluted in Hank's balanced salt solution for 1.5 h at 37 °C to induce apoptosis. The cells were washed three times with Hank's balanced salt solution, and then cultured in culture medium containing the reagents until assay. Similarly, tumor necrosis factor-α (TNF-α, 5-20 ng/ml; Sigma) was added to the medium until assay after 24-h pretreatment with the reagents tested. EC viability and apoptosis were evaluated at 24 h after H_2O_2 treatment, or at 72 h after TNF-α treatment. The effects of temocapril (1–100 μmol/l) and captopril (1–100 μmol/l) were examined by adding them into the medium throughout the experiments. The effect of a specific p38 MAP kinase inhibitor, SB203580 (10 μmol/l; Calbiochem), was examined by treating ECs with SB203580 for 1 h before H_2O_2 treatment.

2.2. Cell viability

Cell viability was estimated using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) assay [17]. Briefly, 1 mg/ml MTT (final concentration) was added to the well and incubated for 2 h at 37 °C. The medium was removed and cells were lysed with 2-isopropanol containing 0.04 mol/l HCl. The absorbance measured at 595 nm was used to calculate the relative cell viability ratio.

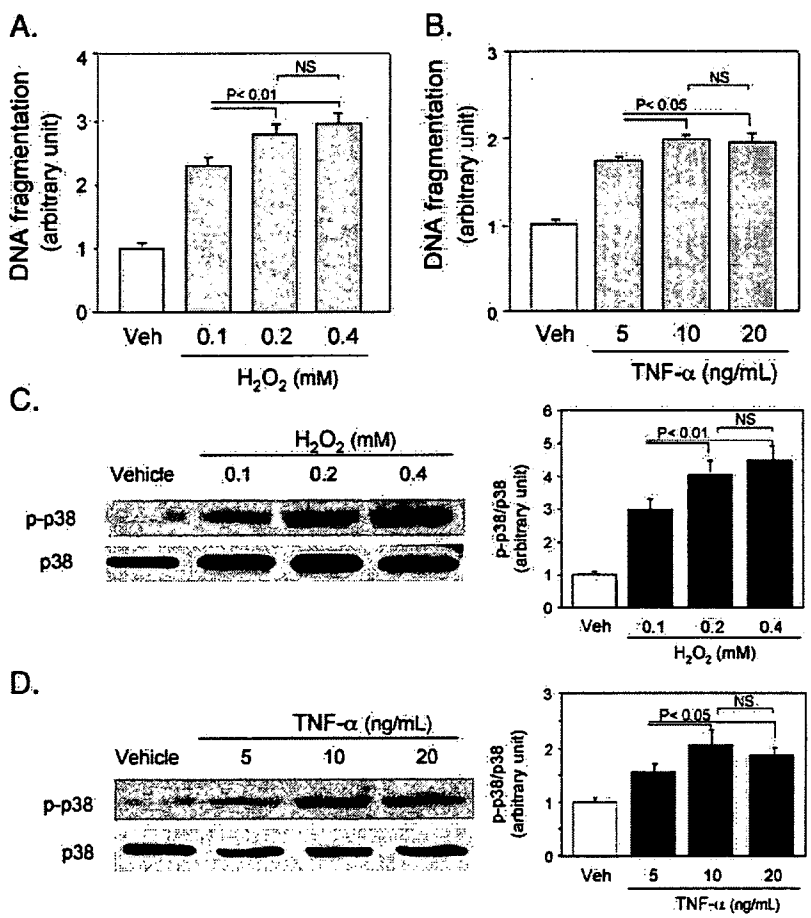

Fig. 1. Dose-dependent effects of H_2O_2 (A, C) and TNF-α (B, D) on EC apoptosis (A, B) and p38 MAP kinase activity (C, D). A and B, apoptosis was evaluated 24 h after H_2O_2 treatment (for 1.5 h) or 72 h after addition of TNF-α by means of DNA fragmentation ($n=3$). C and D, the activity of p38 MAP kinase was evaluated by immunoblotting using the specific antibody against the phosphorylated form of the kinase (p-p38) at 30 min after addition of H_2O_2 or TNF-α. Right panels show the results of densitometric analyses of immunoblotting (mean±SEM, $n=3$). NS, not significant. Values are expressed as mean±SEM ($n=3$).

2.3. Evaluation of EC apoptosis and formation of 8-isoprostane

For quantitative determination, EC apoptosis was measured as DNA fragmentation. DNA fragmentation was evaluated by histone-associated DNA fragments using a photometric enzyme immunoassay (Cell Death Detection ELISA, Roche), according to the manufacturer's instructions. Briefly, attached cells were harvested with trypsin, and the cell suspension was pelleted by centrifugation. Floating and attached cells were lysed. After centrifugation, the supernatant was measured by ELISA.

Formation of 8-isoprostane (8-*iso* prostaglandin $F_{2\alpha}$) was measured using a commercially available EIA kit (Cayman Chemical). Culture supernatants were diluted with EIA buffer when necessary, and were applied to EIA according to the manufacturer's instructions.

2.4. Immunoblotting

The cells were washed twice with ice-cold phosphate-buffered saline and lysed in lysis buffer (25 mmol/l Tris/HCl, pH 7.5, 25 mmol/l NaCl, 0.5 mmol/l EGTA, 10 mmol/l NaF, 20 mmol/l β-glycerophosphate, 1 mmol/l Na_3VO_4, 1 mmol/l

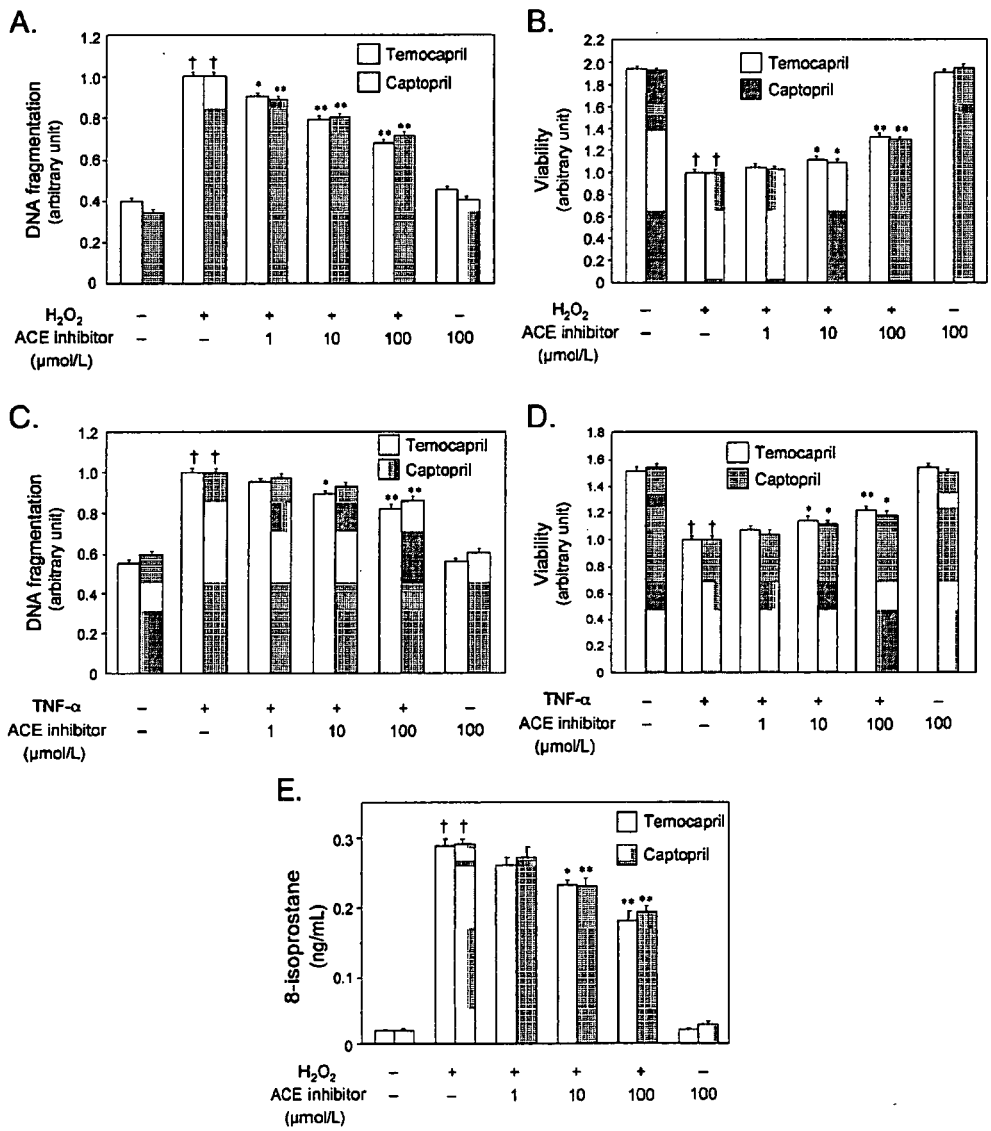

Fig. 2. Effects of ACE inhibitors on H_2O_2-induced (A, B) and TNF-α-induced (C, D) EC apoptosis and the effects of ACE inhibitors on H_2O_2-induced 8-isoprostne formation (E). Temocapril, captopril or their vehicle was added to the culture medium 24 h before H_2O_2 or TNF-α treatment until assay. Apoptosis (A, C) and cell viability (B, D) were evaluated 24 h after H_2O_2 treatment (0.2 mmol/l for 1.5 h) or 72 h after TNF-α treatment (10 ng/ml for 72 h) by means of DNA fragmentation ($n=3$) and MTT assay ($n=8$), respectively. 8-Isoprostane concentration (E; $n=3$) in the culture supernatant was measured 3 h after H_2O_2 treatment. A and B, †$P<0.01$ vs. H_2O_2 (−). *$P<0.05$, **$P<0.01$ vs. H_2O_2 (+)+ACE inhibitor (−). C and D, †$P<0.01$ vs. TNF-α (−). *$P<0.05$, **$P<0.01$ vs. TNF-α (+)+ACE inhibitor (−). Values are expressed as mean±SEM. Similar results were obtained in three independent experiments.

PMSF, and 10 μg/ml aprotinin) at 4 °C. After sonication and centrifugation at 15,000 rpm, the supernatant was used for the following immunoblotting. The lysate (20 μg protein per lane) was separated on 12% SDS-polyacrylamide gel, electroblotted onto nitrocellulose membrane, and immunoblotted with specific primary antibodies, both of which were purchased from Cell Signaling Technology (Beverly, MA). The antibodies used in this study were anti-phospho-p38 MAP kinase (phospho-p38 28B10 #9216) and anti-p38 MAP kinase (#9212). Antibodies were detected by means of a horseradish peroxidase-linked secondary antibody using an enhanced chemiluminescence system (Amersham Pharmacia Biotech). Densitometric analysis was performed using an image scanner and analyzing software (NIH image ver. 1.61). The activity of each kinase was evaluated by calculating the ratio of the amount of the phosphorylated form to that of the total form.

2.5. Data analysis

The values are expressed as mean±SEM in the text and figures. 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. Dose-dependent effects of H_2O_2 and TNF-α on EC apoptosis and p38 MAP kinase activity

Increasing concentrations of H_2O_2 and TNF-α were applied to examine the effects on EC apoptosis and p38 MAP kinase activity. Based on the literature [18] and time–response experiments (data not shown), EC apoptosis was evaluated at 24 h after H_2O_2 treatment for 1.5 h, or at 72 h after addition of TNF-α. The activity of p38 MAP kinase, as measured by immunoblotting using the specific antibody against the phosphorylated form of the kinase, was evaluated at 30 min after addition of H_2O_2 or TNF-α, based on time–response experiments (data not shown). As shown in Fig. 1A–D, the effects of H_2O_2 and TNF-α were

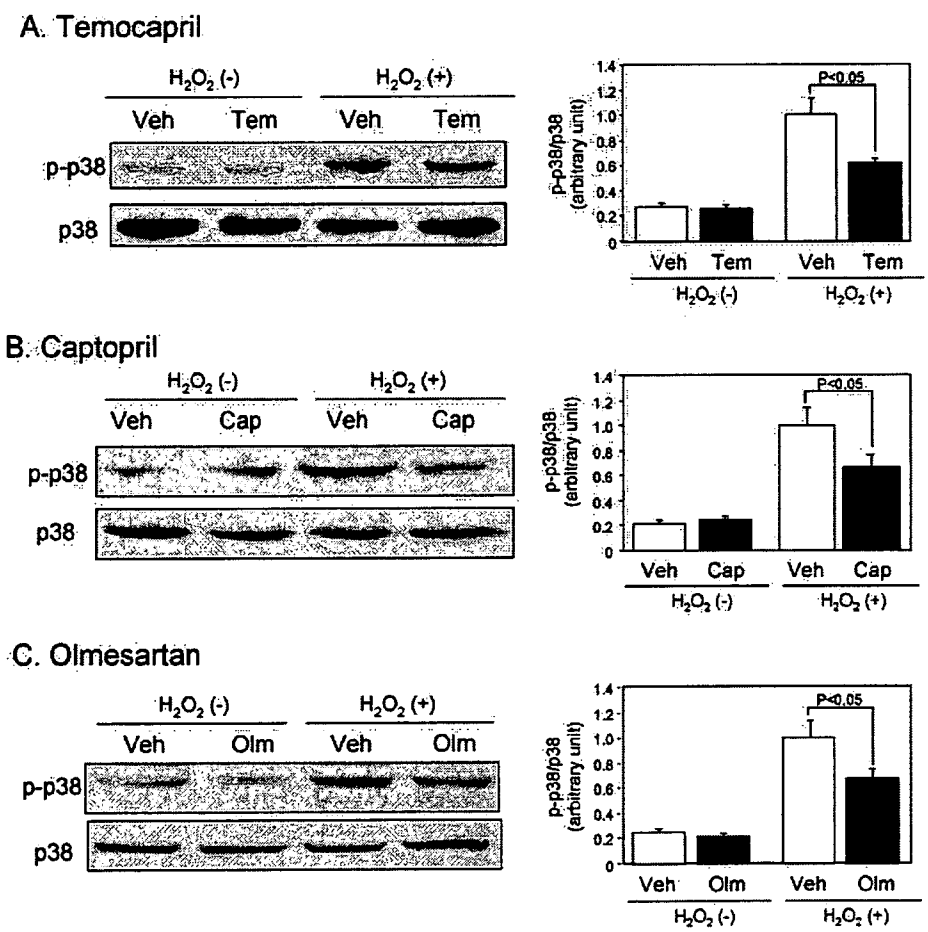

Fig. 3. Effects of temocapril (A), captopril (B) and olmesartan (C) on p38 MAP kinase activity at 30 min after exposure to H_2O_2. Temocapril (100 μmol/l), captopril (100 μmol/l), olmesartan (10 μmol/l) or its vehicle was added to the culture medium 24 h before H_2O_2 treatment until assay. Right panels show the results of densitometric analyses of immunoblotting (mean±SEM, $n=3$).

dose dependent, but there was no significant further increase in EC apoptosis and p38 MAP kinase activity by H_2O_2 of >0.2 mmol/l or by TNF-α of >10 ng/ml. Based on these data, the following experiments were examined using 0.2 mmol/l H_2O_2 or 10 ng/ml TNF-α.

3.2. Effect of ACE inhibitors on EC apoptosis

EC apoptosis, as measured by DNA fragmentation, was significantly attenuated by temocapril and captopril in a dose-dependent manner (Fig. 2A). Reflecting this effect, cell viability was ameliorated by addition of temocapril and captopril in a dose-dependent manner (Fig. 2B).

We also tested using TNF-α whether anti-apoptotic effects of ACE inhibitors would be specific to H_2O_2 or not. As shown in Fig. 2C, both temocapril and captopril effectively inhibited EC apoptosis in a dose-dependent manner. This was associated with the recovery of cell viability by the ACE inhibitors (Fig. 2D). Throughout the experiments, the effects of temocapril were comparable to those of captopril.

To confirm the antioxidant effects of temocapril and captopirl, the formation of 8-isoprostane, a marker of oxidative stress, was measured. Temocapril and captopril restrained 8-isoprostane formation induced by H_2O_2 in a dose-dependent manner (Fig. 2E).

3.3. Effect of ACE inhibitor on p38 MAP kinase activity

Next, the effects of ACE inhibitors on p38 MAP kinase activity were examined because the kinase has been implicated in the cell signaling leading to apoptosis [14,19,20]. As shown in Fig. 3A,B, temocapril and captopril decreased the activity of p38 MAP kinase at 30 min after H_2O_2 treatment by approximately 30–40% without any change in the total protein. An AT1 receptor blocker, olmesartan, showed similar effects on p38 MAP kinase activity (Fig. 3C).

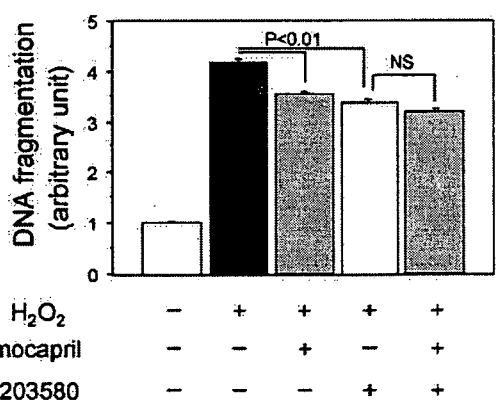

Fig. 4. Effects of temocapril and SB203580 on H_2O_2-induced EC apoptosis. Temocapril (100 μmol/l) or its vehicle was added to the culture medium 24 h before H_2O_2 treatment until assay. SB203580 (10 μmol/l) or its vehicle was added to the culture medium for 1 h before H_2O_2 treatment. EC apoptosis was determined by DNA fragmentation 24 h after H_2O_2 treatment. NS, not significant. Values are expressed as mean±SEM ($n=3$). Similar results were obtained in three independent experiments.

Finally, the effect of a p38 MAP kinase inhibitor, SB203580, was examined. SB203580 reduced H_2O_2-induced EC apoptosis by 20%. More importantly, SB203580 partially but significantly inhibited the effect of temocapril on apoptosis (Fig. 4). Taking these results together with the pro-apoptotic action of p38 MAP kinase, it is suggested that p38 MAP kinase is involved in the effect of temocapril on EC apoptosis.

4. Discussion

A number of investigations have shown that angiotensin II induces oxidative stress in ECs. Angiotensin II stimulates the production of reactive oxygen species in ECs by upregulating the subunits of NAD(P)H oxidase, gp91 phox [21] and p47 phox [22]. It has been reported that the renin angiotensin system contributes to endothelial dysfunction in patients with renovascular hypertension [23]. Conversely, it has been shown experimentally that ACE inhibitors can reduce the production of reactive oxygen species in pathological conditions such as peripheral arteries in rats with chronic heart failure [24], rat diabetic nephropathy [25] and kidney mitochondria in aged rats [26]. In the clinical setting, 4-week treatment with ramipril, in patients with coronary artery disease, diminished the response of endothelium-dependent vasodilation to intracoronary administration of antioxidant vitamin C in parallel with improvement of basal endothelium-dependent vasodilation [27], indicating that ACE inhibitors can improve endothelial function in association with a reduction of oxidative stress.

In the present study, we investigated EC apoptosis, an important process that leads to endothelial dysfunction and atherosclerosis [14,15], and showed that ACE inhibitors, temocapril and captopril, attenuated EC apoptosis induced by H_2O_2 as well as by TNF-α. This result indicates that anti-apoptotic effects of ACE inhibitors are not specific to H_2O_2, but might be attributable to the anti-oxidant action of ACE inhibitors, because reactive oxygen species are known to be involved in TNF-α-induced EC apoptosis [28,29]. Reduction in 8-isoprostane formation by temcapril and captopril further supports the anti-oxidant effects of ACE inhibitors. It is not likely that the anti-apoptotic effects of ACE inhibitors were mediated through nitric oxide production via the inhibition of bradykinin degradation [11], because a nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester, did not influence the effect of temocapril on EC apoptosis (data not shown). Rather, the effects of ACE inhibitors are likely to be mediated through inhibition of angiotensin II production, as was demonstrated by the effect of olmesartan on p38 MAP kinase.

Reactive oxygen species activate many kinds of intracellular signaling, resulting in the transcription of numerous genes and the modulation of cellular function [30]. As

previously reported [31–33], extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and Akt in addition to p38 MAP kinase were activated in ECs by exposure to H_2O_2 (data not shown). Of these serine/threonine kinases, we focused on p38 MAP kinase because p38 MAP kinase is pro-apoptotic signaling, while ERK and Akt are anti-apoptotic, and JNK is anti- or pro-apoptotic depending on conditions [14,19,20]. We found that both temocapril and captopril inhibited the activity of p38 MAP kinase induced by H_2O_2. Although p38 MAP kinase is activated by stress and cytokines and acts on various target proteins, little is known about the downstream signaling [19,20,34]. However, EC apoptosis was effectively blocked in studies using a p38 MAP kinase inhibitor [35,36] and a dominant-negative form of p38 MAP kinase [35], indicating that activation of p38 MAP kinase leads to EC apoptosis. As a matter of fact, a p38 MAP kinase inhibitor, SB203580, partially inhibited H_2O_2-induced EC apoptosis in the present study. More importantly, SB203580 partially but significantly inhibited the effect of temocapril on apoptosis, further implying the role of p38 MAP kinase in the effect of temocapril. However, the partial effects of SB203580 also suggest the role of other pathways than p38 MAP kinase. We should perform future studies to determine the exact mechanism underlying H_2O_2-induced EC apoptosis.

In summary, we found that ACE inhibitors attenuated oxidative stress-induced EC apoptosis in culture. Furthermore, it was suggested that p38 MAP kinase was critical in the inhibitory effect of temocapril on EC apoptosis. These findings provide a mechanistic insight into the effects of ACE inhibitors, which have been used for the treatment of cardiovascular disease.

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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.*

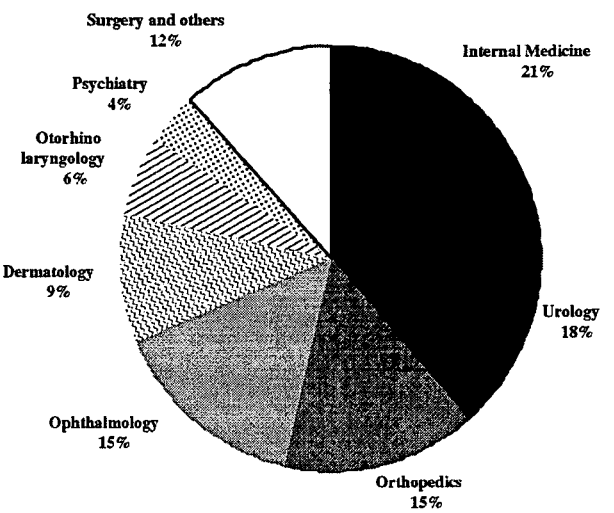

Figure 1 Distribution of specialist referrals.

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

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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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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.

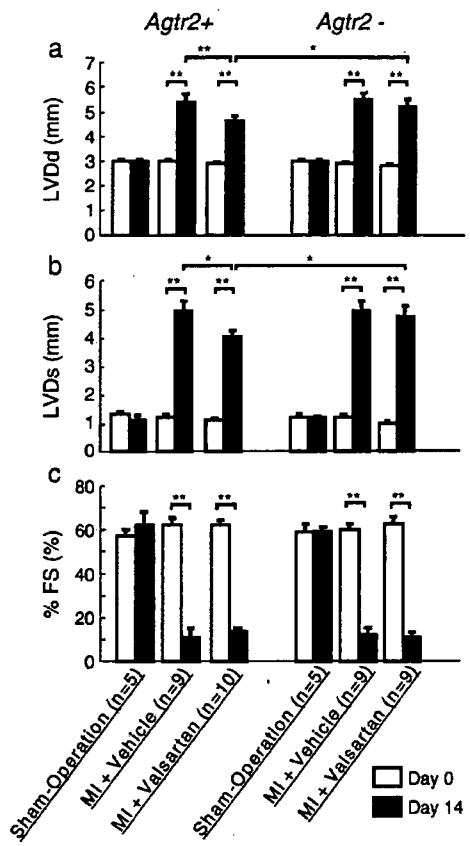

Fig. 1. Changes in (a) end-diastolic LV dimension (LVDd), (b) end-systolic LV dimension (LVDs), and (c) percentage of fractional shortening (%FS) of LV dimensions on day 0 (open column)and day 14 (closed column). Each data point is the mean of pooled data from 5–10 mice. MI; myocardial infarction.**$P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

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

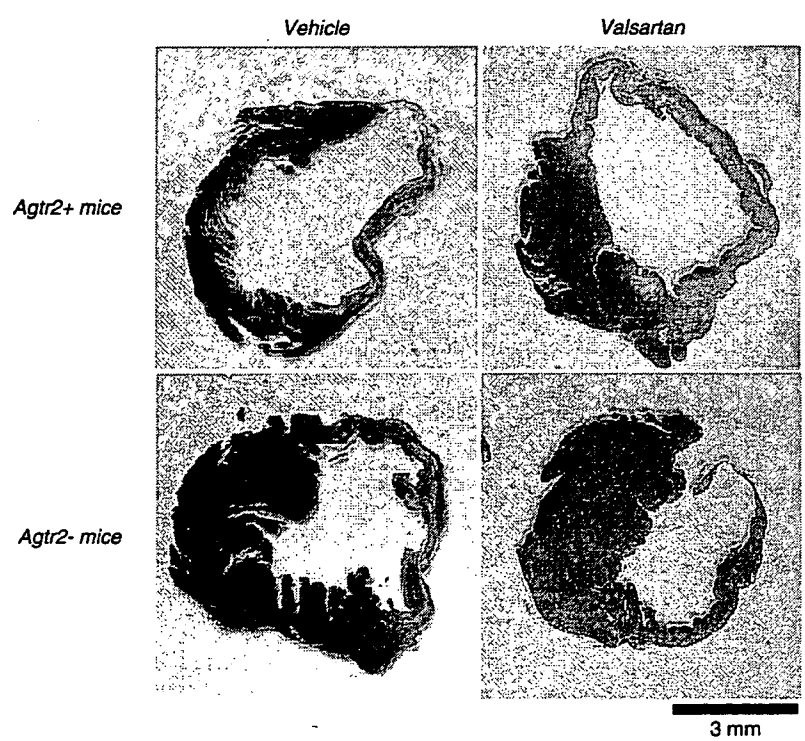

Fig. 2. Transverse sections of LV (post-MI 14 days) from Agtr2+ and Agtr2− mice treated with a vehicle or valsartan. Masson's trichrome staining. Excised LVs were cut into 4 transverse slices, embedded in paraffin, and the sections were cut. The second slices from the apical side are shown. The infarct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

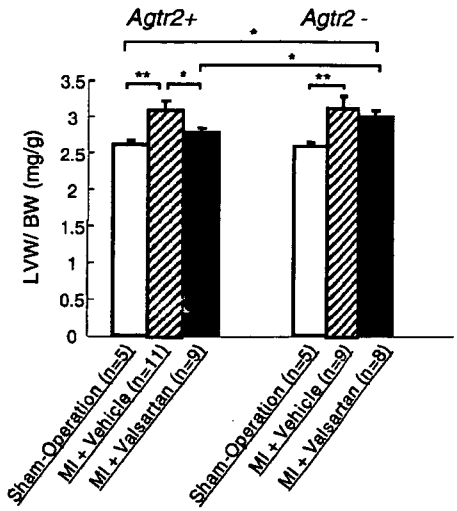

Fig. 3. Left ventricle/body weight ratio(LVW/BW) on day 14 was significantly increased both in *Agtr2+* and *Agtr2−* mice treated with a vehicle. Valsartan significantly decreased LVW/BW in *Agtr2+* mice but not in *Agtr2−* mice. Each data point is the mean of pooled data from 5–11 mice. MI; myocardial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

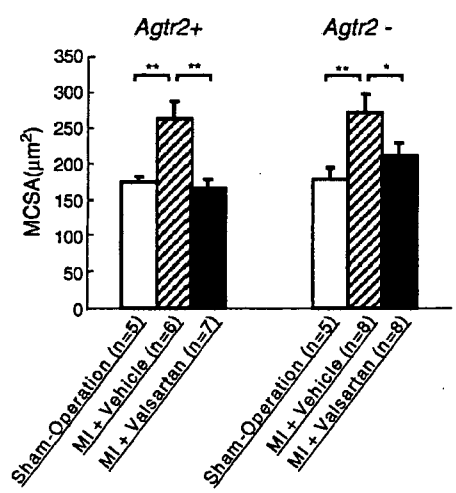

Fig. 4. Myocyte cross-sectional area (MCSA) on day 14 was significantly increased both in *Agtr2+* and *Agtr2−* mice treated with a vehicle. Valsartan significantly decreased MCSA in both strains of mice. Each data point is the mean of pooled data from 5–11 mice. MI; myocardial infarction, $^{**}P<0.001$,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

among the groups. The infarct tissues consist of thin layer of fibrotic tissue with few myocytes and occupy only little area in the total myocardium, explaining unexpectedly small values of infarct areas in comparison with the values of infarct lengths.

Left ventricle/body weight ratio (LVW/BW, Fig. 3) and MCSA (Fig. 4) were not different between sham-operated *Agtr2+* mice and *Agtr2−* mice, indicating that the lack of AT2R did not affect normal myocardial growth. In vehicle-treated *Agtr2+* and *Agtr2−* mice, LVW/BW ($n=11$ and 9, respectively) and MCSA ($n=6$ and 8, respectively) were significantly increased 14 days after surgery to similar extents, suggesting that AT2R does not play a major role in the development of myocardial hypertrophy without pharmacological intervention.

Treatment with valsartan significantly decreased both LVW/BW ($n=10$) and MCSA ($n=7$) in *Agtr2+* mice. In *Agtr2−* mice, valsartan reduced LVW/BW ($n=8$) and MCSA ($n=8$) to a lesser extents than those in *Agtr2+* mice, where only the reduction in MCSA was statistically significant. These results indicate that the anti-hypertrophic effect of valsartan is mediated by stimulation of AT2R and partly by blockade of AT1R.

As shown in Fig. 5, lung/body weight ratio (Lung W/BW), an index for pulmonary congestion, was significantly larger in vehicle-treated *Agtr2−* mice ($n=9$) than in vehicle-treated *Agtr2+* mice ($n=11$). In these vehicle-treated groups, parameters of LV morphology and function were not different (Figs. 1, 3 and 4)), suggesting that factors other than LV function are responsible for the deterioration of pulmonary congestion in *Agtr2−* mice. In other words, the lack of AT2R in the kidney and vasculature may be involved in the mechanism of the pulmonary congestion of *Agtr2−* mice. Treatment with valsartan significantly reduced LungW/BW similarly in the two strains of mice 14 days after surgery, suggesting that this effect of valsartan was mediated by

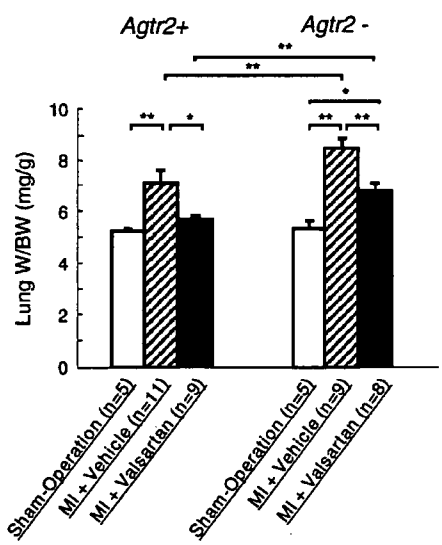

Fig. 5. Lung weight/body weight ratio (Lung W/BW) on day 14 was significantly increased both in *Agtr2+* and *Agtr2−* mice treated with a vehicle. Valsartan significantly decreased Lung W/BW in both strains of mice. However, Lung W/BW was not restored to the level of sham-operated group in *Agtr2−* mice. Each data point is the mean of pooled data from 5–11 mice. MI; myocardial infarction, $^{**}P<0.001$, $^{*}p<0.05$.

AT1Rs in the cardiac- and extra-cardiac tissues. Taken together, the results suggest that deletion of AT2R leads to deterioration of post-infarction congestion and valsartan attenuates the LV remodeling and heart failure through stimulation of AT2R and blockade of AT1R.

MI increased IFI in both *Agtr2−* and *Agtr2+* mice, but valsartan treatment did not affect IFI during the study period (data not shown).

Discussion

LV remodeling after MI is a complex process consisting of acute phase infarct expansion and subsequent myocyte hypertrophy and interstitial fibrosis in the residual myocardium, all of which lead to LV dysfunction. In the present study, effects of valsartan on LV remodeling during the first 2 weeks after MI were examined in *Agtr2+* and *Agtr2−* mice. Valsartan effectively inhibited cardiomyocyte hypertrophy, LV dilatation, and pulmonary congestion, thereby improving survival rate. On the other hand, in *Agtr2−* mice, valsartan had no effect on LV dilatation and had only a limited effect on cardiomyocyte hypertrophy, suggesting that effects of valsartan on post-infarction remodeling were largely mediated by AT2R.

Consistent with our observations, Xu et al. (2002) previously reported that AT2R activation during AT1R blockade plays an important role in the therapeutic effect of ARB in post-MI LV remodeling. Those authors treated AT2R-deficient mice with valsartan from the 4th week after MI for a period of 20 weeks and then demonstrated its benefits on interstitial fibrosis, cardiomyocyte hypertrophy and LV dysfunction, which are mainly mediated by AT2R. Similarly, Liu et al. (2004) demonstrated the role of kinins as a partial mediator of AT2R signaling in a rat post-MI heart failure model using a similar protocol focusing on the late phase of remodeling. Voros et al. (2006) provided support for these observations by results obtained by using a different experimental model, transgenic mice overexpressing AT2R in the heart. In those mice, AT2R overexpression and treatment with an AT1R antagonist had equivalent beneficial effects on the LV remodeling. All of those studies have established the benefit of an AT1R antagonist and the importance of AT2R as a mediator of its effects on late phase post-MI remodeling. On the other hand, relatively little is known about the roles of Ang II and the benefit of AT1R antagonist during the early period after MI, when the risks of LV dysfunction, arrhythmia, and cardiac rupture are high (Pfeffer and Braunwald, 1990). In such a time period, administration of an AT1R antagonist could be harmful because of hypotension (Pourdjabbar et al., 2005) or other causes. In the present study, we demonstrated that valsartan is effective for inhibiting acute phase LV dilatation and dysfunction and that most of its effects are dependent on AT2R. Consistent with our observation, the VALIANT trial (Pfeffer et al., 2003) demonstrated safety of starting valsartan as early as day 1 post-MI. Taken together, valsartan is effective to inhibit both of the early and late phases of post-MI LV remodeling and its effects are mediated by AT2R.

In our previous study (Oishi et al., 2003), we demonstrated that the survival rate of *Agtr2+* mice during a period of 14 days

after MI was significantly higher than that of *Agtr2*− mice. In the present study, the survival rate of vehicle-treated *Agtr2*+ mice during a period of 14 days was also higher than that of vehicle-treated *Agtr2*− mice (41% vs. 33%). Valsartan improved the survival rates in both strains of mice to the same extents (41% to 61% in *Agtr2*+ mice and 33% to 52% in *Agtr2*− mice). However, neither the difference between the mortality rates in the two strains nor the improvement in survival rate by valsartan did reached statistical significance. In the present study, we induced larger MI than that in our previous study (Oishi et al., 2003) in order to accomplish earlier progression of LV dilatation and heart failure after MI. The infarct length in the present study was 65%, which is larger than the values (30–55%) reported in previous studies (Fuchs et al., 2003; Matsushima et al., 2006). The mortality rate of wild-type mice in this study was approximately 2-fold higher than that previously reported in this model (Harada et al., 1999; Liu et al., 1997; Xu et al., 2002). Such a severe MI may have overwhelmed the beneficial effects of AT2R and/or valsartan treatment, explaining, at least in part, the failure to detect statistical difference in the survival rates between the two mouse strains and valsartan's effects on it. Similarly, this large MI caused extremely low LV contraction (% FS, Fig. 1c) in both strains of mice, which may have been too severe to be restored by valsartan.

In the present study, the beneficial effect of valsartan on LV remodeling was abolished in *Agtr2*− mice (Figs. 1 and 4), suggesting that the effect of valsartan on LV remodeling was fully mediated by AT2R in the heart. However, valsartan treatment also improved the pulmonary congestion (Fig. 5) and the survival of *Agtr2*− mice, suggesting that (1) the pulmonary congestion and the survival rate were not determined solely by the extent of LV remodeling and that (2) some of the beneficial effects of valsartan in *Agtr2*− mice were mediated in part by AT1R *in the heart as well as* in the extra-cardiac tissues such as the vasculature and the kidney. It is plausible that the dominant receptor subtype that mediates the Ang II action may be AT1R in the vasculature and the kidney, which may explain the similar improvement in heart failure by valsartan in *Agtr2*+ and *Agtr2*− mice.

However, it should be noted that the extent of pulmonary congestion (Fig. 5) was more severe in *Agtr2*− mice than in *Agtr2*+ mice. This difference may be attributed to the absence of AT2R. Since AT2Rs in the kidney and systemic vasculature are involved in the mechanisms of natriuresis (Carey et al., 2000) and vasorelaxation (Akishita et al., 1999; Carey et al., 2000), respectively, a deletion of AT2R might cause volume overload and increase in systemic vascular resistance, leading to the deterioration of systemic hemodynamics after MI.

The effects of ARBs may change depending on the tissue content of Ang II and the expression levels of AT1R and AT2R. In the failing heart, tissue level of Ang II is increased and the increase in Ang II level is further facilitated by ARB administration (Spinale et al., 1997). MI results in upregulation of AT1R (Matsubara, 1998) and AT2R (Adachi et al., 2003; Matsubara, 1998). It has been also reported that ARBs upregulated AT2R (Jugdutt and Menon, 2004). These findings may explain the effectiveness of ARB in post-MI remodeling, particularly through its action on AT2R.

An unexpected result was that IFI was not improved by valsartan. There is a consensus that administration of ARBs inhibits interstitial fibrosis after MI both in rodents (Liu et al., 2004, 1997; Voros et al., 2006; Xu et al., 2002) and humans (Di Pasquale et al., 1999). However, previous studies (Harada et al., 1999; Liu et al., 1997; Voros et al., 2006) suggest that it takes approximately four weeks until significant fibrosis develops after MI in rodents. In the present study, focusing on the early phase of post-MI LV remodeling, we observed LV dilatation during a period of two weeks after MI, a period that was not sufficient to study post-MI interstitial fibrosis. Furthermore, in the present study, the infarct size was large, resulting in a delay in the healing process involving fibrosis formation.

A growing body of evidence (Dickstein and Kjekshus, 2002; Pfeffer et al., 2003) suggests that ARBs are important agents for treating heart failure. However, the advantage of this class of drugs over angiotensin converting enzyme inhibitors is not well understood. Likewise, possible differences in the pharmacological actions and clinical benefit among ARBs are not well elucidated. In the present study, we demonstrated an important action of valsartan to stimulate AT2R, which is associated with additional cardioprotection to a simple blockade of AT1R, particularly during early days after MI. It is of note that the cardioprotective role of AT2R was undetectable until valsartan was administered, suggesting that AT2R need to be "stimulated" by valartan to function as a cardioprotective mechanism. Precise studies on the effect of valsartan and other ARBs may be helpful for improving treatment of cardiovascular diseases.

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